Chapter 2

Materials and Methods

2.1 Materials

$sPLA_2$ assay kit was purchased 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) for coagulation analysis. Uniplastin and Liquicelin for PT and APTT were purchased from Tulips diagnostics (India). Bovine serum fibrinogen, Tween 20 for TBST (Tris buffer saline Tween 20) and BCIP/NBT (chromogenic substrate), Ringers solution tablets (calcium chloride, 0.12 g/L, potassium chloride, 0.105 g/L, sodium bicarbonate, 0.05 g/L, sodium chloride, 2.25 g/L) of ¼ strength), 4-vinylpyridine, ammonium biocarbonate, purified bovine thrombin, bupivacaine hydrochloride (sodium channel blocker) and quinine hydrochloride dihydrate (potassium channel blocker) and barbital were purchased from Sigma Aldrich (USA). High range standard protein ladder (10-250 kDa) was purchased from Thermo Scientific (USA). Polyvalent antivenom manufactured by Bharat Serums and Vaccines Limited (Batch no. AO5315011) (India) was purchased from local vendor. Nitrocellulose membrane was purchased from Whatman (USA). HEK 293 and L6 rat skeletal muscle cell line were procured from National Centre for Cell Sciences (Pune, India). DTT was purchased from Gold Biotechnology (USA) and Protease max from Promega (USA). All protein sequencing reagents were purchased from Applied Biosystems and guanidinium hydrochloride was purchased from Alfa aesar (USA).

C_{18} Reverse phase HPLC columns were purchased from Waters (USA) and Phenomenex (USA). Acetonitrile (HPLC grade) and tri-fluro acetic acid (HPLC grade) were purchased from Merck (Germany). Syringe filters (0.2µ) were purchased from Genetix, Biotech Asia Pvt. Ltd. (India) and Whatman, GE healthcare (UK). Disposable syringes (1 and 2ml) for filtration of samples and venom injection into experimental animals were purchased from Romsons (Romsons junior, India). Silver staining kit was purchased from Thermo scientific (USA), Ampicillin for bacteriacidal activity assay was purchased from Merck, the bacterial strains used

were Gram positive bacteria (*Staphylococcus aureus)* and gram negative bacteria *(Pseudomonous aureginosa).*

All other chemicals used for preparation of buffers and solutions were of analytical grade and procured either from Merck (Germany) or Sigma-Aldrich, (USA).

2.1.1 Collection of snake venom

N. kaouthia was captured from its natural habitat (Jamugurihat, Sonitpur, Assam, India) and its venom was extracted. For extraction of the venom, the snake was allowed to bite into a sterile beaker covered with para-film and venom released under natural condition was collected. Squeezing and pressing on neck was avoided and venom was extracted according to the method followed by Mackessy 1988 [222]. After extraction, the snakes were released into its habitat. The permission for extraction of snake venom from Assam 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) (appendix-V).

2.1.2 Preparation and storage of venom samples

The extracted crude venom from *N. kaouthia* in the sterile beaker was carefully transferred to sterile eppendorf tube by pipetting. The sterile milking beaker was rinsed with 200µl of 20mM Tris HCl, pH 7.4 to reduce loss of venom sample and transferred to a separate eppendorf tube for immediate desiccation. Further the venom collected was freeze dried using a freeze dryer (Hahntech, India) and stored at -20◦C until further use.

2.1.3 Animals

Swiss albino mice of both sexes weighing 40 ± 3gm were used for animal studies and were obtained from central animal facility, University of Mysore. All animal experiments were performed in Department of Studies in Biochemistry, University of Mysore, Mysore, Karnataka, India. The animals were housed in well ventilated cages maintaining 12hrs light and dark cycles. Health condition of the animals was inspected in daily basis by animal care taker (trained personal from the animal

centre) and depending on the physiological status, the animals with proper health condition were chosen for all the experiments. Food and drinking water for the animals were obtained from the animal facility centre. The animal housed cages were cleaned and replaced with fresh husks every after 2days of housing. All the experiments were performed as per the Ethical Guidelines of Animal Ethical Committee of University of Mysore, Mysore, India (Animal ethical committee approval no, UOM/IAEC/25/2011 (enclosed in appendix-V).

Common Asian toads *(Duttaphrynus melanostictus)* were used for neurotoxicity studies. The species is under least concern (LC) category of IUCN conservation status. Toads of either sex were captured and collected from nearest cultivation areas, ponds and other water bodies. Some toads were also captured from under street lamps at evening. The expeditions for capturing and collecting toads were seasonal and were done in March to May. Toads collected were immediately weighed and only those weighing 30-35 grams were kept and rest were released in its natural habitat. Weighing of toads was followed by finding an uniform size between the captured toads. Toads with uniform size and approximately similar weight were chosen for further experiments. The experiments were performed following the ethical guidelines of Tezpur University Animal Ethical Committee (TUAEC) (Animal ethical committee approval no, DoRD-Pro/TUAEC/10/56/15/Res-02 (enclosed in appendix-V).

