Chapter 3

Biochemical and biological characterization of crude Naja kaouthia venom

3.1 Introduction

In India the "Big Four", the Indian cobra (*Naja naja*), the common krait (*Bungarus caeruleus*), the Russell's viper (*Daboia russelli*) and the saw scaled viper (*Echis carinatus*) are considered to be medically important snakes which are responsible for most of the fatal bites. However, in addition to "Big Four", medically important snakes in a particular geographical area might be present which needs to be addressed. This is important for clinical diagnosis for treatment with antivenom and for production of effective antivenom.

N. kaouthia from northeast India is one of the most prevalent snakes responsible for most of the snakebite cases. According to WHO, *N. kaouthia* belongs to Category 1 as it is a highly venomous snake which cause numerous bites. The clinical symptoms of cobra bites are generally neurotoxic, leading to flaccid paralysis and death by respiratory failure. Moreover, bites from *N. kaouthia* of Asian origin are reported to exhibit symptoms of coagulopathy [15]. In India, *N. kaouthia* venom of West Bengal have been studied extensively [15, 87, 88, 221, 254]; however, biochemical and biological property of northeast India origin venom has not been documented.

Hence, in the present study the biochemical and biological characterization of the crude *N. kaouthia* venom from northeast India has been undertaken to understand the post-envenomation effects. Further, the effectiveness of commercially available polyvalent antivenom in neutralizing some biochemical and biological properties of the crude venom was studied *in vitro*.

3.2 Results

3.2.1 Collection of venom, preparation and storage

Crude *N. kaouthia* venom of northeast India is not supplied by the commercial vendors, eg. Irula snake firm, Chennai, India. Hence for our studies, *N. kaouthia* from its natural habitat were captured at Jamugurihat, Sonitpur, Assam (Figure 3.1). Jamugurihat (26.73^{o}N 92.93^{o}E) is situated at north bank of river Brahmaputra, a small town in Sonitpur district. The major occupation in the area is agriculture and many are engaged in tea cultivation. Vegetation cover and close proximity to the Nameri National Park (protected area) makes the area biologically diverse (Figure 3.1). The amphibians and reptile populations are also high due to its cultivation lands and also close proximity of the national park (Figure 3.1).

We captured the snake from its natural habitat and extracted. In each exctraction, 0.5 to 0.7ml of crude venom was obtained and 6 extractions were done in 2 seasons from different cobras captured in the same geographical location. The pooled crude venom was lyophilized and pale yellow crystals of dried crude venom were obtained after lyophilization. The dried venom was weighed (0.31gm) and was stored at -20°C until further use.

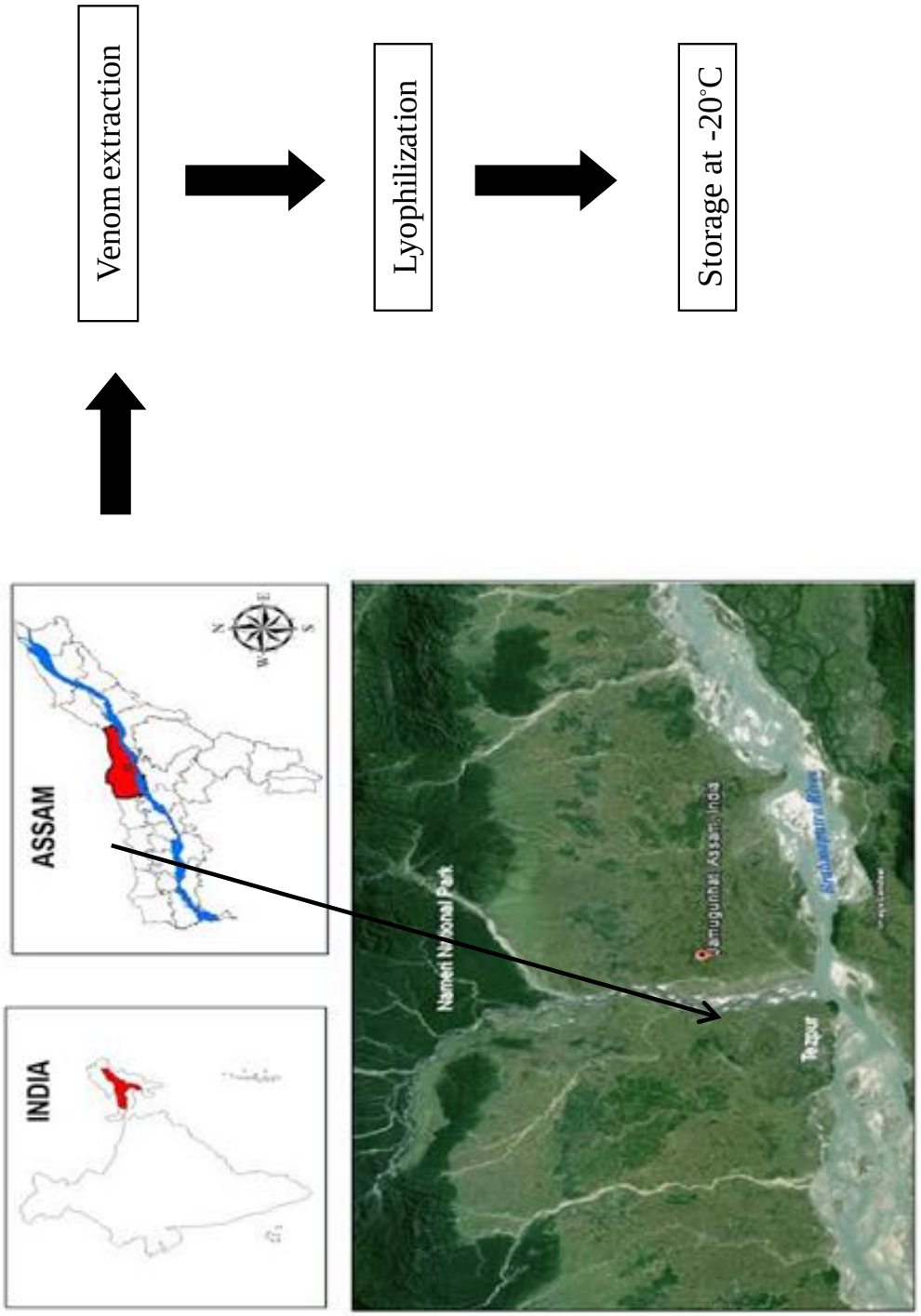

Figure 3.1: Geographical location of Jamugurihat, Assam, India: *N. kaouthia* was captured in wild

3.2.2 Determination of total protein content

Lyophilized (2.051mg) crude *N. kaouthia* venom was reconstituted in 0.5ml of 20mM Tris-Cl buffer (pH 7.4) and total protein content was determined according to Lowry's method [223]. A standard curve of absorbance (y axis) versus concentration of protein (x axis) was plotted using BSA as a standard (Figure 3.2). 5µl of dissolved crude venom was used as unknown to determine the protein content. The regression (R^2= coefficient of multiple determinations) was calculated to be 0.984.

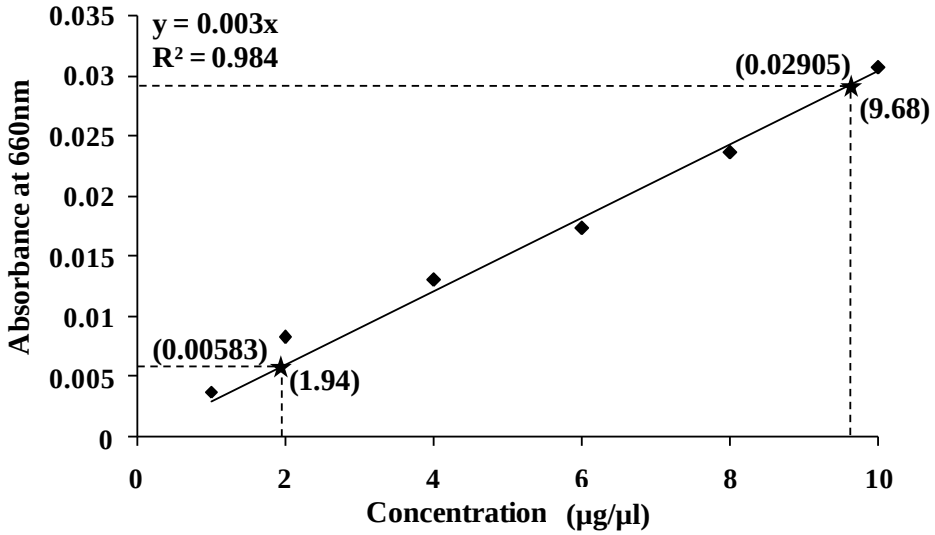

Figure 3.2: BSA standard curve. 0.02905 is the absorbance of the crude venom sample where protein content was calculated to be 9.68µg/5µl. Simultaneously, concentration of crude venom protein at 1µl was found to be 1.94µg and absorbance was 0.00583.

The absorbance for crude venom (5µl) at 660 nm was found to be 0.02905 after deducting the blank value and concentration of protein was calculated using y= 0.003x, where y is the absorbance and x represents concentration of protein (Figure 3.2).

Therefore, concentration of protein present in 5µl, 0.02905= 0.003x (y= 0.003x)

$$x = \frac{0.02905}{0.003}$$

$$= 9.68$$

Concentration of crude venom protein in 1µl $= \frac{9.68}{5}$

$$=1.936$$

$$=1.94\mu g$$

Total protein in crude venom (%) = $\frac{\text{Concentration of protein (µg/µl) x100}}{\text{Initial amount of crude venom dissolved (mg)}}$

$= \frac{1.94 \times 100}{2.051}$

$= 94.6$

Hence, concentration of protein in crude venom was found to be 1.94µg/µl and total percentage of protein present in the crude venom was determined to 94.6%.

3.2.3 SDS-PAGE (Sodium dodecyl sufate polyacrylamide gel electrophoresis)

Crude *N. kaouthia* venom was subjected to SDS PAGE to visualize the various proteins present based on their molecular mass (Figure 3.3). Electrophoresis was conducted in reduced and non-reduced condition and molecular mass of various protein bands were determined using a standard molecular weight marker (10-230kDa) (Figure 3.3.).

