Chapter 6

Functional characterization of purified Nk-3FTx

6.1 Introduction

3FTxs exhibit potent toxic effect to the prey or the victims physiology and studies on these proteins have contributed significantly in understanding venom toxicity [308]. They are involved in inducing neurotoxicity, cardiotoxicity, cytotoxicity, anticoagulant effect, myotoxicity etc [85, 150, 157, 302, 309, 310]. 3FTx are target specific, which helped in deciphering molecular details of many physiological processes, eg. α-bungarotoxin, a 3FTx from *B. multicinctus* venom helped in isolation of nicotinic acetylcholine receptor (nAChR) and aided in understanding of myasthenia gravis [308, 311]. They are also helpful in determining distribution of several specific receptors, ion channels, identification of subtypes of receptors etc [312–315].

Many snake venom components induce neurotoxicity in prey or the victim's physiology by affecting neuro-muscular junctions, various ion channels responsible for propagation of stimulus, affects neurotransmitters and others [67, 145, 195, 316, 317]. Snake venom components affecting ion channels may be responsible for various post envenomation effects such as paralysis and cardiac arrest [309, 318]. A neuron has several ion channels, which are associated with transmission of neuronal stimulus from one nerve to another. The propagation of stimulus is by a chemically mediated process involving neurotransmitters followed by conversion of chemical signal to electrical signal. These electrical signals are transferred via voltage-gated specific channels.

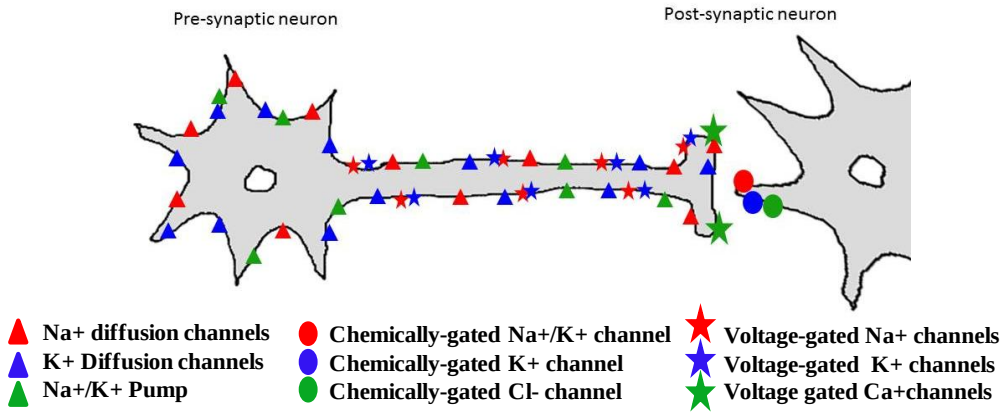

Figure 6.1: Various ion channels present on a neuron

Action potential is an electrical event where change in electrical potential of a cell associated with impulse passage. Voltage gated ion channels generate it. When depolarization in trans-membrane occurs, there is an inward flow of sodium ion via sodium channels which changes the electrochemical gradient. This is an explosive process resulting in a large upswing in the membrane potential due to influx of sodium ions and the sodium ion channels rapidly inactivate. Again potassium ion channels are activated and to maintain the electrochemical gradient there is an outward current of potassium ions and obtains the resting state of the nerve axon. Soon after an action potential, a transient negative shift called after hyperpolarization or refractory period, due to additional potassium currents. This mechanism prevents an action potential to revert.

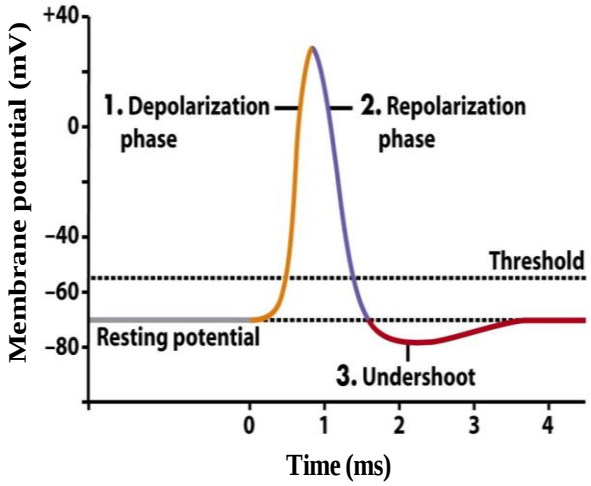

Figure 6.2: Action potential of a neuron (www.uic.edu)