2.2 Methods

2.2.1 Determination of protein content

Protein concentration of the crude *N. kaouthia* venom/purified toxin (Nk-3FTx) was determined according to Lowry's method using BSA (Bovine serum albumin) as a standard [223]. The principle of Lowry's method is based on the reactivity of the peptide nitrogen's with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin Ciocalteu phosphomolybdic phosphotungstic acid (FC reagent) to heteropoly-molybdenum blue by the copper-catalyzed oxidation of aromatic acids. The method is also sensitive to lower concentration ranging from 0.10 to 2mg of protein per ml. For preparation of the standard curve, various concentrations of BSA (0.2 to 10mg/ml) dissolved in H_2O were mixed with 250µl of

alkaline $CuSO_4$ and incubated for 10mins. Further 25µl of FC reagent was added and incubated at room temperature for another 30mins. The absorbance of the reaction mixture was measured at 660nm using a MultiSkan GO multi plate reader (Thermo scientific, USA). Samples tested were in triplicates and the optical density versus BSA concentration was plotted on excel sheet to prepare the standard curve. 5µl of the crude venom/purified protein was taken to determine the concentration. Samples were tested in triplicates and the mean ±SD was considered for determining the concentration using the BSA standard curve.

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

SDS-PAGE was performed according to the method described by Laemmli et al. [224]. 12.5% resolving gel containing 2.5% glycerol was prepared using commercially available acrylamide/bisacrylamide. Both reduced and non-reduced samples were analyzed in SDS-PAGE. For reduction of crude *N. kaouthia* venom/purified protein (Nk-3FTx), samples were incubated at 100^{o}C for 3min with 5% loading dye containing β-mercaptoethanol. For analyzing non-reduced samples, it was incubated with loading dye devoid of β-mercaptoethanol. 20µg (in 20µl) of reduced/non-reduced crude venom/purified protein were loaded into different wells of stacking gel. Electrophoresis was carried out at a current of 15mA in the stacking gel and was increased to 20mA as the dye reached the separating gel. The current was maintained at 20mA until the dye front (bromophenol blue) reached the bottom of the gel. After electrophoresis, the gel was removed from the plate and stained with 0.25% coommassie brilliant blue R-250 in methanol: acetic acid: water (40:10:50 v/v/v) for 4-5 hrs under constant gentle shaking. Destaining was peroformed using methanol: acetic acid: water (40:10:50 v/v/v) until the protein bands were visible. The destained gels were observed using G-Box Image analyzer (Syngene, UK) and the molecular mass of the protein bands under reduced and non-reduced condition was analyzed by comparing the migration of the standard protein marker (10-250kDa, high range).

2.2.3 Phospholipase A_2 (PLA_2) activity

2.2.3.1 Colorimetric method

PLA_2 activity was assayed by colorimetric method using $sPLA_2$ assay kit (Cayman Chemical Company, USA) as per manufacturer's instructions. The Cayman Chemical secretory PLA_2 ($sPLA_2$) assay kit uses 1,2-dithio analog of diheptanoyl phosphatidylcholine which serves as a substrate for most PLA_2s from bee and cobra venoms, pancreatic, etc. but not for PLA_2s found in cytosol [225, 226]. PLA_2 hydrolyses the thio-ester bond at the *sn*-2 position and the free thiols released is detected using DTNB (5, 5-dithio-*bis*-(2-nitrobenzoic acid)). Briefly, in a 96-well microtitre plate, 10 µl of venom/purified protein (0.001, 0.01 and 0.1 µg/µl) were added followed by 10 µl of DTNB (5, 50- dithio-bis-(2-nitrobenzoic acid)) and 5 µl assay buffer. The reaction was initiated by adding 200 µl of substrate solution. After gentle shaking the optical density was measured every minute at 405 nm using a, MultiSkan GO multi plate reader (Thermo scientific, USA). 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 phosphatidylcholine hydrolyzed per min per milligram of enzyme.

2.2.3.2 Turbidometric method

Turbidometric method of PLA_2 activity assay was performed as described by Joubert and Talijaard [227] and as modified by Doley and Mukherjee [88]. One egg yolk was suspended in 250ml of 0.9% NaCl containing 0.02% (w/v) sodium azide. Sodium azide was added to avoid any fungal contamination. The mixture was stored at 4^oC and used within 3 days post preparation. For the experiment, 1ml of the egg yolk suspension was mixed with 20mM Tris-Cl, pH 7.4 until the OD at 740nm was 1. Various amounts of crude venom/purified protein (Nk-3FTx) (0.01, 0.1, 0.2, 0.4, 0.6, 0.8 and 1µg) in a final volume of 60µl were mixed with 170µl of reaction mixture on a microtitre plate. The decrease in absorbance at 740nm was measured in a MultiSkan GO multi plate reader (Thermo scientific, USA) for 10mins at an interval of 30s. Reaction mixture with 20mM Tris-Cl, pH 7.4 was considered

as blank. The experiment was conducted in triplicates and values are ±SD of three independent experiments. One unit of PLA_2 activity is defined as a decrease in 0.01 absorbance in 10min at 740nm [88, 227].