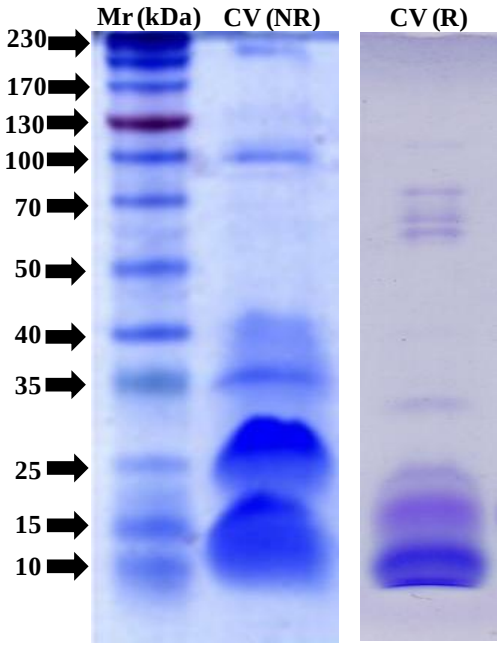

Figure 3.3: SDS PAGE of crude *N. kaouthia* venom. 15µg of crude venom was loaded for electrophoresis. Mr (kDa): Molecular weight marker, CV (NR): crude venom under non-reduced condition. CV (R): crude venom under reduced condition.

The crude venom was found to consist of major percentage of proteins at molecular mass range of 35 to < 10kDa under non-reduced condition. However, when the venom was electrophorsed under reduced condition a smear of proteins between 25kDa to

15kDa was observed (Figure 3.3). An intense thick band of protein smear was also observed at 10kDa and less than 10kDa under reduced condition. Further, a smear of protein bands was observed at 35 to 40kDa range under non-reduced condition which however under reduced condition a single homogeneous band was observed near 35kDa (Figure 3.3). Two single homogeneous bands were observed at a molecular mass range of 100kDa and near 230kDa under non-reduced condition (Figure 3.3).

While under reduced condition a faint band at 100kDa and three other protein bands were observed near 70kDa (Figure 3.3). The SDS-PAGE profile of crude *N. kaouthia* venom suggests the presence of low molecular weight proteins (3FTx family, kunitz-type serine protease inhibitors, PLA_2 and its isoforms) and larger molecular weight proteins , (proteases and L-amino acid oxidases).

3.2.4 Lethal dose (LD_{50}) determination of crude *N. kaouthia* venom

The lethal dose of crude *N. kaouthia* venom was found to be 0.148mg/kg body weight of mice. Calculations for determining LD_{50} are given in table 3.1 and table 3.2. Figure 3.4 shows the survival time of experimental animal against doses of venom injected. Out of the seven experimental mice, the two animals injected with 1mg/kg and 0.75mg/kg venom survived up to ~100 to 110mins (Figure 3.4). Mouse M5, injected with 0.5mg/kg of crude venom was found to survive ~300mins.

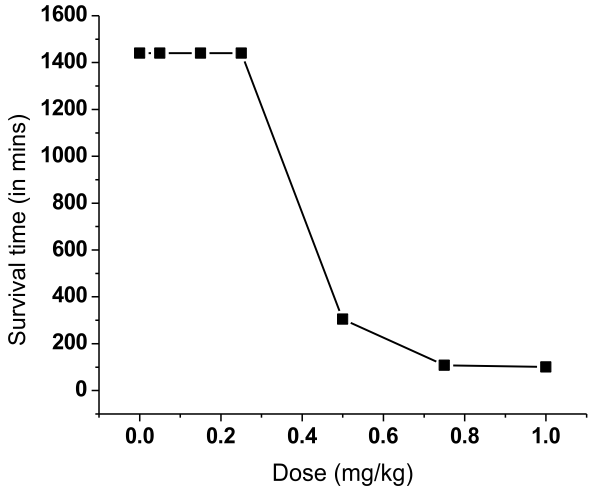

Figure 3.4: Survival time of experimental mice after injection of crude *N. kaouthia* venom (i.p.).

Table 3.1: Calculation of LD_{50} according to Meier and Theakston, 1986

Sl no.	Animal no. (A)	Weight (in gm)	Injection Volume (µl)	IP Dose (mg/kg)	IP² dose	Venom (µg/gm)	Time of Injection (hr)	Time of Death (hr)	Survival Time (mins)	Dose/ Time (B)	(Dose/ Time) ² (C)	D/T*IP dose (F)
1	MF1	42	150	Control	NA	NA	16:07	NA	1440	0	0	0
2	MF2	45	150	0.05	0.0025	2.25	16:12	NA	1440	3.47222	1.206E-09	1.74E-06
3	MF3	41	150	0.15	0.0225	6.15	16:13	NA	1440	0.00010	1.085E-08	0.00002
4	MF4	39	150	0.25	0.0625	9.75	16:14	NA	1440	0.00026	3.014E-08	4.34E-05
5	MF5	40	150	0.5	0.25	20	16:16	21:21	305	0.00164	2.687E-06	0.00082
6	MF6	43	150	0.75	0.5625	32.25	16:18	18:06	108	0.00694	4.823E-05	0.00521
7	MF7	34	150	1	1	34	16:23	18:04	101	0.00990	9.803E-05	0.0099
8	AVG	40.6		2.7	1.9					0.019	0.0002	0.016

LD_{50} of crude *N. kaouthia* venom of northeast India was found to be **0.148mg/kg** on Swiss

Table 3.2: Calculation of LD_{50} according to Meier and Theakston, 1986

1	**Number of animals used (A)**	7
2	**Sx Sum of Dose of venom injected/Survival time for all mice (B)**	0.018797
3	**Sx Sum of (D/T) ² of all mices (C)=**	0.00015
4	**Sy Sum of doses of venom injected (D)=**	2.7
5	**Sy Sum of squares of doses of venom injected (E)=**	1.9
6	**Sxy (F) = B*D =**	0.01599
7	**G = C- B^2/A**	9.85255
8	**H= F - B*D/A**	0.00874
9	**J= E-D^2/A**	0.85857
10	**K= H^2/G**	0.77527
11	**L= J-K**	0.08331
12	**M= B/A**	0.00269
13	**N= D/A**	0.3857
14	**P= H/G**	88.706
15	**LD 50 (Q)= N-m*p**	0.148

LD_{50} was determined by the formulae: $LD_{50}(Q) = N-m*p$
$= 0.3857-0.00269*88.706$
$= 0.148mg/kg$

3.2.5 Behavioral study

Behavioral study of experimental animals post-venom injection helps in understanding the nature of toxicity of the venom. In the present experiment, the experimental animals were injected with 50% of LD_{50} (0.074mg/kg) of crude *N. kaouthia* venom from northeast India via intra-peritoneal route.. The following manifestations on the experimental animals were observed soon after venom injection:

a) After the venom injection the first manifestation of intoxication became evident at 3-7mins where the mice are trying to remain near to the drinking water source, and frequent drinking of water was observed.

b) The experimental animal stayed in a corner of the cage separated from the other healthy mice and was disheveled. Sluggishness in locomotion was observed. There was no reaction towards weak sound and only responded to loud sound and abrupt jerky movements. Difficulties in breathing were also observed.

c) From 15-20mins, heavy difficulty in breathing was seen. There was no defecation however salivation was prominent. Paralysis of lower limbs and facial whiskers were observed which acts as active sensory organs for the mice to navigate through their environment.

d) At 25-45mins, the Experimental animal suffered significant respiratory disorder and was completely paralyzed.

e) The physiological condition almost remained constant with severe breathing problem and paralysis. However, the animals were found to be alive up to 80-90mins.

f) The experimental animals succumbed at ~90-100mins.

Above behavioral observation of mice upon injection of crude *N. kaouthia* venom suggests neurotoxic nature.

3.2.6 Hemorrhagic activity assay

The crude *N. kaouthia* venom (15µg) from Northeast India was not observed to exhibit any hemorrhagic activity in the experimental animal when injected intra-dermally (i.d.) (Figure 3.5). The experimental animal was sacrificed and dorsal surfaces of the skin were removed and inner surface was checked for hemorrhagic activity. The crude *N. kaouthia* venom did not induce any hemorrhage however; the site of injection has a small spear of hemorrhagic spot of less than a centimeter (Figure 3.5B). The control animal was injected with 20µl of saline and hemorrhage was not observed as well (Figure 3.5A).

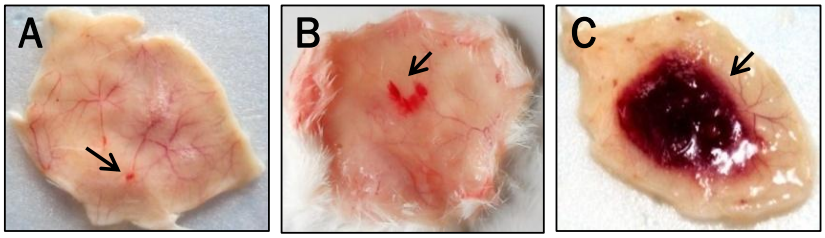

Figure 3.5: Hemorrhagic activity assay of crude *N. kaouthia* venom. A: Control (20µl of saline), **B:** Crude *N. kaouthia* venom (15µg), **C:** Saw scaled viper venom (3µg) (Positive control), the arrow indicates site of injection

The animal injected with 3µg of crude *Echis carinatus* venom (positive control) showed a significant hemorrhage and the hemorrhagic spot was measured manually to ~4.7cm (Figure 3.5C). However, at higher amount the venom might show hemorrhage which might involve influence of other major toxins such as PLA_2.

3.2.7 Edema inducing activity assay

Crude *N. kaouthia* venom was found to exhibit edema inducing activity when tested on experimental animals. The effect of crude venom on local inflammation was tested in 6

sets (n=3) of experimental animal and injected with 0.5µg to 20µg of crude venom into the right leg of the mice and left leg received 0.9% NaCl (Table 3.3) which served as vehicle. Weight of the legs from the ankle was taken and the minimum edema dose (MED) of the crude venom was calculated and found to be 10±2.15µg. Minimum edema dose (MED) is defined as the amount of protein required to cause an edema ratio of 120% [231] (Figure 3.6). The edema ratio was determined using:

$$\text{Edema ratio (in \%)} = \frac{\text{Weight of edematous leg (gm) X 100}}{\text{Weight of normal leg (gm)}}$$

Table 3.3 Edema inducing activity of crude *N. kaouthia* venom

Sl no	Injections (i.d.)	Mouse no. (M)	Amount of venom (µg)	Normal leg weight (gm)	Edematous leg weight (gm)	Edema ratio (%)	Average of edema ratio (%)
1	Saline	M1	0	0.12	0.112	93.3	99.2
2	Saline	M2	0	0.12	0.125	104.2	
3	Saline	M3	0	0.13	0.13	100	
4	CVNK	M4	0.5	0.12	0.134	111.7	107.7
5	CVNK	M5	0.5	0.13	0.133	102.3	
6	CVNK	M6	0.5	0.12	0.131	109.2	
7	CVNK	M7	2	0.14	0.137	97.85	105.8
8	CVNK	M8	2	0.11	0.125	113.6	
9	CVNK	M9	2	0.12	0.127	105.8	
10	CVNK	M10	5	0.12	0.132	108.3	111.6
11	CVNK	M11	5	0.12	0.139	115.8	
12	CVNK	M12	5	0.14	0.144	110.8	
13	CVNK	M13	10	0.13	0.157	120.8	120.3
14	CVNK	M14	10	0.12	0.142	118.3	
15	CVNK	M15	10	0.12	0.146	121.7	
16	CVNK	M16	15	0.11	0.162	147.3	137.6
17	CVNK	M17	15	0.12	0.164	137	
18	CVNK	M18	15	0.13	0.167	128.5	
19	CVNK	M19	20	0.12	0.221	184.2	184.7
20	CVNK	M20	20	0.13	0.233	179.2	
21	CVNK	M21	20	0.13	0.248	190.8	