Compound sensory nerve action potential (SNAP) is a result of phase summation and cancellation of single fiber potentials (SFAPs) along with amplitudes that depends on

the fiber diameter [319]. The conduction velocity are determined by summation of SFAPs of different fiber diameters [319].

In the previous chapter, we described the isolation and purification of Nk-3FTx. Further, we characterized Nk-3FTx to determine its molecular mass and primary structure. The studies led us to confirm Nk-3FTx belonging to 3FTx family. It has four conserved disulfide bonds which is the conserved molecular organization of 3FTx family. The presence of fifth disulfide bond in first loop of Nk-3FTx confirmed it as a non-conventional 3FTx. The toxin showed similarity with a melanoleuca toxin CM-9a reported from the venom of Thailand cobra *N. naja kaouthia* [191].

The present chapter discusses the biochemical and biological properties of Nk-3FTx. Further, we analyzed its effect in behavioral changes on experimental animals and performed *in-vitro* neurotoxicity studies using isolated sciatic nerve.

6.2 Results

6.2.1 Biochemical and biological activities

Purified Nk-3FTx was found to be devoid of any PLA_2, direct, indirect hemolytic and caseinolytic activity as compared to crude venom at similar venom amounts (Figure 6.3).

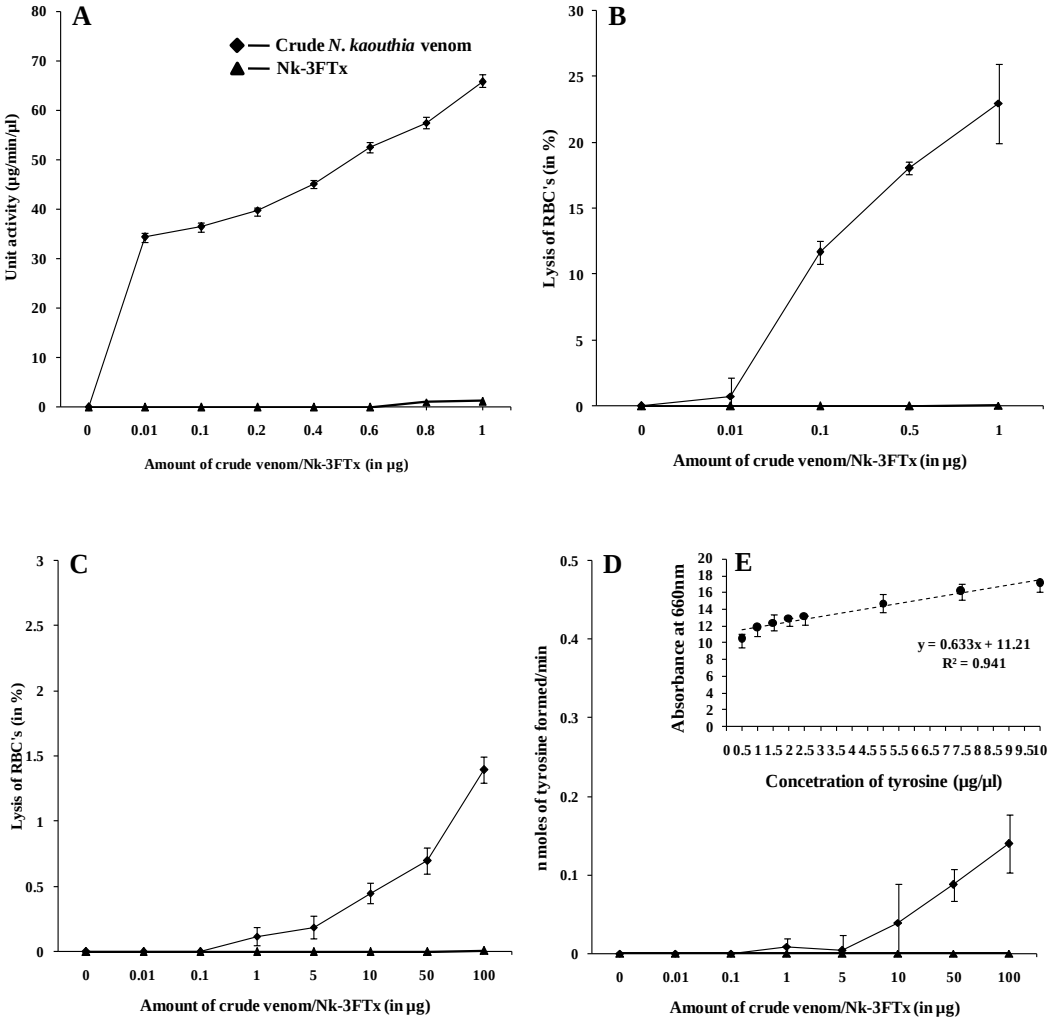

Figure 6.3: Various *in-vitro* biochemical and biological tests of Nk-3FTx. A. PLA_2 activity assay of Nk-3FTx, one unit of PLA_2 activity is defined as decrease in 0.01 absorbance in 10 mins at 740 nm. **B.** Indirect hemolytic activity assay of Nk-3FTx in dose-dependant manner. **C.** Direct hemolytic activity assay of Nk-3FTx dose dependently. **D.** Proteolytic activity of Nk-3FTx on casein in dose dependently. One unit of protease activity is defined as *n* mole equivalent of tyrosine formed per min per ml. **E.** Tyrosine standard curve. All the experiments were compared with activities of crude venom. Values shown are from three independent experiments and ±SD.