2.2.4 LD_{50} determination

LD_{50} of crude *N. kaouthia* venom of Northeast India was determined according to the method of Meier & Theakston [228]. The method was developed for use in small scale experiments conducted in biology laboratories. This method significantly reduced the number of animal used and also reduced animal sufferings. As the method involves lesser number of animal uses, it was found to be ethical, scientific and economic and if satisfactory results obtained it was suggested to avoid the classical method of LD_{50} determination. In the present experiment various amounts of crude venom dissolved in 150µl of PBS and injected intra-peritoneally (i.p.) to the experimental mice. Group of six male Swiss albino mice were used for each dose and were observed for 24hrs. Control animals were injected with similar volume of PBS.

2.2.5 Behavioral study of the experimental animals

The experimental animals used during lethal dose determination of crude *N. kaouthia* venom/ purified toxin (Nk-3FTx) were also observed for behavioral changes such as paralysis of lower limbs, difficulties in breathing and others post injection. A constant observation of the experimental animals was made followed by noting down the behavior change when observed.

2.2.6 Edema inducing activity assay

Determination of edema-inducing activity of crude *N. kaouthia*/ purified toxin (Nk-3FTx) venom was performed according to the method described by Yamakawa et al., [229] and as modified by Vishwanath and co-workers [230]. 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 which served as vehicle After 2.5hrs the mice were sacrificed with an overdose of anesthesia (barbitone 30mg/kg, i.p.). However, in case of Nk-3FTx, only 15µg of the toxin was used to determine edema on the experimental animal. The legs

were removed at ankle joints and weighed. Minimum edema dose (MED) is defined as the amount of protein required to cause an edema ratio of 120% [231]. The experiments were done in triplicates and results are expressed as the mean ±SD. Edema ratio was determined using:

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

2.2.7 Hemorrhagic activity

Hemorrhagic activity assay was done as described by Kondo and co-workers [232]. Three male albino mice were injected via intra-dermal (i.d.) route with 15µg crude *N. kaouthia* venom/ purified toxin (Nk-3FTx) in 30µl of PBS. The control animals were injected with saline. We used 3µg of *Echis carinatus* venom in 30µl of PBS which served as positive control. The mice were kept under observation for 3hrs and sacrificed by injecting high dose of barbitone (30mg/kg), intra-peritoneal (i.p.). The dorsal surfaces of the skin were removed and inner surface was checked for any hemorrhagic spot. The minimum hemorrhagic dose (MHD) is defined as the concentration of venom required to induce a hemorrhagic spot of 1cm diameter at the injection site.

2.2.8 Myotoxicity studies

Release of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in the blood were measured spectrophotometrically using AGAPPE kit (AGAPPE diagnostics, Switzerland). Groups of six male albino mice were injected (i.m.) with 15µg crude *N. kaouthia* venom/ purified toxin (Nk-3FTx) dissolved in 40µl of PBS, and controlsreceived 40µl of PBS. After 3hrs mice were anesthetized and 0.5ml of blood samples were drawn by cardiac puncture [233]. The serum obtained by centrifugation was diluted with PBS at 1:20 ratio. The CK and LDH activity were measured in 10µl of plasma according to the manufacturer's protocol of AGAPEE kit (AGAPPE diagnostics, Switzerland) and were expressed in Units/liter (U/l).

2.2.8.1 Lactate dehydrogenase assay (LDH)

Determination of lactate dehydrogenase in serum was done according to the method of McQueen [234]. The reduction of pyruvate with NADH to form NAD catalyzed by LDH was monitored. Rate of oxidation of NADH to NAD is measured as a decrease in absorbance. Decrease in absorbance at 340nm is proportional to LDH activity of the sample.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-Latate} + \text{NAD}^+$$

The experiment was conducted as instructed in the manufacturer's protocol of AGAPPE kit (AGAPPE diagnostics, Switzerland). Blood was collected from the experimental mice by cardiac puncture and allowed to clot and serum was obtained. 0.01ml of serum was collected and added to 1ml of working reagent prepared by mixing 4 volume of reagent 1 (80mmpl/L Tris buffer, pH 7.4, 1.6mmol/L Pyruvate, 200mmol/L Sodium Chloride) with 1 volume of reagent 2 (240µmol/L NADH) provided in the kit. The mixture is incubated for 1 minute at 37^{o}C and change in absorbance was measured per minute for 3 mins at 340 nm. The average value out of the three separate experiments was considered and results shown are ±SD. LDH activity in the sample was calculated by:

$$\textbf{LDH activity (U/L)} = (\Delta \text{ OD / min}) \times 16030$$

Where, 16030 is the extinction coefficient as determined by AGAPPE for the reaction.