CVNK- Crude *N. kaouthia* venom

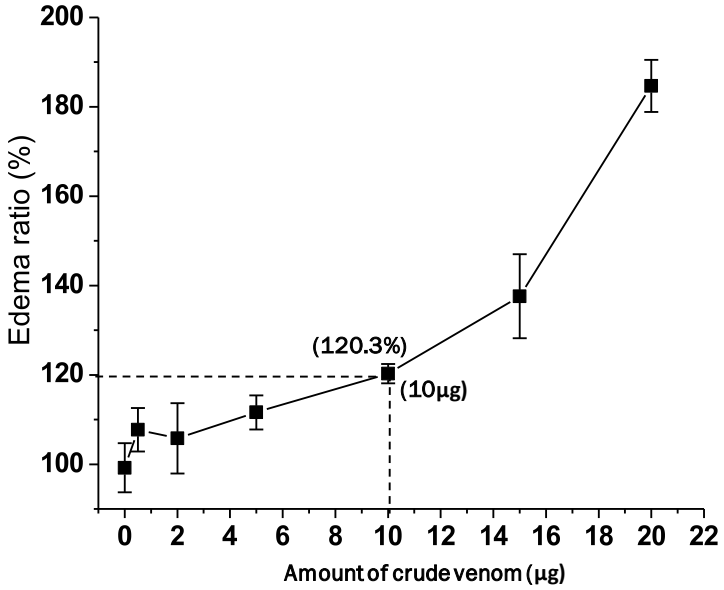

Figure 3.6: Edema inducing activity of crude *N. kaouthia* venom. At 10µg of crude venom the edema ratio was calculated to 120.3%. Minimum edema dose (MED) is defined as the amount of protein required to cause an edema ratio of 120%.

3.2.8 Phospholipase A_2 (PLA_2) activity assay

SDS-PAGE of crude *N. kaouthia* venom from Northeast India clearly suggests the presence of this enzymatic protein component. However, surplus presence of PLA_2 s in elapid venom is also known from literature [84, 87, 255–257]. The enzymatic activity of PLA_2 enzymes in the crude venom was tested *in-vitro* by colorimetric and turbidometric methods.

3.2.8.1 Colorimetric method:

Crude *N. kaouthia* venom in various concentrations viz. 0.001, 0.01 and 0.1µg/µl was tested. Bee venom (0.01µg/µl) was tested as positive control. The activity was expressed as micromoles of diheptanoyl Thiol-PC hydrolyzed/min/mg of enzyme. At 0.01µg/µl of crude *N. kaouthia* venom, the PLA_2 activity was found to be 7.584µmol/min/mg when assayed with $sPLA_2$ kit (Figure 3.7).

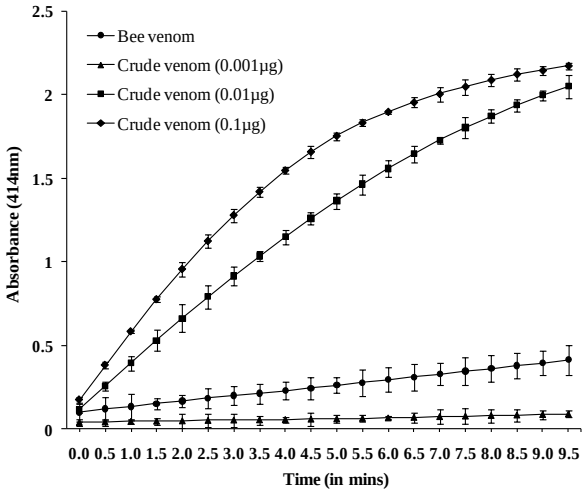

Figure 3.7: PLA_2 activity assay of crude *N. kaouthia* venom by colorimetric method. Bee venom PLA_2 served as positive control.

3.2.8.2 Turbidometric method:

Various amounts of crude venom (0.01µg, 0.1µg, 0.2µg, 0.4µg, 0.6µg, 0.8µg and 1µg) were tested for PLA_2 activity using egg yolk as a substrate. One unit of PLA_2 activity is defined as a decrease in 0.01 absorbance in 10min at 740nm [88]. The crude *N. kaouthia* venom was found to show a significant increase in unit activity in a dose-dependant manner (Figure 3.8). At 1µg, the PLA_2 activity was 64.9µg/min/mg.

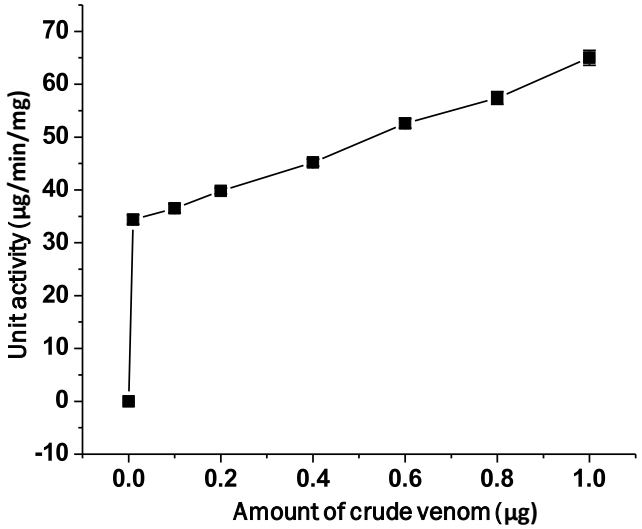

Figure 3.8: PLA_2 activity assay of crude *N. kaouthia* venom by turbidometric method.

3.2.9 Indirect hemolytic activity assay

Crude *N. kaouthia* venom was tested on washed RBC's with egg yolk as free phospholipid substrate for PLA_2 enzyme. Various amounts of crude venom were checked (0.01µg, 0.1µg, 0.5µg and 1µg) and a dose-dependent increase in activity was observed (Figure 3.9). At 1 µg of crude venom, 23% hemolysis of washed RBC's was observed.

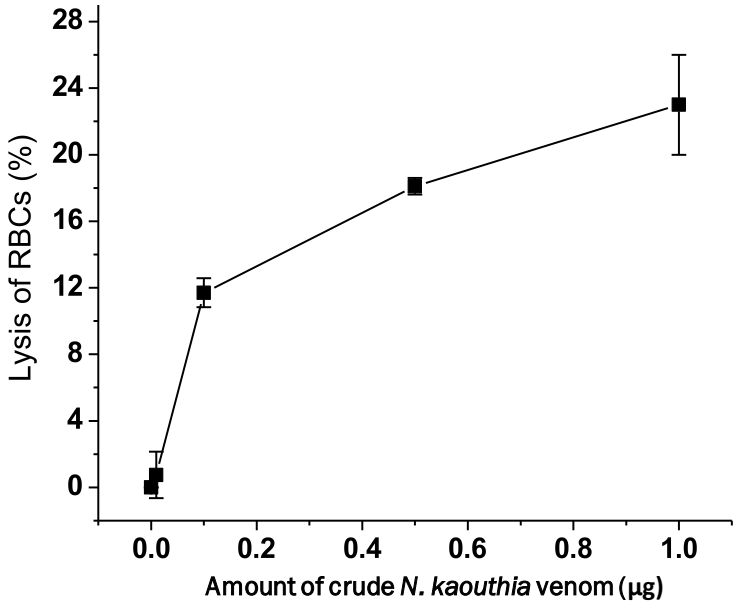

Figure 3.9: Indirect hemolytic activity assay of crude *N. kaouthia* venom

3.2.10 Direct hemolytic activity assay

Lytic activity of crude venom on washed RBC's could not be observed at lower doses (0.01µg, 0.1µg, 1µg), and at higher doses (5µg, 10µg, 50µg, 100µg), the activity was negligible. At 100µg, lysis of washed RBC's was found to be 1.4% (Figure 3.10).

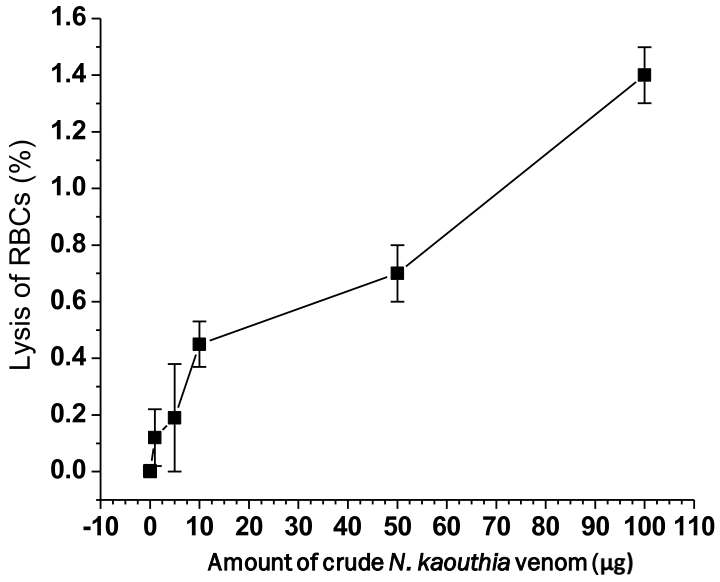

Figure 3.10: Direct hemolytic activity assay of crude *N. kaouthia* venom

3.2.11 Caseinolytic activity assay

Crude *N. kaouthia* venom was tested for proteolytic activity on casein. A standard curve was plotted for tyrosine (Figure 3.11A). The crude venom was found to show less proteolytic activity even at 100µg (Figure 3.11B). The amount of tyrosine liberated was 0.14 ±0.02moles by 100µg of venom in 1min. One unit of protease activity is defined as *n* mole equivalent of tyrosine formed per min per ml.