The PLA_2 activity of Nk-3FTx was found to be negligible even at 1µg of the purified protein (Figure 6.3A) whereas the crude venom exhibited 65.8µg/min/µl unit activity with 1µg (Figure 6.3A). The crude venom was found to exhibit a significant indirect hemolysis to the washed RBC's at 1µg (23%) which is associated with venom PLA_2 activity. However, Nk-3FTx was found to be devoid of such activities (Figure 6.3B).

Further, the crude venom was found to exhibit direct hemolytic activity in a dose-dependant manner, the extent of hemolysis was found to be less. At 100µg of crude venom the percentage lysis of washed RBC's were found up to 1.4% (Figure 6.3C). In contrast, Nk-3FTx did not show any such activity (Figure 6.3C). Caseinolytic activity by crude venom was also found to be only at higher concentration where 0.14 moles of tyrosine equivalent was liberated when treated with 100µg/µl (Figure 6.3D). The concentration of tyrosine liberated was calculated from the tyrosine standard curve (Figure 6.3E). However, Nk-3FTx was not observed for caseinolytic activity even when treated up to 100µg (Figure 6.3D).

Nk-3FTx did not show any hemorrhagic or edema inducing activity (Table 6.1) on experimental mice (Figure 6.4). There was a small smear of blood observed when crude venom was injected but this might be a condition in hemorrhage which is negligible as compared to mice injected with *Echis carinatus* venom (positive control) (Figure 6.4B). The minimum hemorrhagic dose (MHD) is defined as the concentration of venom required to induce a hemorrhagic spot of 1 cm diameter at the injection site. Nk-3FTx did not exhibit any hemorrhagic activity as observed in experimental mice (Figure 6.4C). However, when the experimental animal was injected with 3µg of saw scaled viper venom (positive control), a significant hemorrhagic spot of 4-5cm was observed (Figure 6.4D). The animals injected only with normal saline served as control and hemorrhage was absent.