2.2.8.2 Creatine Kinase activity assay (CK)

CK activity assay of crude *N. kaouthia* venom was performed according to the method of Witt and Trendelenburg [235]. CK catalyses the conversion of creatine and utilizes adenosine triphosphate (ATP) to create phosphocreatine (PCr) and adenosine diphosphate (ADP) [236]. The procedure involves measurement of CK activity in the presence of an antibody to CK-M monomer (CK-M: Creatine kinase-muscle). This antibody completely inhibits the activity of iso-enzymes CK-MM (Creatine kinase

expressed in skeletal muscle, 98% and in heart muscle, 70%) [237] and half of the activity of CK-MB (Creatine kinase expressed in heart muscle 25-30%) [238], while not affecting the B subunit activity of the CK-MB and CK-BB (Creatine kinase expressed in brain and smooth muscle, including vascular and uterine tissue) [239, 240]. In the present experiment, CK method is used to quantitatively determine CK-B activity. The CKMB activity is obtained by multiplying the CK-B activity.

The experiment was conducted according to the manufacturer's protocol of AGAPPE kit (AGAPPE diagnostics, Switzerland). Serum was obtained as explained in section 2.2.8. In brief, 0.04ml of serum was added to 1ml of working reagent prepared by mixing 4 volume of reagent 1 (125mmol/L of Imidazole, pH6.7, 25mmol/L of D-Glucose, 25mmol/L of N-Acetyl-L-cysteine, 12.5 mmol/L of magnesium acetate, 2.55mmol/L of NADP, 2.025mmol/L of EDTA, hexokinase >6800 U/L) with 1 volume of reagent 2 (250 5mmol/L of Creatinine phosphate, 15.2 5mmol/L of ADP, 25mmol/L of AMP, 103mmol/L of diadenosinepentaphosphte, G-6-PDH >8800U/L) provided in the kit. The mixture is incubated for 5mins at 37^{o}C and change in absorbance was measured for 3mins at 340nm. The experiment was conducted in triplicates and results shown are ±SD. The CK activity of the sample was calculated by the following formulae:

CK activity (U/L) = (Δ OD / min) x 8254

Where8254 is the extinction coefficient as determined by AGAPPE for the reaction.

2.2.9 Direct and indirect hemolytic activity assay

Fresh goat blood was collected from local butcher shop in citrated tube (0.11M tri sodium citrate) at 9:1 ratio (blood: citrate) according to Doley and Mukherjee [88]. Further the tubes were centrifuged (1,122 x g) for 20mins to separate the platelet poor plasma and red blood cells (RBCs). The obtained RBC pellet was washed 4-5 times with 0.9% NaCl by centrifugation [241]. The RBC pellet was re-suspended in 0.9% saline to a final concentration of 10% (V/V). Various amounts of crude *N. kaouthia* venom/ purified toxin (Nk-3FTx) (0.01-100µg) in 20µl of 0.9% NaCl was incubated for 60 min at 37°C with 150µl of 10% RBC to final volume of 2ml with

0.9% NaCl. The tubes were centrifuged (2739 x g) for 10 mins and the absorbance of the supernatant was measured at 540nm in a MultiSkan GO, UV-Vis spectrophotometer (Thermo scientific, USA). The hemolysis caused by dH_2O was considered as 100% hemolysis. For Indirect hemolytic assay 20µl of egg yolk solution was added to the reaction mixtures at the time of incubation.

2.2.10 Caseinolytic assay

2.2.10.1 Tyrosine standard curve

For the standard curve a stock solution of 100µg/ml of tyrosine was prepared. Various concentrations of tyrosine (0.5to 10µg/µl) were made and amount of tyrosine was estimated by Lowry's method [223]. One unit of protease activity is defined as *n* mole equivalent of tyrosine formed per min per ml.

2.2.10.2 Digestion of casein

Crude *N. kaouthia* venom was tested for casienolytic activity according to the original method of Ouyang and Teng[242] and as modified by Doley and Mukherjee [88] in *in-vitro*. Briefly, 1.0ml of 1 % (w/v) of casein in 20mM Tris-Cl buffer, pH 7.4 was incubated with various amounts of venom protein (1, 5, 10, 50 and 100µg) for 1hr at 37°C. Further, the reaction was stopped by addition of 0.5 ml of 10% (w/v) ice-cold TCA and centrifuged (2739 x g) for 10mins in Heraeus Multifuge X1R (Thermo Scientific, USA). The supernatant with the digested protein was transferred to a fresh tube and release of estimated using Lowry's method [223]. The caseinolytic activity of crude *N. kaouthia* venom at various concentrations was determined from the standard curve by calculating the amount of equivalent tyrosine released in the sample solution.