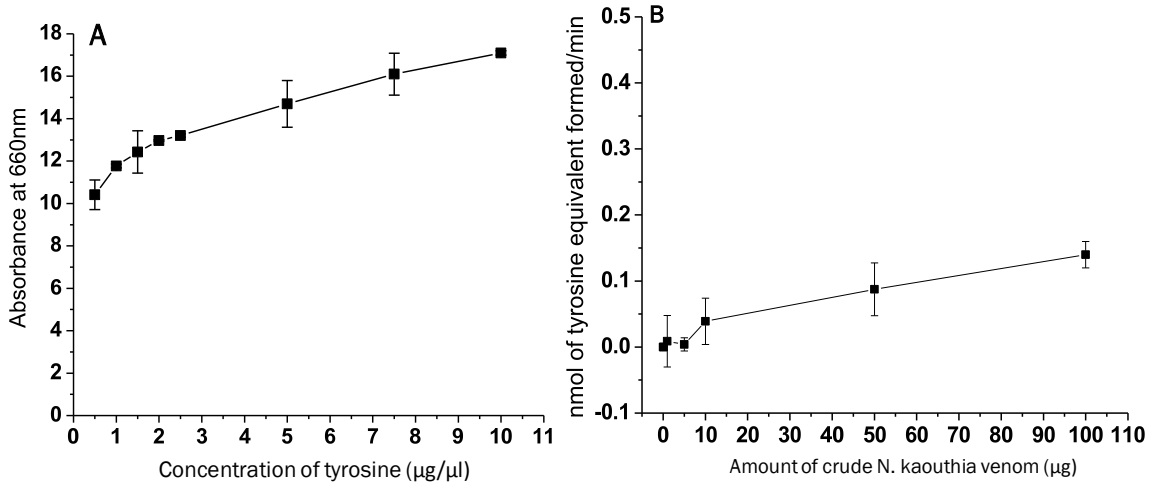

Figure 3.11: Caseinolytic activity of crude *N. kaouthia* venom: A: Tyrosine standard curve. **B:** Proteolytic activity of crude venom on casein in dose dependently. One unit of protease activity is defined as *n*mole equivalent of tyrosine formed per min per ml.

3.2.12 *In-vitro* coagulation activities

The venom showed anticoagulant activity in a dose-dependent manner. The delay in clotting (recalcification time) of human plasma was found to be significant when it was incubated with 1µg crude venom, the plasma did not form clot up to 500s whereas the normal clotting time was 126.5sec (Figure 3.12). To test the effect of crude venom in external pathway of blood coagulation, prothrombin time (PT) was tested. It was found that the PT of citrated plasma was increased dose-dependently. At 4.2µg/ml, clot formation was delayed up to 500sec (Figure 3.12). However, while testing the intrinsic path way of blood coagulation using APTT test on plasma, the clotting time did not increase significantly up to 0.042µg/ml but when the amount was increased to 4.2µg/ml the plasma did not form clot (Figure 3.12).

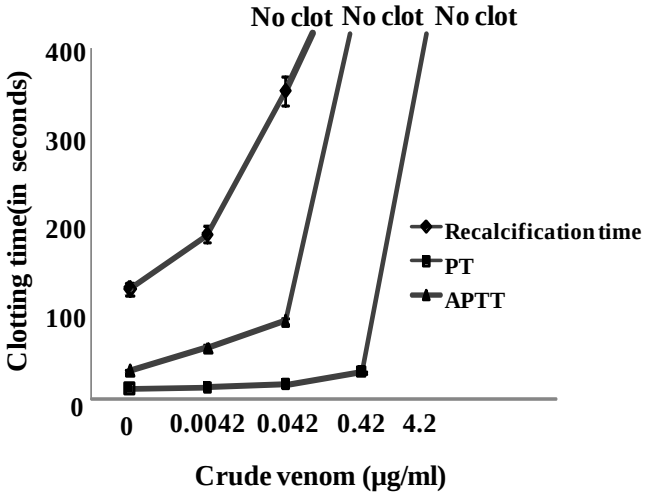

Figure 3.12: Dose-dependent anticoagulant activity of crude *N. kaouthia* venom on citrated human plasma. Effect of crude venom on recalcification time, prothrombin time test (PT) and activated partial thrombin time test (APTT), No clot- The experiment was set to observe blood clot only up to 400s. Above the given limit, all treatments (plasma+venom) which exceeded 400s were considered as no clot. The results are means ±SD of three experiments.

3.2.13 Whole blood coagulation analysis

3.2.13.1 Analysis by Rotem® Analyzer

In the 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 (Figure 3.13). 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 3.4). 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 3.4).

Table 3.4: Thromboelastometry analysis of whole blood

Parameters	PBS	Crude venom (µg/ml)		
		0.1	1.0	10
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*

*NCF- No clot formations, the results are expressed as the mean ± SD

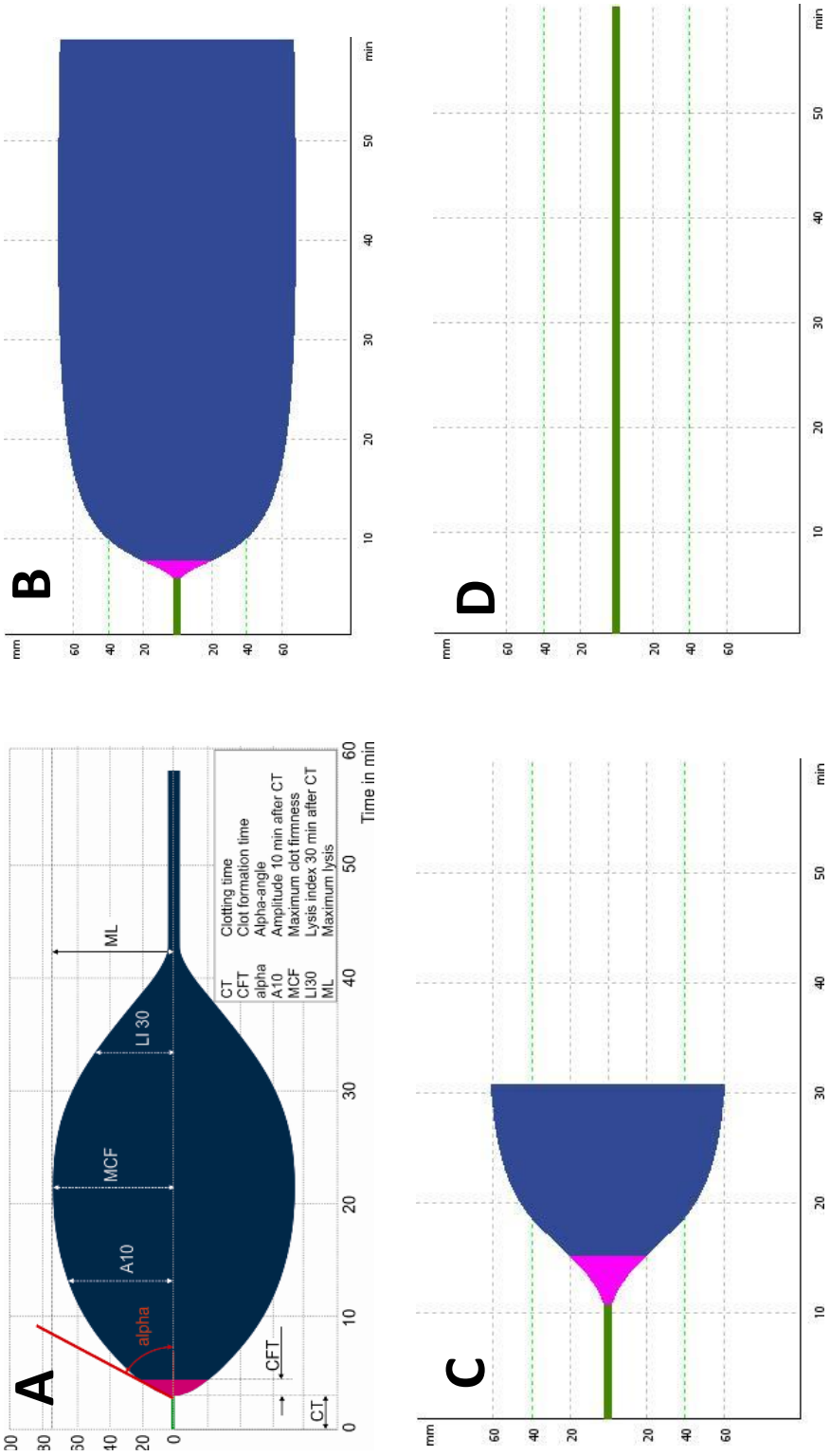

Figure 3.13: Graphical representation of clot formation on Rotem® analyzer. A. Normal description of the various parameters (http://www.rotem.de).**B.** Normal clot formation in donor with PBS.**C.** Clot formation in presence of 0.1 µg of *N. kaouthia* venom. **D.** Clot formation in presence of 1 µg of *N. kaouthia* venom. Dark green bar indicates the clotting time. Pink color in the graph represents the clot formation time. Dark blue color indicates clot strength. The graph represents one representative of the assay.

3.2.13.2 Sonoclot analysis

To confirm further the effect of crude venom on whole citrated blood a Sonoclot coagulation and platelet function analyzer was used. The continuous curve or the sonoclot signature describes the whole coagulation process *in-vitro*, from formation of the fibrin, polymerization of the fibrin monomer, platelet interaction and clot retraction followed by lysis. The results are shown in table 3.5. Activated clotting time (ACT) increases dose dependently and at 10 µg/ml concentration it was recorded to be 591secs. The clot rate is the period during which fibrinogen forms the fibrin gel. At 0.1 µg/ml concentration, the clot rate was similar to normal clot rate (normal range 9-35) (Figure 3.14) but with increase in concentration the clot rate decreased due to depletion of fibrinogen. However up to 1.0 µg/ml concentration the platelet function was found to be normal but at 10 µg/ml the platelet function was nil, which might be due to non-availability of fibrinogen (Figure 3.14).

Table 3.5 Sonoclot analysis of whole blood

Parameters	PBS	Crude venom (µg/ml)		
		0.1	1.0	10
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 the mean ± SD

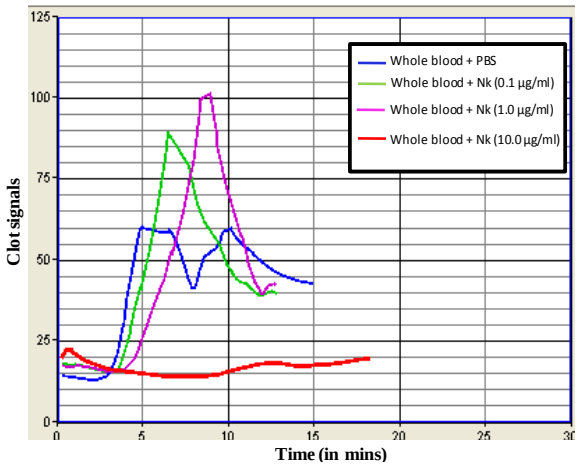

Figure 3.14: Sonoclot graph of crude *N. kaouthia* venom on citrated human whole blood.