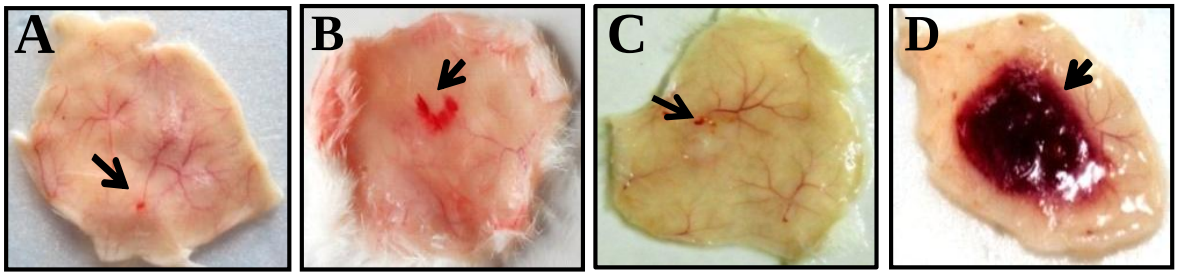

Figure 6.4: Hemorrhagic activity. A: Control (30µl of saline), **B:** Crude *N. kaouthia* venom (15µg), **C:** Nk-3FTx (20µg), **D:** *Echis carinatus* venom (Saw scaled viper) (3µg) (Positive control), the arrow indicates the site of injection of venom.

The edema inducing activity is calculated as explained in section 2.2.6. Minimum edema dose (MED) is defined as the amount of protein required to cause an edema ratio of 120% [230]. 15µg crude venom was found to exhibit edema inducing activity with an edema ratio of 199.3% when injected subcutaneously in the paw of experimental animal (Table 6.1). However, 15µg of Nk-3FTx was found to exhibit an edema ratio of 94.33% which was significantly less in comparison with the crude venom activity (Table 6.1). The edema ratio observed from Nk-3FTx confirms its non-edema inducing property.

Table 6.1 Edema inducing activity of Nk-3FTx

Sl no	Venom	Mice no. (M)	Normal leg weight (gm)	Edematous leg weight (gm)	Edema ratio (%)	Average (%)
1	CVNK	M1	0.13	0.29	223	199.3
2	CVNK	M2	0.14	0.27	192.9	
3	CVNK	M3	0.132	0.24	182	
4	Nk-3FTx	M4	0.133	0.13	97.7	94.33
5	Nk-3FTx	M5	0.143	0.15	105	
6	Nk-3FTx	M6	0.137	0.11	80.3	

NKCV: *N. kaouthia* crude venom. M: mice.

To determine any cytotoxic effect of Nk-3FTx, HEK293 and L6 rat skeletal muscle cell lines were used. Nk-3FTx did not display any cytotoxic effect on HEK 293 cells even up to 100ng/ml concentration (Figure 6.5). However, HEK 293 cells treated with silver nitrate ($AgNO_3$) and crude venom exhibited altered morphology and cell death (Figure 6.6A). Moreover crude venom treated cells were found to detached from surface of the culture plate (Figure 6.6A). In contrast, the L6 cells treated with crude venom did get inhibited; however, no significant altered morphology and cell detachment was observed (Figure 6.6B). The L6 cells treated with Nk-3FTx showed moderate cytotoxicity at 100ng/ml concentration after 24hrs (Figure 6.5).

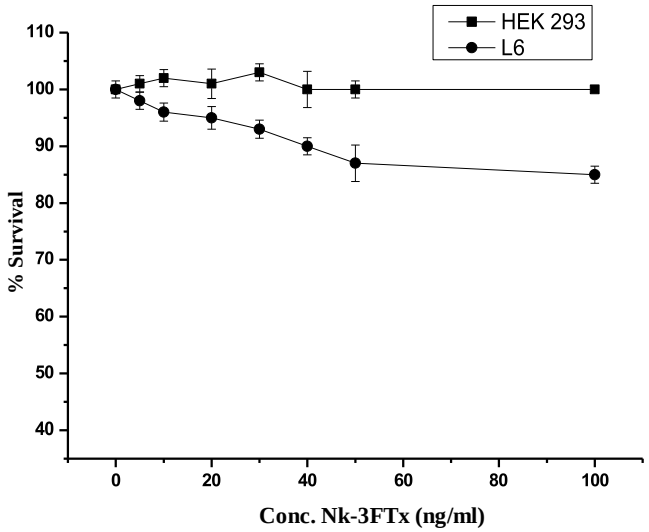

Figure 6.5: Dose dependent effect of Nk-3FTx on HEK 293 and L6 rat skeletal muscle cell lines. Cell count was done by vital staining with trypan blue dye and finally counting on a haemocytometer. The experiment was conducted in triplicates and results are mean ±SD.