2.2.11 Fibrinogenolytic activity

Fibrinogen is a soluble, large and complex glycoprotein which helps in formation of a blood clot [243]. The glycoprotein has two sets of disulfide bridged Aα, Bβ and γ chains. Each molecule has two outer D domains which is connected to E domain (a central domain) by coiled coil segment [244–246]. The experiment was conducted to

observe any effect of crude *N. kaouthia* venom on various chains of bovine fibrinogen. In brief, bovine fibrinogen (2mg/ml) was dissolved in 50mM TrisHCl buffer, pH 7.4, 0.15M NaCl for overnight. Various amounts of crude venom from (1-10µg) in 150 µl of buffer (Tris-Cl buffer, pH 7.4, 0.15M NaCl) were incubated with 300µl of dissolved fibrinogen for different time intervals (0, 30, 60, 120, 240, 480, 960 and 1440mins) at 37°C [242]. The collected mixtures at various time intervals were then run on a 12.5% SDS-PAGE to observe any changes in the protein bands of fibrinogen. Staining was done with 1.2% coommassie brilliant blue R250 and destained with Methanol: Acetic acid: Water (40:10:50) to observe any effect on the Aα, Bβ and γ chains of bovine fibrinogen.

2.2.12 *In-vitro* coagulant assays

2.2.12.1 Recalcification time

Recalcification time test was performed according to the method described by Doley and Mukherjee [88]. Fresh blood from healthy human donor was collected in 3.2% tri-sodium citrate at 9:1 ratio (blood: citrate). The tubes containing the citrated blood were centrifuged (1,122 x g) for 20mins and platelet poor plasma (PPP) was separated from the whole blood were harvested. To assay the recalcification time, various concentrations of crude *N. kaouthia* venom (0.0042 to 4.2µg/ml)/ purified toxin (Nk-3FTx) in 50µl of PBS was pre-incubated with 150µl of human PPP for 3mins at 37^{o}C for 3mins. Soon after the incubation period, 100µlof 50mM $CaCl_2$was added to initiate the clot formation and clotting time was measured using a coagulation analyzer (STAGO, France). The clotting time of plasma with PBS was considered as normal clotting time.

2.2.12.2 Prothrombin time (PT) test

Human plasma was prepared as explained in section 2.1.12a. Various concentrations of crude *N. kaouthia* venom / purified toxin (Nk-3FTx) (0.0042 to 4.2µg/ml) were incubated with plasma to determine its effect on PT of harvested PPP. The assay was performed using PT reagent (NEOPLASTINE® CL PLUS) obtained from STAGO (France) and according to the manufacturers' protocol. The specified concentrations of

crude *N. kaouthia* venom in 50µl of PBS and 50µl of human PPP at 37^{o}C for 2mins. Further, 100µl of PT reagent was added to initiate the clot formation and clot formation time was determined using a coagulation analyzer (STAGO, France). The clotting time with PBS was considered as normal clotting time. The experiment was conducted in triplicates and results are ±SD of these independent experiments.

2.2.12.3 Activated partial thromboplastin time (APTT) test

Preparation of PPP from human blood was done as explained in section 2.1.12a and the experiment was followed according to Doley and Mukherjee [88]. Activated partial thrombin time was determined using APTT reagent obtained from STAGO (France) according to the manufacturers' protocol. Various concentrations of crude *N. kaouthia* venom/ purified toxin (Nk-3FTx) (0.0042 to 4.2µg/ml) were assayed to determine its effect on APTT of harvested PPP. The crude venom was pre-incubated with 50µl PBS, 50µl of human PPP and 50µl of APTT reagent for 3mins at 37^{o}C on a coagulation analyzer (STAGO, France). Further, 50µl of 0.025M $CaCl_2$ was added to the reaction mixture to determine clot formation time. The clot formation time with PBS was considered as normal clotting time.

2.2.13 Whole citrated blood analysis

2.2.13.1 Thromboelastometry analysis

Fresh human whole blood was collected from healthy human volunteer and collected in 3.2% tri sodium citrate at 9:1 (blood: citrate) ratio. Various concentrations of crude *N. kaouthia* venom (0.1, 1.0 and 10µg/ml) in 20µl of PBS were mixed with 20µl of 200mM $CaCl_2$. To the reaction mixture 320µl of whole citrated blood was added and clot formation was observed over 30mins. 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 [247, 248]. CT functions of whole blood treated with only PBS was considered as control.

2.2.13.2 Sonoclot analysis

Citrated human whole blood was collected as explained in section 2.2.12.1. Various concentrations of crude *N. kaouthia* venom (0.1, 1 and 10µg/ml) in 20µl of PBS was added to 20µl of 200mM $CaCl_2$. To the reaction mixture 320µl of citrated human blood added. A glass bead activated test (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) [249, 250]. 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. USA).

2.2.14 Bacteriacidal activity assay

Bacteriacidal activity of crude *N. kaouthia* venom was tested using Gram +ve *(Staphylococcus aureus)* and Gram –ve *(Pseudomonous aureginosa)* by well and disc diffusion method on LB agar media plate. Gentamicin 10mg/ml in saline was used as a positive control and saline alone served as blank.

2.2.14.1 Well diffusion

LB agar as a media was used. It was weighed, autoclaved and poured in sterile petridishes. The media was then allowed to solidify; 100µl of Gram +ve & Gram –ve bacteria are inoculated in separate petri dishes and spreaded using sterile glass beads. The plate was allowed to dry and wells were made by using gel puncher. Further, various amounts of crude *N. kaouthia* venom (1µg, 5µg and 10µg) in a final volume of 10µl of saline were loaded to the wells and incubation for overnight microbial growth. Zone of clearance of microbial growth by crude venom was observed after 16hrs.