3.2.14 Fibrinogenolytic activity

The crude *N. kaouthia* venom was tested for fibrinogenolytic activity using purified bovine fibrinogen. Various amounts of crude venom (1µg, 2.5µg, 5µg and 10µg) treated with bovine fibrinogen in a time dependant experiment for 24hrs. Fibrinogen incubated with 1, 2.5 and 5µg of crude venom did not degrade Aα, Bβ and γ chain of fibrinogen as observed on SDS-PAGE (Figure 3.15A, 3.15B and 3.15C). However, when the amount of venom was increased to 10µg, clear digestion of α chain of fibrinogen was observed after 16hrs of incubation (Figure 3.15D).

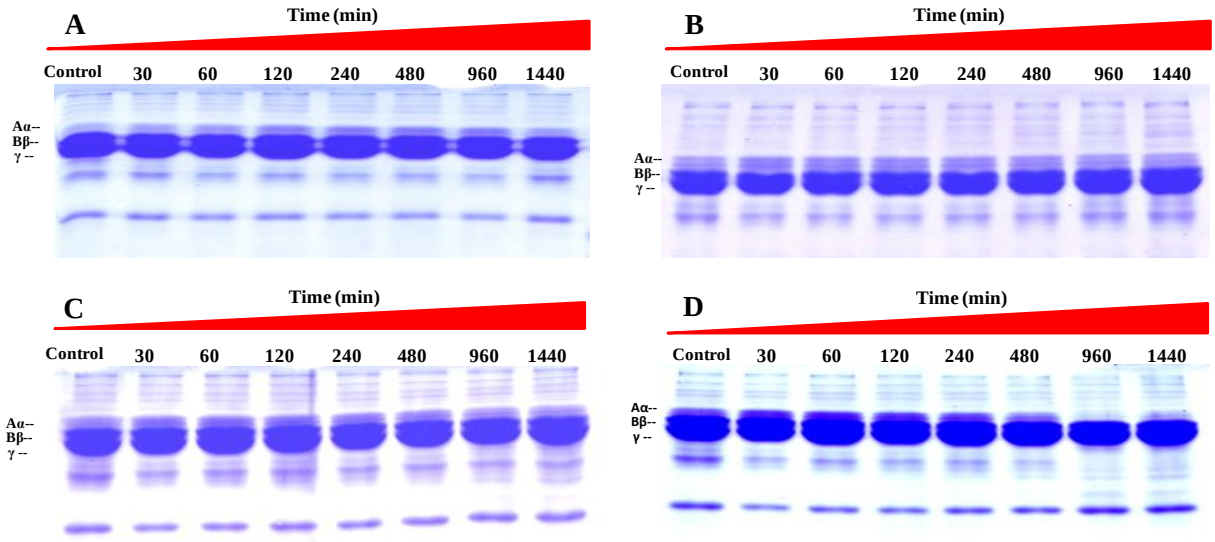

Figure 3.15: Degradation of fibrinogen by crude *N. kaouthia* venom on SDS-PAGE at various time intervals. A. Bovine fibrinogen (reduced) after incubation with 1µg, **B.** Bovine fibrinogen (reduced) after incubation with 2.5µg, **C.** Bovine fibrinogen (reduced) after incubation with 5µg, **D.** Bovine fibrinogen (reduced) after incubation with 10µg.

3.2.15 Bacteriacidal activity

The microbial strains used are *Staphylococcus aureus* (Gram +ve) *and Pseudomonous aureginosa* (Gram –ve). 1, 5 and 10µg of crude venom were used for both well and disc diffusion methods.

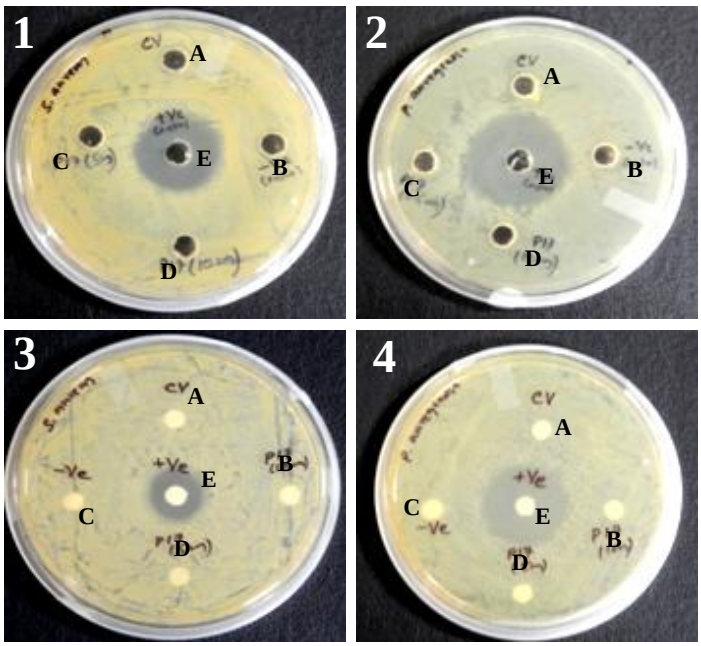

Figure 3.16: Bactericidal activity assay (well and disc diffusion method) of crude *N. kaouthia* venom (V) on Gram +ve *(S. aureus)* and Gram –ve bacteria *(P. aureginosa).* 1 & 2 (Gram +ve bacteria & Gram –ve bacteria) **A-** V(1µg), **B-** 20mM TrisCl, pH 7.4,**C-** V(5µg), **D-** V(10µg), **E-** Gentamicin (10µg). **3 & 4** (Gram +ve bacteria, & Gram –vebacteria), **A-** V(1µg), **B-** V(5µg),**C-** 20mM TrisCl pH 7.4, **D-** V(10µg), **E-** Gentamicin (10µg).

After 16rs of incubation the bacterial plates were checked and compared with the blank (20mM TrisCl, pH 7.4) and positive control (gentamicin, 10µg). No zones of clearance in the bacterial colonies were observed in both the methods (Figure 3.16). The crude *N. kaouthia* venom from Northeast India was not found to elicit any bacteriacidal activity when tested up to 10µg.

3.2.16 Myotoxicity studies

Myotoxic activities of snake venom are mainly associated with presence of snake venom metalloproteases (SVMPs), PLA_2 and hyaluronidases[71, 106, 258–260]. Although crude *N. kaouthia* venom was not found to exhibit bacteriacidal, hemorrhagic and significant edema inducing activity, the presence of PLA_2 might be involved in myotoxicity. Hence, release of tissue creatine kinase (CK) and lactate dehydrogenase (LDH) on treatment of crude *N. kaouthia* venom to experimental animals was determined in this study.

5µg and 15µg of crude *N. kaouthia* venom was injected (i.p.) on experimental mice (n=3) and after 3hrs mice were sacrificed and blood was collected by cardiac

puncture. CK and LDH were determined as per the manufacturer's protocol of AGAPPE kit and as explained in section 2.2.8. The values for CK and LDH level are given in Table 3.6.

Table 3.6: Myotoxicity of crude *N. kaouthia* venom

Mouse no.	parameters	OD at 340nm	Average	Unit (U/l)
1	CK	0.790	0.804	6.636 ±0.0006
2		0.821		
3		0.801		
1	LDH	1.637	1.637	26.3 ±0.016
2		1.637		
3		1.638		

*****Values shown are subtracted from blank

3.2.17 Cytotoxicity

We have tested crude *N. kaouthia* venom of Northeast India on HEK 293 and L6 rat skeletal muscle cell lines to examine its cytotoxic effect. Cytotoxicity of crude venom was determined colorimetrically using MTT method. Crude *N. kaouthia* venom was found to exhibit cytotoxicity to both the cell lines in a dose-dependant manner (Figure 3.17).

The crude *N. kaouthia* venom on HEK 293 cell lines was observed to exhibit cytotoxicity from 2.5ng/ml (~40% survival) and significantly resulted death of the cells from 50ng/ml (~21% survival) to 100ng/ml. At 100ng/ml, the percentage survival of the tested cells was ~1%. Incase of L6 rat skeletal muscle cell line, significant decreases in viable cells were observed at 2.5ng/ml (~55% survival) which further decreased significantly at 100ng/ml (~28% survival).

We also observed morphological changes of the cells treated with crude venom. The cells were observed under an inverted microscope at 10X objective (Figure 3.18). The cells were compared with the normal cells treated with 0.9% NaCl and cells treated with $AgNO_3$ (positive control) for cell detachment and structural changes (Figure 3.18).

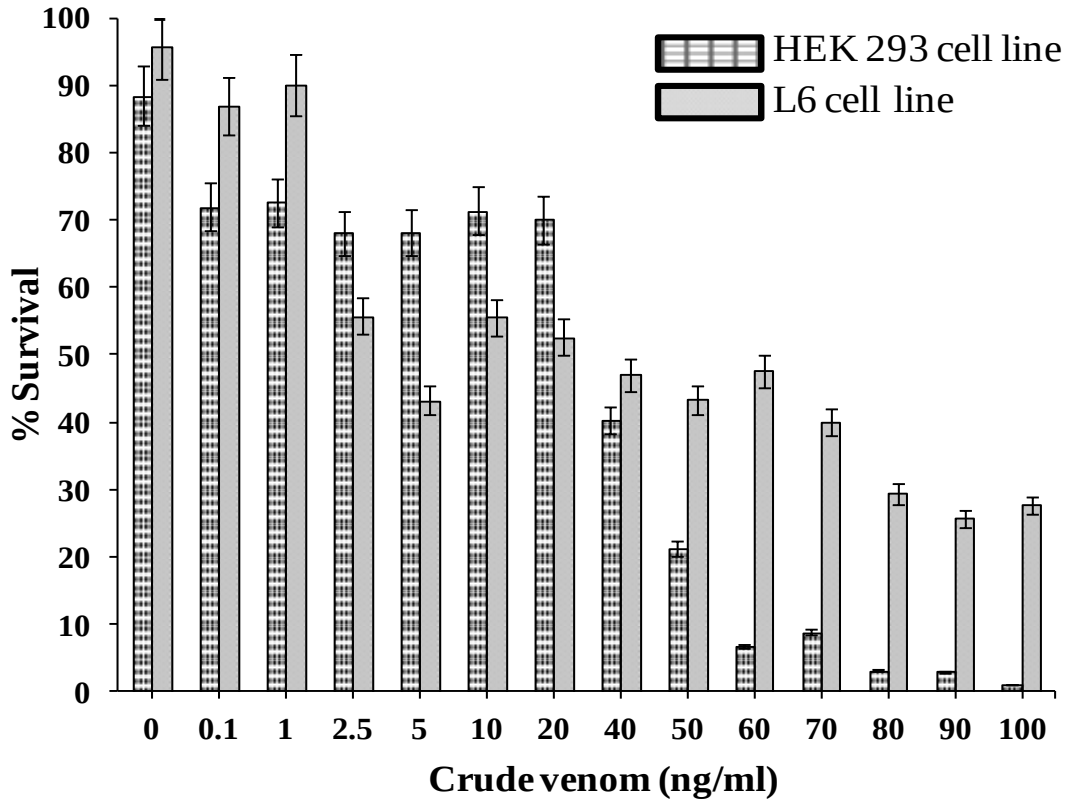

Figure 3.17: Effect of crude *N. kaouthia* venom on HEK293 and L6 rat skeletal muscle cell lines by MTT assay. Various concentrations of crude venom were assayed for cytotoxicity assay (0.1-100ng/ml); cells treated with only sterile 0.9% NaCl served as control. $p < 0.05$.