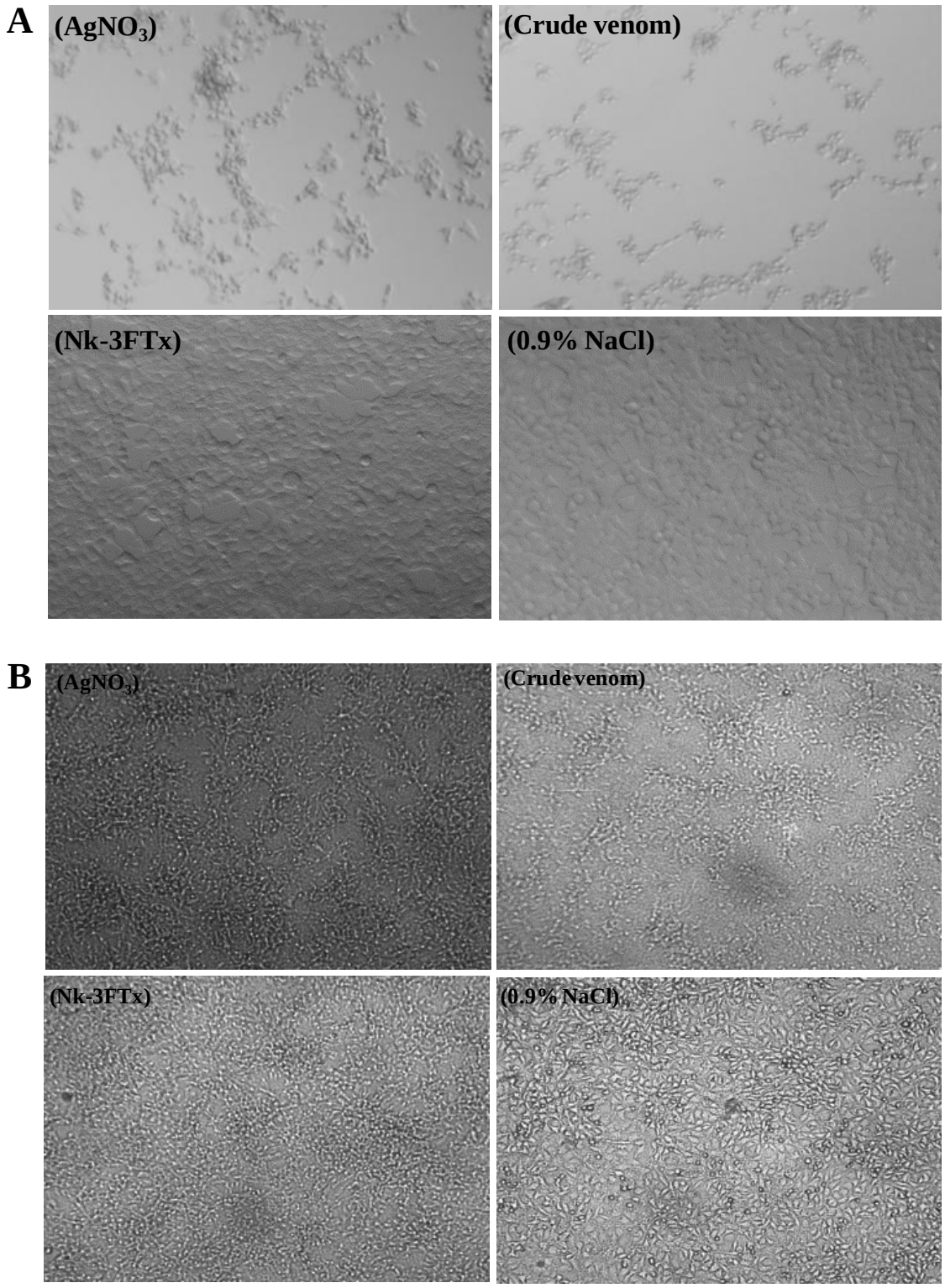

Figure 6.6: Representative cell images of HEK 293 (Panel A) and L6 rat skeletal muscle cell lines (Panel B). Cells treated with 100ng/ml of crude venom and Nk-3FTx is compared. Cells only treated with 0.9% NaCl served as blank and $AgNO_3$ treated cells served as positive control.