2.2.14.2 Disc diffusion

Uniform disc of 1cm in diameter of blotting paper were made and autoclaved. The sterile discs were then allowed to soak the final solution (10µl) containing the crude *N. kaouthia* venom (1µg, 5µg and 10µg). Further, the discs were dried in an incubator at 37°C for 1hr and carefully placed on the surface of the LB agar

plate under a laminar air flow. The plates were kept overnight at 37°C in an incubator for microbial growth and observed for any zone of clearance after 16hrs of incubation.

2.2.15 Neutralization studies

Neutralization of crude *N. kaouthia* venom by polyvalent antivenom (Bharat Serums and vaccines limited (Batch no.AO5315011), India) was performed to determine the effect of polyvalent antivenom on various biological activities of the venom sample in *in-vitro*. Various biological activities viz. phopholipase A_2 activity, indirect hemolytic activity, recalcification time, PT, APTT and fibrinogenolytic activities were considered for the neutralization study by polyvalent antivenom. Various amount of polyvalent antivenom was pre-incubated with 1µg of *N. kaouthia* venom in a final volume of 20µl. The ratio of venom: antivenom was maintained at 1:1, 1:10 and 1:100 (v/v). The mixture was incubated for 1hr at 37°C and assays were performed as described in previous sections. The percentage inhibition was calculated by considering the activity in absence of polyvalent antivenom as 100%.

2.2.16 Western blotting

Cross reactivity of crude *N. kaouthia* venom with commercially available polyvalent antivenom (from Bharat Serums and vaccines limited (Batch no.AO5315011), India) was performed as described by Gutiérrez et al. [251]. Crude *N. kaouthia* venom was run on glycine 12.5% SDS PAGE. Soon after the run, the gel was carefully removed and soaked in transfer buffer (20mM TrisCl, pH 7.4, Glycine, 20% Methanol, SDS) for 15mins with nitrocellulose membrane (previously soaked in 100% methanol for 2mins). The sponges and blotting papers were also soaked in transfer buffer for 5mins. The blotting sandwich was made and whole cassette was soaked in the transfer buffer. Eletrophoresis at 100V, 300mA for 90mins was run and the cassette was opened in transfer buffer. Further the gel was blocked with 20ml of TBST (Tris buffer saline tween 20) and 1% BSA for 1hr. Blocking of gel was repeated thrice. The blot was further incubated with primary antibodies (Polyvalent antivenom, Bharat serums, India) at 1:4000 dilutions. Later the blot was washed with TBST for 15mins and the process was repeated twice. Rabbit anti-horse IgG was made at 1:1000 ratio (Rabbit anti-horse IgG:Blocking buffer (1% BSA in TBST) and

incubated with the blot for 2hrs and washed thrice with TBST. Finally, the blot was covered with 1 to 3ml of pre-mixed BCIP/NBT solution for color development with continuous shaking. The reaction was stopped by using 1% acetic acid once the color development was satisfactory. Further, the blot was dried and documented in G-Box, Image analyzer (Syngene, UK).

2.2.17 Cytotoxicity

Exponentially growing HEK293 (human embryonic kidney cells) and L6 (rat skeletal muscle cells) cell lines at 10^6/ml were incubated with various concentrations (0.1 to 100ng/ml) of crude *N. kaouthia* venom/ purified toxin (Nk-3FTx) for 24hrs at 37˚C with 5% CO_2 in a CO_2 incubator (Eppendorf, USA). Cell count was performed on haemocytometer after vital staining with trypan blue dye. MTT assay was performed to observe the cytotoxic effect of the crude *N. kaouthia* venom. 20µl of MTT (5mg/ml) was incubated for 3.5hrs prior to the end of 24hrs of incubation with crude *N. kaouthia* venom at 37˚C in a class-II biosafety cabinet (Model no- LCB-0123,Daihan Labtech, Korea). The class-II biosafety cabinets provide protection to both samples and environment where makeup air is HEPA (high efficiency particulate arrestance) filtered. The formazan granules formed by viable cells are dissolved in 150µl MTT solvent and agitated for 15mins. Absorbance at 570nm was measured using a MultiSkan Go spectrophotometer (ThermoScientific, USA). The crude *N. kaouthia* venom treated HEK 293/L6 cells were observed under an inverted microscope (Zeiss, USA); the cells were observed at 10X and checked for any changes in their shape, size, density and adherent property.

2.2.18 Neurotoxicity

2.2.18.1 Dissection and isolation of sciatic nerve from common Asian toads

Preparation, dissection and isolation of sciatic nerve was performed according to Katsuki et al. [252]. During dissection, efforts were taken to minimize both animal sufferings and animal number. Common Asian toads *(Duttaphrynus melanostictus)* of similar weight and uniform size of either sex were decapitated and then pithed. The sciatic nerve (length 3-5cm; 0.5-1mm diameter) was carefully dissected from lumber plexus to the knee joint in

Ringer's solution. Utmost care was taken so that the nerve was not touched by fingers, cut muscle, frog skin or with any metal instruments. The nerve was kept moist all the time during the procedure. Finally, the dissected nerve was tied with sterile surgical thread at knee joint and placed on nerve chamber (AD Instruments, PowerLabs, Sydney, Australia). Throughout the experiment the nerve was kept moist in a nerve chamber using Ringer's solution.