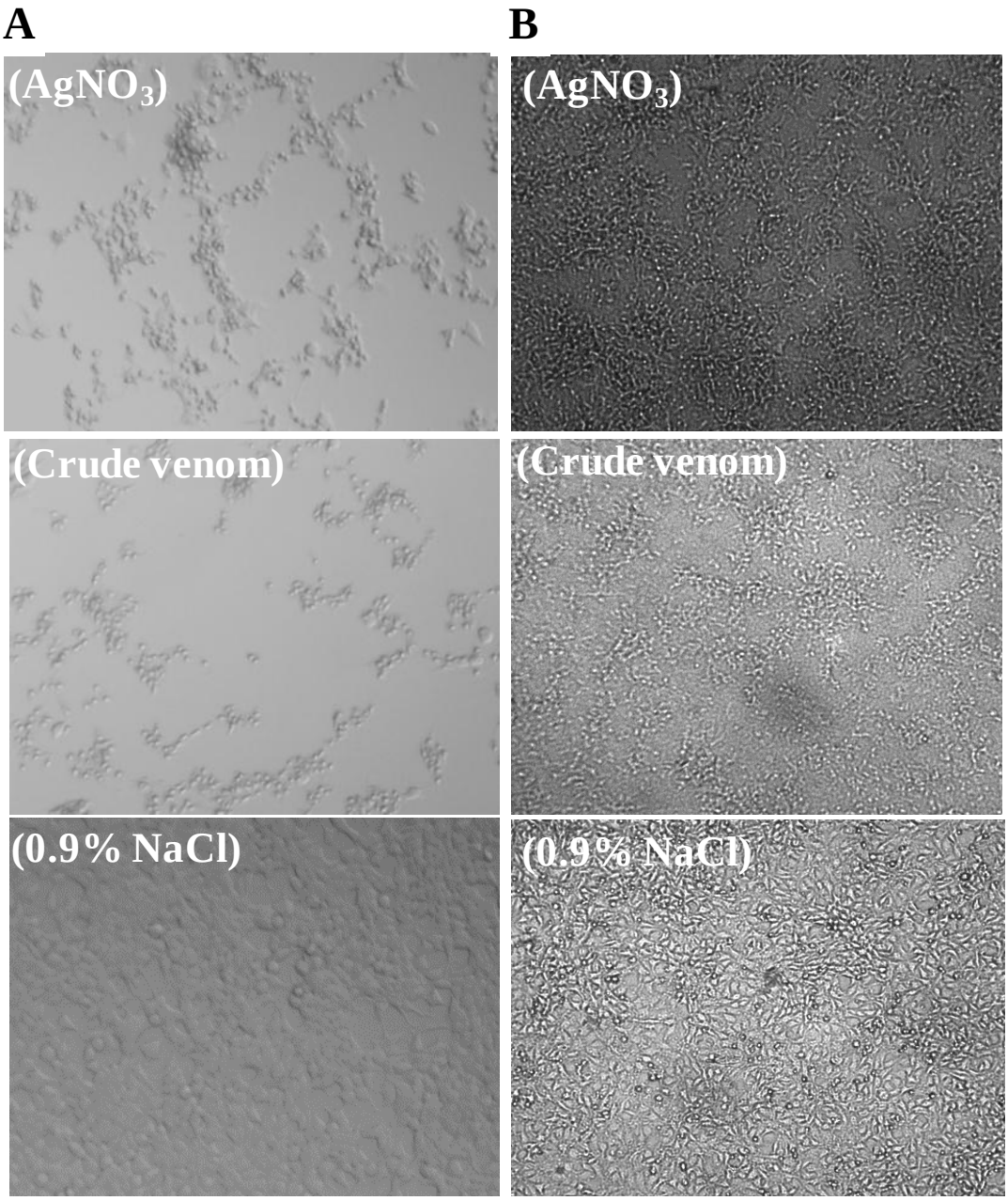

Figure 3.18: Representative cell images of HEK 293 (Panel A) and L6 rat skeletal muscle cell lines (Panel B): Exponentially growing cells were washed with PBS and treated with 300mM AgNO3, crude venom (100ng/ml) 24hrs at 37˚C with 5% CO_2 in a CO_2 incubator. Cells treated with 0.9% NaCl was considered as control. MTT assay was performed to observe the cytotoxic effect. The experiment was conducted in triplicates and results are mean ±SD.

3.2.18 Neurotoxicity

Neurotoxicity study of crude *N. kaouthia* venom was carried out using isolated sciatic nerve from common Asian toads as an experimental model. In the present experiments we tested the effect of crude venom on compound action potential (CAP) of sciatic nerve. The sciatic nerve of common Asian toad was dissected out from lumber plexus to the knee joint and prepared as explained in section 2.2.18.1 and placed on the nerve chamber (Figure 3.19). Further the nerve was stimulated with amplitude of 1V for duration of 0.2ms..

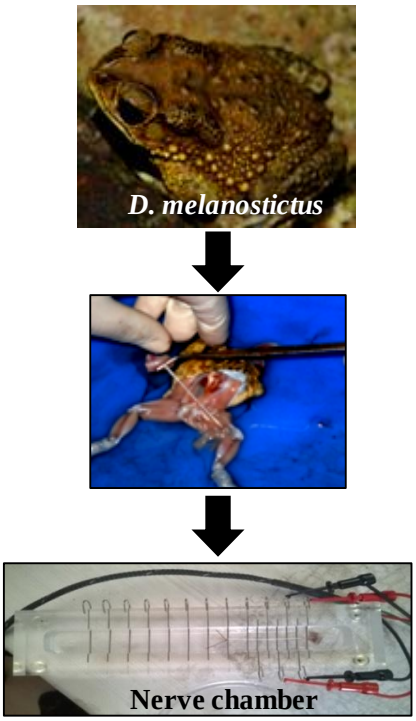

Figure 3.19: Dissection and isolation of sciatic nerve from common Asian toad.

The CAP of the dissected nerve was measured in a nerve chamber and was found to be 9.9mV at proximal electrode and 9.8mV at distal electrode (Figure 3.20A). Further, the sciatic nerves were treated dose-dependently and CAP was checked and determined. CAP of isolated sciatic nerve was found to be decreased at increasing concentration of crude venom. At 1319.4nmol/L concentration of crude venom the CAP of the proximal electrode was recorded up to ~3.01mV and distal electrode potential was found to be less than 1mV (Figure 3.20G). The values were compared to the control nerve and it was found that the crude venom was significantly affecting the CAP.

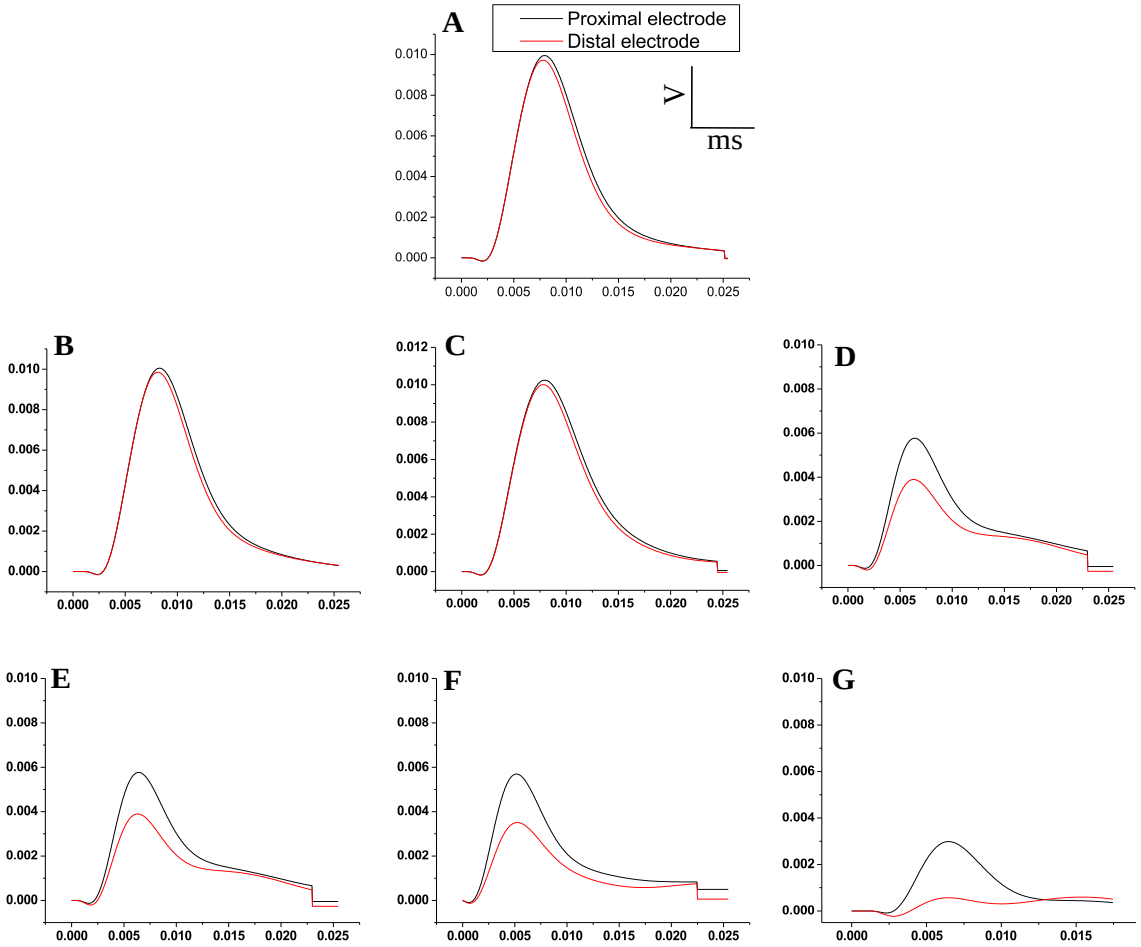

Figure 3.20: Effect of crude *N. kaouthia* venom on CAP of toad sciatic nerve. A: Nerve only treated with Ringer's solution as control. **B-G:** Dose-dependent treatment of sciatic nerve by crude *N. kaouthia* venom at 0.0132nmol/L to 1319.4nmol/L of concentration and determination of CAP. The values are ±SD of 3 independent experiments, $p < 0.05$.

Reduction in amplitude of CAP by crude *N. kaouthia* venom treatment on isolated sciatic nerve suggests its neurotoxic effect.