The effect of Nk-3FTx on blood coagulation cascade was studied using both goat plasma and human plasma. However, we observed a slight deviation from clotting time of human plasma when treated with Nk-3FTx as compared to normal clotting time (Table 6.2). Nk-3FTx was found to delay the calcium clotting time in dose-dependant manner. At 5.0µg/ml of Nk-3FTx the clotting time was delayed up to 147.6 ±4.01 seconds when the normal clotting time was recorded at 114.7 ±2.2 seconds (Table 6.2). However, the delay in clotting time by Nk-3FTx was found negligible when compared with anticoagulation by crude venom. When tested for extrinsic pathway of blood coagulation (PT), Nk-3FTx was found to delay the clotting time up to 23.75 ±0.07 seconds. The PT normal clotting time for plasma was recorded at 21.25 ±0.64 seconds (Table 6.2). This result suggests that Nk-3FTx might not affect in extrinsic pathway of blood coagulation. However, Nk-3FTx was found to slightly delay the APTT clotting time up to 68.5 ±3.45 seconds when normal clotting time was recorded at 42.1 ±1.93 seconds (Table 6.2).

Nk-3FTx exhibited a mild anticoagulation effect which was observed in calcium clotting time and APTT. However, delay in clotting time was insignificant for the entire coagulation cascade pathway when compared with the crude venom anticoagulation activity.

Table 6.2: Effect of Nk-3FTx on clotting time of citrated human plasma. Clotting time of plasma in presence of Tris buffer was considered as normal clotting time. Values represented are mean ±SD of three independent experiments. PT: Prothrombin time; APTT: Activated partial thromboplastin time.

	NCT	0.005 µg/ml	0.05 µg/ml	0.5 µg/ml	5.0 µg/ml
Re-calcification time (in secs)	114.7 ±2.2	115.6 ±0.603	116.1±0.87	120.7±1.44	147.6 ±4.01
PT (in secs)	21.25 ±0.64	21.1±0.38	21.7±0.4	22 ±0.3	23.75 ±0.07
APTT (in secs)	42.1 ±1.93	42 ±0.35	43.15±8.7	47.1 ±1.4	68.5 ±3.45

NCT- Normal clotting time

Further, blood coagulation experiments were repeated using citrated goat plasma and clotting time was compared between crude venom and Nk-3FTx at similar concentrations. The crude venom showed a significant anticoagulation effect in all of

the tested coagulation experiment viz. calcium clotting time, PT and APTT. At similar crude venom/Nk-3FTx concentration the calcium clotting time of Nk-3FTx was found to be negligible as compared to crude venom. At 0.42µg/ml of crude venom there was no clotting of citrated plasma; however Nk-3FTx showed delay in clotting time up to 170 ±1.1 seconds even at 42µg/ml (Figure 6.7A). The normal value for calcium clotting time was recorded at 112 ±1.17 seconds (Figure 6.7A) and the effect of Nk-3FTx was found insignificant when compared with crude venom.