2.2.18.2 Recording of compound action potential (CAP) and determination NCV (Nerve conduction velocity)

The experiment was performed in *in-vitro* by treating the dissected nerve with various concentrations of crude *N. kaouthia* venom (0.132nmol/L to 1319 nmol/L)/ purified toxin (Nk-3FTx) for 2mins. CAP was measured in a nerve chamber (AD Instruments, Powerlabs, Australia) equipped with 17 stainless steel electrodes. CAP obtained was monitored by a dual Bio Amp/stimulator. In brief, standard techniques for extracellular recordings were followed. The dissected sciatic nerve was externally stimulated with a frequency of 1Hz where pulses at 0.1ms duration were used to determine the compound action potential [252]. A setup comprised of two male BNC (Bayonet Neill–Concelman) connectors to three micro-hooks constructed of gold-plated beryllium copper used to stimulate the nerve. CAP of sciatic nerve in nerve chamber was detected at two points separated by a distance of 3cm. The nerve end with lumber plexus of spinal cord was connected with proximal recording electrode and electrode at nerve end connecting knee joint acted as distal recording electrode. Flow of stimulus was recorded at two points to determine NCV on the dissected nerve. The procedure was completed within a time interval of 2mins per nerve treated to avoid drying. CAP recorded was analyzed by data software SCOPE (Powerlabs, Australia). Nerve only treated with Ringer's solution served as control for the experiment. Nerve treated with bupivacaine hydrochloride (BH) (voltage gated sodium channel blocker) and quinine hydrochloride dihydrate (QH) (voltage gated potassium channel blocker) were considered as positive controls. The experiment was performed

in triplicate and results shown are ±SD. NCV of the sciatic nerve was determined using the following formula:

$$NCV\ (m/s) = \frac{\text{Distance between the electrodes (in meters)}}{\text{Time taken by the stimulus (in seconds)}}$$

2.2.19 Scanning electron microscopy

The isolated sciatic nerves from toad were treated with crude venom and checked for any morphological changes under scanning electron microscope (SEM). Crude *N. kaouthia* venom to a final concentration of 0.1µg/ml, 1µg/ml and 10µg/ml in Ringer's solution were incubated with isolated sciatic nerve for 2mins. The sciatic nerves were primarily fixed with 2.5% of gluteraldehyde for 3hrs and carefully relocated on washing buffer (Ringer's Solution). The nerves were then washed 2-3 times with Ringer's solution and further fixed with osmium tetroxide (OsO_4) for 4hrs as secondary fixation procedure. Later, the fixed nerves were dehydrated in increasing percentage of ethanol (40%<50%<60%<70%<80%<90%<95%).

Further the fixed nerves were sliced at 1mm thickness using a RMC microtome cutter, X and XL (Boeckeler instruments, USA). The sliced nerve sections were first fixed on a holder and coated with platinum ions. The coated nerve section was finally mounted on a JSM 6390 LV Scanning electron microscope (GEOL, Japan). The nerves were observed at 2000X for any morphological changes.

2.2.20 Liquid chromatography-mass spectrometry (LC/MS) of crude venom

Lyophilized crude *N. kaouthia* venom (2mg) was dissolved in 20µl of MilliQ water. Prior to fractionation of the crude venom, the separating Symmetry C18 analytical column was equilibrated with 0.1% (v/v) trifluoroacetic acid attached to a Accela LCQ fleet ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) for MS analysis. The crude *N. kaouthia* venom mixture was eluted using a linear gradient of 80% (v/v) acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 50µl/min. Electrospray ionization mass spectrum was acquired in positive ion mode with an orifice potential of 80V. Nitrogen was used as curtain gas with a flow rate of 0.6liters/min and as nebulizer gas with a pressure setting of 100 psi. Full scan

data were acquired over the ion range from 500 to 3000 m/z with step size of 0.1 Da. Data was analyzed by Promas for Xcaliber.