3.2.19 Scanning electron microscopy (SEM)

In the present experiment various molar concentrations of crude *N. kaouthia* venom (13.194nmol/L, 131.194nmol/L and 1319.4nmol/L) sciatic nerve and later fixed and observed under SEM for any morphological changes as explained in section 2.2.18. Sciatic nerve is a collection of other smaller nerves. Therefore, the thickness of some nerves in the control nerve and treated nerves were measured and compared for any changes. It was observed that there were changes in the morphology of the sciatic

nerve and thickness of the sciatic nerve were also reduced which might be due to crude venom activity. At 1319.4nmol/L of crude venom concentration the thickness of the sciatic nerves were measured to an average of 0.91µm (Figure 3.21D), whereas the nerve treated only with Ringer's solution had an average thickness of nerves to 1.3µm (Figure 3.21A). The thickness of nerves at various crude venom treatments are given in table 3.7.

Table 3.7: Thickness of sciatic nerves at various concentrations of crude *N. kaouthia* venom treatment

Sl no	Conc. (mg/ml)	Thickness of nerve (in µm)	Avg(in µm)
1	Control	1.46	1.3
2		1.08	
3		1.25	
4		1.28	
5	0.1	1.28	1.22
6		1.12	
7		1.40	
8		1.08	
9	1	0.92	1.026
10		0.95	
11		1.02	
12		1.12	
13		1.12	
14	10	0.81	0.91
15		0.89	
16		0.94	
17		1.00	

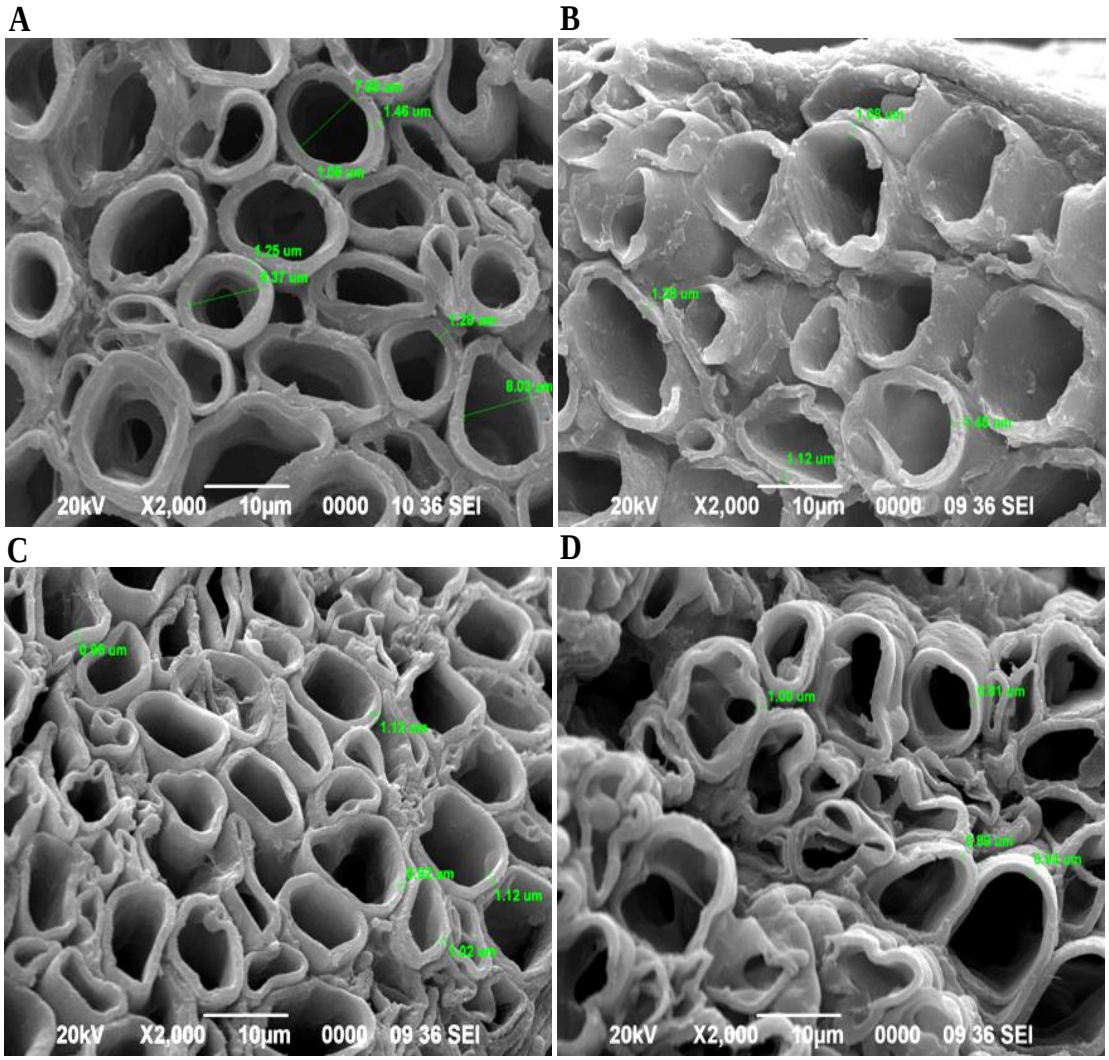

Figure 3.21: SEM images of sciatic nerve (cross sections) after crude *N. kaouthia* venom treatment. A: Untreated nerve. Nerve treated with crude venom, **B:** 0.1mg/ml, **C:** 1mg/ml, **D:** 10mg/ml.

3.2.20 Neutralization studies

Various amounts of crude *N. kaouthia* venom were incubated with polyvalent antivenom in various ratios (venom: antivenom) (µg:µg) and checked. Some of the effects of polyvalent antivenom on biochemical and biological properties of *N. kaouthia* venom are shown in Table 3.8.

Table 3.8: *In-vitro* neutralization of whole venom activity by commercially available polyvalent antivenom

Activity	**% inhibition by ASV* (polyvalent) (venom:asv (µg))**		
	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

***ASV- Anti Snake venom**

It was found that, at 1:1 ratio, the polyvalent antivenom was not able to neutralize the PLA_2 activity of the crude venom but at 1:100 ratios, 97.38 ± 4.8% inhibition was observed. Similarly, the indirect hemolytic activity of the crude venom was also inhibited as observed in case of PLA_2 activity. When the concentration of the polyvalent antivenom was increased 100 fold, indirect hemolytic activity was completely neutralized. Neutralization of anticoagulant property of crude *N. kaouthia* venom was observed at 1:1 ratio (49.34% inhibition) . However, 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, the degradation of fibrinogen α band by crude *N. kaouthia* venom was observed to be neutralized at 1:1 ratio (Figure 3.22).

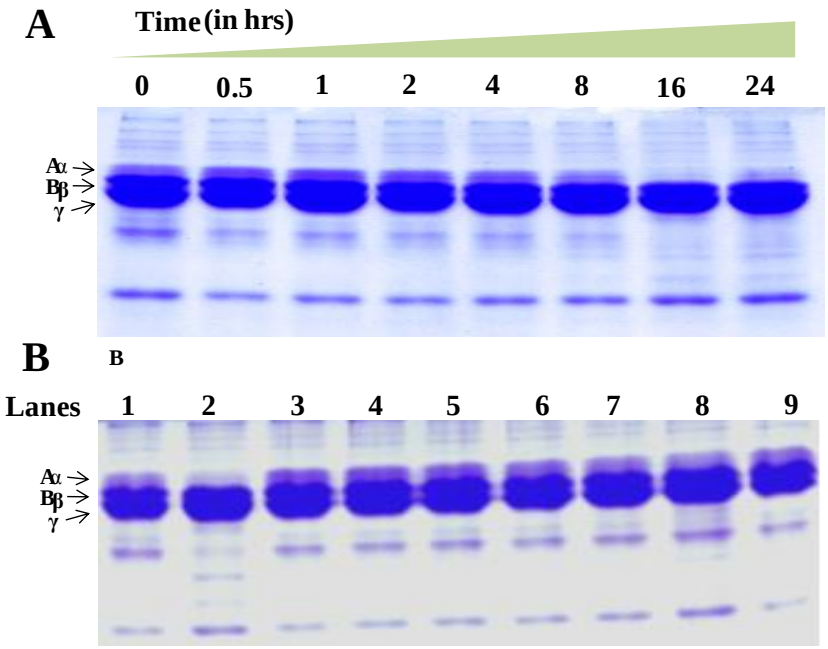

Figure 3.22: Neutralization of fibrinogenolytic activity of crude *N. kaouthia* venom by commercially available polyvalent antivenom. A. SDS-PAGE of bovine fibrinogen (reduced) after incubation with 10µg crude *N. kaouthia* venom at various time intervals. **B.** Inhibition of fibrinogenolytic activity of *N. 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 24hrs 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 2hrs; **Lane 6.** After 4hrs; **Lane 7.** After 8hrs; **Lane 8.** After 16hrs; and **Lane 9.** After 24hrs.

3.2.21 Western blotting

The ability of antivenom to bind specific snake venom toxins can be best studied by Western immunoblotting. The Coommassie staining of the SDS-PAGE of reduced crude venom indicated the presence of venom proteins in the molecular mass range from >10kDa to ~80kDa. Majority of the crude *N. kaouthia* venom protein appears to be at 8 to 12kDa (Figure 3.23A).

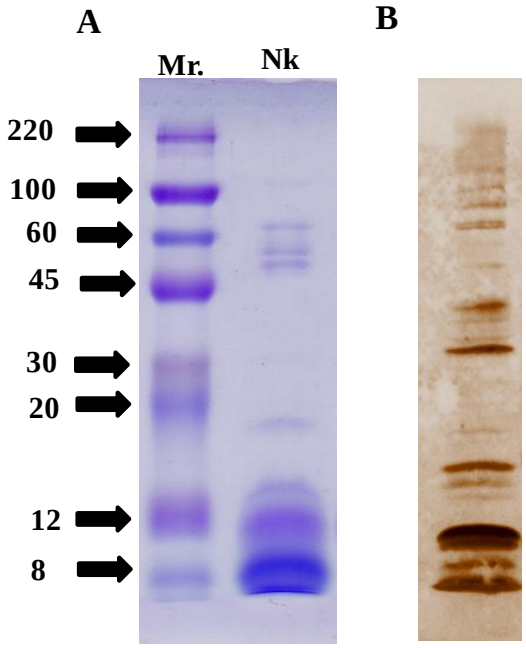

Figure 3.23: Immunoreactivity of snake venom toxins to commercially available polyvalent antivenom**. A)** SDS PAGE of crude *N. kaouthia* (Nk) venom. **B)** Western blot of crude *N. kaouthia* venom using commercially available polyvalent antivenom. Various bands on the blot suggest the detected antigens (snake venom proteins) by the secondary antibodies. Mr.- Molecular weight markers, in Dalton.