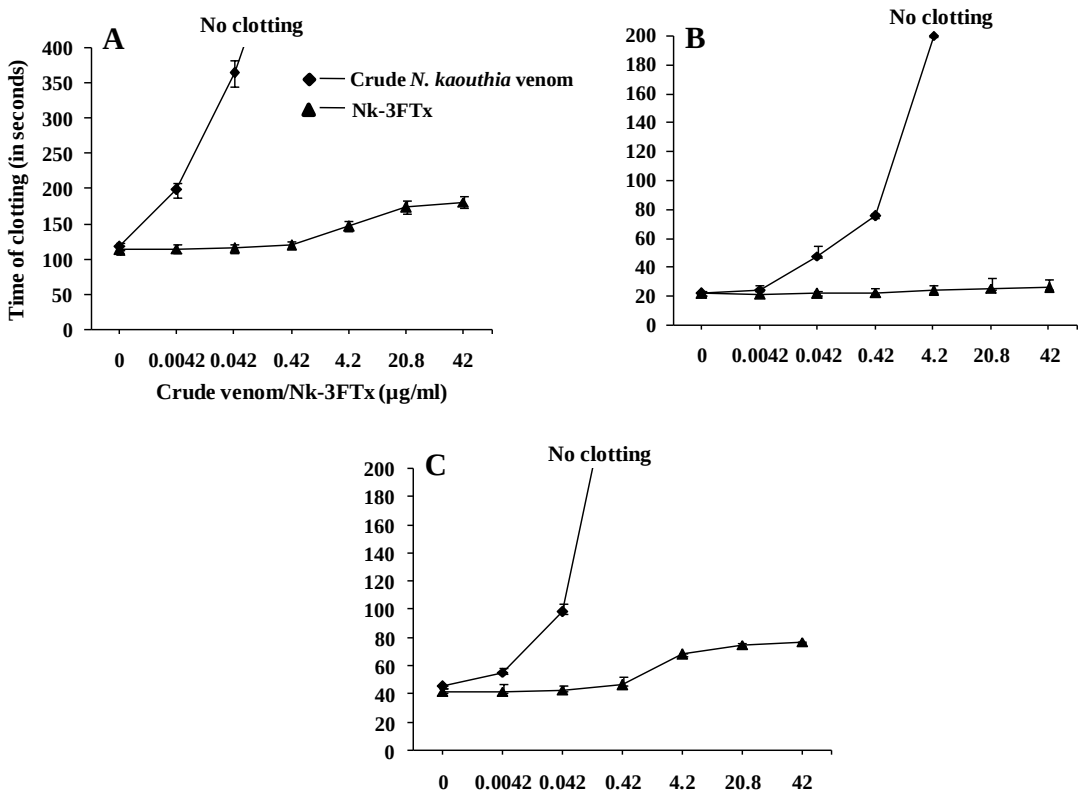

Figure 6.7: Comparison of anticoagulant effect of Nk-3FTx and crude *N. kaouthia* venom on clotting time of goat plasma. A. Calcium clotting time, **B.** Prothrombin time (PT), **C.** Activated partial thromboplastin time (APTT). Clotting time of plasma in presence of Tris buffer was considered as normal clotting time. Values represented are mean ±SD of three independent experiments. PT: Prothrombin time; APTT: Activated partial thromboplastin time.

PT test of goat plasma by crude venom also showed a significant delay in clotting time. There was no clotting of the plasma at 20.8µg/ml of crude venom (Figure 6.7B), however treatment of Nk-3FTx at 42µg/ml to citrated plasma delayed the coagulation time only up to 22.3 ±1.2 seconds (Figure 6.7B) whereas the normal

clotting was recorded at 20.3 ±0.29 seconds. The crude venom significantly delayed the clotting time when checked for APTT on citrated goat plasma. Clotting time was delayed up to 97 ±1.27 seconds (Figure 6.7C) when treated at 0.042µg/ml of crude venom. However, no clotting was observed when the concentration was increased up to 0.42µg/ml (Figure 6.7C). Nk-3FTx as observed in case of human plasma did delayed the clotting time of citrated goat plasma in dose-dependent manner (Figure 6.7C). At 42µg/ml of Nk-3FTx, the clotting time was recorded up to 79.3 ±0.31 seconds (Figure 6.7C). When compared with crude venom the effect of Nk-3FTx was found to be insignificant

6.2.2 Behavioral study

Nk-3FTx was injected intra-peritoneally (0.01mg/kg, 0.05mg/kg, 0.1mg/kg) into the experimental mice to observe any behavioral changes. The purified protein was found to be neurotoxic in nature. The behavioral changes of the experimental animals injected with Nk-3FTx are similar to that of the crude venom injected animals. However, the effects of neurotoxicity were observed only after 1hr post injection. The following manifestations on the experimental animal were observed after Nk-3FTx injection:

a) Frequent drinking of water was observed after 30mins of injection of Nk-3FTx; the drinking behavior the mice were normal.