2.2.21 Fractionation of crude venom

The crude venom was fractionated using a Symmetry C18 column (5µ, 300Å, 4.6x250mm) (Waters, USA). Briefly, 2mg of crude *N. kaouthia* venom sample was dissolved in 20mM Tris Cl, pH 7.4 and kept at 4^{o}C overnight to dissolve. Prior to the start of fractionation, the column was washed and pre-equilibrated with 0.1% TFA in Milli-Q (Buffer A) for 10 column volume. The dissolved venom was filtered using 0.2µ syringe filter and loaded in the HPLC system. Fractionation of crude *N. kaouthia* venom was an optimization process. Various gradients of buffer B (80% Acetonitrile containing 0.1% TFA) at different time intervals were used to obtain a proper fractionation of crude venom. Gradients of buffer B in 0-100% for 150mins, 20-70% for 130mins and 20-50% for 130mins were used. Elution of the bound proteins was detected by Waters dual UV/Vis detector (Waters, USA) at 215nm and 280nm. The peak retention time and percentage area was calculated using Empower 2 data software (Waters, USA). Each peak collected was immediately lyophilized. In brief, the eluted protein peaks were collected and immediately transferred to -80^{o}C to solidify and freeze. The tubes were then connected to a pre conditioned lyophilizer (112N-G, Hahntech, South Korea) at -80^{o}C and vacuum was created using a rotary van vacuum pump (Kolkata, India) for 5-7hrs. When the fractionation process was completed, the tubes were collected and stored at -20^{o}C for further use.

2.2.22 Purification of Nk-3FTx

The lyophilized fractionated protein peaks were reconstituted using 20mM TrisCl, pH 7.4, The peak (Peak 10) containing the 3FTx was rechromatographed on an Aeris WIDEPORE (XB-C18, 3.6µ, 150x2.10mm, 200Å) (Phenomenex, USA). Rechromatography and purification of Nk-3FTx was manually optimized on HPLC machine at various gradients of buffer B. The gradients used for purification of Nk-3FTx were 38-43% for 120mins, 40-45% for 45mins and 40-45% for 45mins. The shallow gradient of 40 to 42% buffer B for 45mins was observed to separate the Nk-3FTx from other impurities and a sharp symmetrical peak was obtained. The total purified Nk-3FTx was pooled together and freeze dried.

2.2.23 Molecular weight determination

Molecular mass of the purified Nk-3FTx was determined by electrospray ionization mass spectrometry (ESI-MS) using an LC Q fleet ion trap mass spectrometry (Thermo Scientific, USA)[137]. Ion spray voltage was set at 4.4KV. Nitrogen was used as a curtain gas at a flow rate of 0.6 l/hr and compressed air was used as a nebulizer. The sample was infused by flow injection at flow rate of 50µl/min. Solvent used was 50% acetonitrile in 0.1% formic acid at a flow rate of 200µl/min. Promas for Xcaliber was used to analyze and decipher the raw mass data.

2.2.24 N-terminal sequencing of the purified protein

N-terminal sequencing was determined by automated Edman degradation process[253] using PPSQ 31 (Shimadzu, USA) with phenlythiohydantoin (PTH) derivative analyzer. Briefly, Lyophilized Nk-3FTx was dissolved in 100µl of denaturant solution (6.0M guanidinium hydrochloride, 0.13M Tris, 1mM EDTA, pH-8.0) containing 0.07M β-mercaptoethanol and incubated at 37˚C for 2hrs. Subsequent blocking of sulfhydral groups was done by adding 4-vinylpyridine and incubating at room temperature for 2hrs. The protein was desalted by RP HPLC and subjected to N-terminal sequencing.

2.2.25 ESI LC-MS/MS (Electrospray ionization liquid chromatography/tandem mass spectrometry)

For ESI-LC-MS/MS analysis of the purified protein, proteolysis was carried out using trypsin with protease Max surfactant (Promega, Madison, WI, USA) according to the manufacturer's instructions. Protein sample (~50µg) was dissolved in 50µl of MilliQ water. To this, 41.5µl of 50mM of ammonium bicarbonate, 2µl of 1% protease Max and 1µl of 0.5M DTT were added. The reaction mixtures were incubated at 56°C for 20mins. Then 2.7µl of 0.55M IAA was added and incubated in dark for 15mins. Finally 1µl of 1% protease Max and 1.8µl of trypsin (1µg/µl in 50mM acetic acid) were added and the reaction mixtures were incubated at 37°C for 3hrs. To stop the reaction 0.5µl of 100% TFA was added and incubated at room

temperature for 5mins. The reaction mixtures were centrifuged at 12,000rpm for 10mins.

The tryptic digests of individual samples was loaded onto Accela LCQ fleet ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) for MS/MS analysis. Each sample (~80µl) was injected into a Hypersil gold C_{18} column (50 x 2.1mm, 1.9µm, Thermo Scientific, Waltham, MA, USA) pre-equilibrated with 0.1% formic acid. Elution was carried out at a flow rate of 200µl/min with a linear gradient of 40%-80% acetonitrile in 0.1% formic acid. The eluent from liquid chromatography (LC) column was directly fed to the mass spectrometer. Ion polarity of the system was set to positive ionization mode. Spectra were obtained in MS/MS mode and MS/MS scan range was set from 500 to 2000m/z. Oxidation of methionine residues and *S*-carbamidomethylation of cysteine residues were set as modification. The MS/MS spectra was analysed by the software proteome discoverer 3.1 using Sequest program.

2.2.26 Statistical analysis

The experimental values in the entire thesis are averages of three independent experiments ±SD. P value and other significant differences in various treatments of crude venom and Nk-3FTx were evaluated using student t test (Microsoft Excel 2007).