The commercially available polyvalent antivenom appeared to detect most of the proteins of crude venom (Figure 3.23B). Immunoreactivity was particularly observed with toxins with a molecular mass range of 2.5 to 7kDa. Also, reactivity to proteins above ~14kDa to 27kDa was too observed. There were toxins which are detected by the polyvalent antivenom and were not visualized by coommassie staining are found to be at ~40kDa which might be due to less sensitive antibodies.

3.3 Discussion

The patho-physiological effect of post-snakebite envenomation varies greatly among various species [261]. Venom variation has been reported to occur at several levels, including inter-family, inter-genus, inter-species, inter-subspecies and intra-species variation [262]. Moreover, variation in venom composition between species and within a species may be influenced by the geographical origin, habitat of the snake, seasonal variation, diet, age and sexual dimorphism [262]. Hence, the variation in clinical manifestations within species can be due to differences in their venom proteins and

biological activities. These envenomation effects and pharmacological effects need specific consideration in terms of treatment. Therefore, understanding the biochemical and biological properties of snake venom from a particular geographic location is important.

The lethality of the crude *N. kaouthia* venom was tested in experimental mice. The LD_{50} was found to be 0.148mg/kg of mice body weight. The reported LD_{50} for *N. kaouthia* of Thailand and Kolkata origin were 0.23mg/kg and0.7mg/kg, respectively [241, 263]. Though the route of injection was different (Kolkata origin venom given via tail vein injection) in these experiments, the lethal dose of Northeast origin venom was less than that of the other geographical locations suggesting it might be more lethal. Although studies on lethality are important information with regard to venoms, certain limitations remain, for example, the health status of the experimental animals, handling of animals while injecting venom and also strains of animal used for the studies [264]. In snake venom there are major proteins families present, eg. PLA_2, which are responsible for the final contribution to lethality but when whole crude venom is used the contribution of the major toxins toxicity level and contribution cannot be calculated [265]. Hence, it is reasonable to have a venom of lesser LD_{50} value which might show its lethal effects *in-vivo* but can be fast in exhibiting its toxicity in an *in-vitro* preparation [266].

The crude *N. kaouthia* venom of Northeast India did not induce hemorrhage in experimental mice. When compared with the Kolkata venom, the results were similar. Hemorrhagic activity is mainly associated with the presence of metalloproteases, which are abundantly found in viper venom[101, 258]. Also, in our experiment the positive control (*Echis carinatus* venom) with 3µg produced a significant hemorrhagic lesion more than 1cm, whereas 15µg of crude *N. kaouthia* venom did not lead to any hemorrhage. Edema-inducing activity of crude *N. kaouthia* venom was observed and at 10µg crude venom the edema ratio was calculate to 120.3%. Edema-inducing activity of crude *N. kaouthia* venom, associated with PLA_2 enzymes, was significant in experimental animals. Hence this venom might not induce inflammation and tissue damage at the site of bite. Other biological activities such as direct hemolytic activity of the crude venom were negligible as the venom showed only 1.4% hemolysis at 100µg. However, crude *N.*

kaouthia venom of Kolkata origin exhibited hemolysis up to 39% at 100µg[241]. Low molecular weight proteins such as cardiotoxins/cytotoxins are primarily responsible for membrane damaging activity [174, 267]. Indirect hemolytic activity of snake venom in presence of egg yolk as a supplement is due to the presence of 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[268].

Elapid venom has been extensively reported to contain PLA_2 as one of the major constituents. The presence of various PLA_2 and its isoforms also have been reported by various researchers [88, 269, 270]. PLA_2 activity by crude *N. kaouthia* venom using diheptanoyl-Thiol-PC as substrate was determined to 7.9±0.24µmol/min/mg. The enzymatic activity of PLA_2in crude *N. kaouthia* venom was also confirmed using egg yolk as a substrate and at 1µg of crude venom PLA_2 unit activity was found to be 64.9µg/min/µl. This clearly suggests the presence of enzymatically active PLA_2 in the crude venom. Due to their property of hydrolyzing phospholipid membranes, PLA_2 enzymes are involved in multiple toxicity in the prey or victim physiology such as neurotoxicity, prolongation of coagulation time and membrane damaging activity [74, 88]. Hence, myotoxicity, neurotoxicity and prolongation of coagulation time can be correlated to the presence of PLA_2 enzymes in crude *N. kaouthia* venom of Northeast India.

The observed differences in the biochemical and biological activities in the venoms of Northeast Indian origin *N. kaouthia* from Jamugurihat of Tezpur, Assam might be due to feeding habits and geographical locations. Both venoms were collected during summers; although in the present study, exact ages of the captured snakes for venom extraction were not known, none of the snakes were either neonate or juvenile. Detailed analysis of *N. kaouthia* venoms from different locations of India need to be carried out to decipher the differences in the venom composition which will help us to understand the variation in biological activities.

Snake venom has been reported to affect the haemostasis process of victim or prey by interacting with various proteins and factors in coagulation process. Due to the presence of high percentage of strong and weak enzymatic proteins such as PLA_2 enzymes, elapid venom is anticoagulant in nature[87, 88]. However, effect of non-

enzymatic proteins and on blood coagulation cascade are also well reported. Cardiotoxins from *Naja nigricollis crawshawii*, hemextin A and hemextin AB complex from *Hemachatus haemachatus* venom, KT-6.9 from *N. kaouthia* were found to be anticoagulant in nature[141, 150, 177]. Crude *N. kaouthia* venom of Northeast India significantly delayed the recalcificaion time, PT and APTT of plasma under *in-vitro* conditions. 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[271]. 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[88, 272]. The Sonoclot and rotem analysis also demonstrated that the *N. kaouthia* venom is anticoagulant in nature. The whole citrated blood analysis by Sonoclot clearly indicated the depletion of fibrinogen 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 [221, 273]. 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 *N. 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.

Cytotoxins or cardiotoxins exhibit wide variety of pharmacological activities on the prey or the victims physiology such as hemolysis, muscle depolarization, muscle fusion, inhibition of protein kinase C and muscle contraction [274, 275]. They are also reported to have cytotoxic activities to certain tumor cells [276]. Earlier, crude *N. kaouthia* venom from eastern India has been reported with anticancer and cytotoxic effect [277]. The crude venom of Northeast Indian *N. kaouthia* was found to exhibit lesser percentage of direct hemolytic activity on washed RBC cells, however

significant inhibitory activity of crude venom was observed on HEK293 and L6 rat skeletal muscle cell lines. They are also reported to have anti-cancerous activities to certain tumor cells [276]. Even though the crude venom did not affect washed RBC_s, presence of various cytotoxins/cardiotoxins cannot be denied. Snake venom cytotoxin interact with the phosphotidylserine group of lipid and bind to membrane; eg CT4 of *N. kaouthia* was studied *in-silico* for its interaction [174]. Kaouthiotoxin (KTX) in *N. kaouthia* showed synergistic cytotoxicity with PLA_2 by non-covalent interaction [254].

Metalloproteinases in snake venom are responsible for pathogenesis including local tissue damage[101]. Apart from exhibiting hemorrhagic property, venom metalloproteinases induces myonecrosis, skeletal muscle damage and microvessel disruption, leading to fibrosis and permanent tissue loss post envenomation [278]. Metalloproteinases are abundant in viper venom and are also reported from elapid venom, such as kaouthiagin and metalloproteinase Nk from *N. kaouthia* [273, 279]. Crude *N. kaouthia* venom from northeast India was found to be myotoxic when tested on the experimental animals. Also, SDS-PAGE of crude *N. kaouthia* venom under reduced condition shows presence of distinct bands at 40 to 60 kDa which might be PIII metalloproteinases responsible for various pharmacological activities such as myotoxicity and cytotoxicity.

Bacteriocidal activity is also related to the membrane-damaging activity. Toxins from various snakes have been reported with bacteriacidal activity, eg. Toxin γ from *Naja nigricollis* venom showed inhibitory activity on the growth of *Staphylococcus aureus* (Gram-positive bacteria) and *Escherichia coli* (Gram-negative bacteria) [280]. Cardiotoxin 3 (CTX3) from *Naja naja atra* showed inhibitory activity for the growth of *Staphylococcus aureus* (Gram-positive bacteria) relative to that of *Escherichia coli* (Gram-negative bacteria)[281], a homodimeric secretory PLA_2 from *Bungarus fasciatus* display potent gram-positive bactericidal activity[282]. However, the crude *N. kaouthia* venom did not affect the growth of either gram positive or gram negative bacteria. Zone of clearance were absent as observed on the microbial plates. This might be due to low percentage of the respective proteins Behavioral studies with experimental animals shows the neurotoxic nature of the crude venom and difficulties in breathing and paralysis are the clear signs of acute neurotoxicity [283].

Elapid venom is known for its neurotoxic effects. PLA_2 and 3FTxs are the two groups of toxins which are responsible for neurotoxicity in prey or the victim[140, 143, 265, 284]. Further tests on CAP of isolated sciatic nerve confirm the neurotoxic behavior of the crude venom. The crude *N. kaouthia* venom was found to decrease the CAP of sciatic nerve in a dose-dependant manner at lower concentrations. The decrease in amplitude of action potential is directly related to the effect of crude venom at various ion channels responsible for a stimulus. Although report of α-cobrotoxin from cobra venom is well documented and is referred as the principal neurotoxin (affects the α subunit of nAChR) responsible for observed neurotoxicity [161], the effects of crude northeast Indian *N. kaouthia* venom needs more study to draw a conclusion. Neurotoxicity by cobra venom might not be restricted to blocking of only nAChRs but also various pumps, channels on a neuron for a successful stimulus. Therefore, apart from binding to nAChRs, the crude venom might be involved in blocking any of these channels responsible for the flow of action potential. Use of crude venom on isolated sciatic nerve may have other delirious effect such as damage to the morphology of the nerve, degradation of myelin sheath, other integral proteins etc. Therefore, morphology of treated nerve was observed under scanning electron microscope. It was found that the neurons present in the sciatic nerve had deformed morphology. Also, the thicknesses of nerves were affected. This might be due to the presence of toxins like PLA_2 which might act on the myelin sheath which is made of phospholipids. The morphology of the venom treated nerve was also degraded which will in turn lead to delayed stimulus propagation and neurotoxicity. This effect of crude venom can be related to the behavioral changes in experimental mice post venom injection. Paralysis of lower limbs might be due to blockade of AChRs [266].

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 [285, 286]. 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 *N. kaouthia* venom proteins. Present study documents that the polyvalent antivenom can neutralize some of tested biochemical and biological activities of *N. kaouthia* venom under *in-vitro* condition. The immune blotting experiment with commercially available polyvalent antivenom shows that the antivenom could detect most of the crude venom components; however a few proteins were not detected as compared to the SDS PAGE, which might be due to venom variation.