b) Effect of Nk-3FTx was not observed till 45mins, however at 50mins post injection the experimental animal was found to have minor difficulties in breathing after careful observation. However, the mice behavior was not conclusive as the mice were absolutely normal in its other activities.

c) Effect of Nk-3FTx was more prominent between 2.5-4hrs post injection. The experimental animal was found to exhibit similar neurotoxic behavioral changes which were observed in case of crude venom injection as explained in section 3.1.5. The mice were disheveled, sluggishness in its movement was observed. Heavy difficulties in breathing were also observed.

c) From 5-6hrs, heavy difficulty in breathing and significant sluggishness (almost paralysis) in lower limbs was observed. No defecation or salivation was noted.

d) At 6-6.5hrs, there was significant respiratory disorder and the mice succumb due to respiratory failure and paralysis.

The behavioral study of experimental mice shows that Nk-3FTx is neurotoxic in nature. The protein did not exhibit any other tested biochemical and biological property when subjected for other *in-vitro/in-vivo* biochemical and biological studies.

6.2.3 Sequence Analysis

Nk-3FTx was not found to exhibit any activity *in-vitro* and *in-vivo* biochemical and biological activities in our previous studies. However, the purified protein was responsible for significant changes in the behavior of experimental animal and its death suggesting its neurotoxic nature. 3FTx have short chain neurotoxins and long chain neurotoxins which are reported to exhibit neurotoxic pharmacological effect in the prey or victims physiology. Also, non-conventional 3FTxs are reported to have various activities such as cytotoxicity and neurotoxicity. Nk-3FTx is a non-conventional 3FTx. Hence, we compared the partial amino acid sequence of Nk-3FTx with other reported 3FTxs with pharmacological activity. This helped us in understanding and proceeding further experiments with Nk-3FTx.

Initially we compared the sequences from reported 3FTxs of *N. kaouthia.* CM-9a (Acc. No. P25679.2) from *Naja naja kaouthia* of Thailand and a tryptophan containing weak neurotoxin (Acc. No. P82935.2) from *N. kaouthia* of Thailand were found to be only two similar non-conventional 3FTx. However, there were substitutions of 6 amino acids at 6 different positions on Nk-3FTx (Figure 6.8). Additionally, a tryptophan (Try36 at tryptophan containing weak neurotoxin) was missing in Nk-3FTx which is present in tryptophan containing neurotoxin (Figure 6.8).

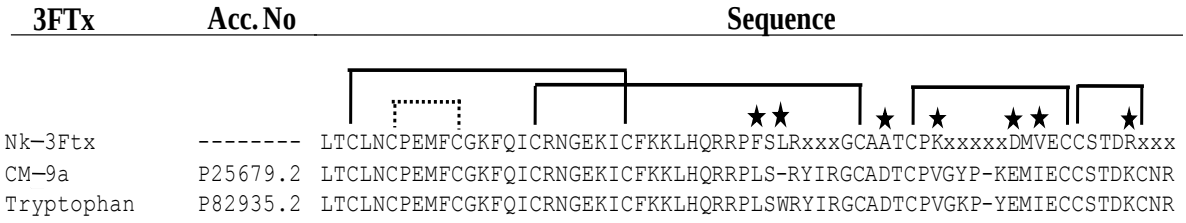

Figure 6.8: Comparison of sequence with highest homology to Nk-3FTx. The amino acids substituted in Nk-3FTx are marked with a star. The solid line represents the four disulfide bridges present in the core. The dotted line represents the fifth disulfide bond between Cys2 and Cys3 present in the first loop.

Nk-3FTx was also compared with a long chain neurotoxin, α- cobratoxin (Alpha-elapitoxin) (P01391.1) from *N. kaouthia* of Thailand. However, the crucial amino acid responsible for binding to AChR were found to be absent in case of Nk-3FTx except Arg37 (Figure 6.9). The residues Try29, Asp31 and Phe33 were replaced by His29, Arg31 and Pro33 in Nk-3FTx (Figure 6.9). Also, the extended C-terminal residues in long chain neurotoxin was absent in Nk-3FTx which is a characteristic in long chain neurotoxin (Figure 6.9).

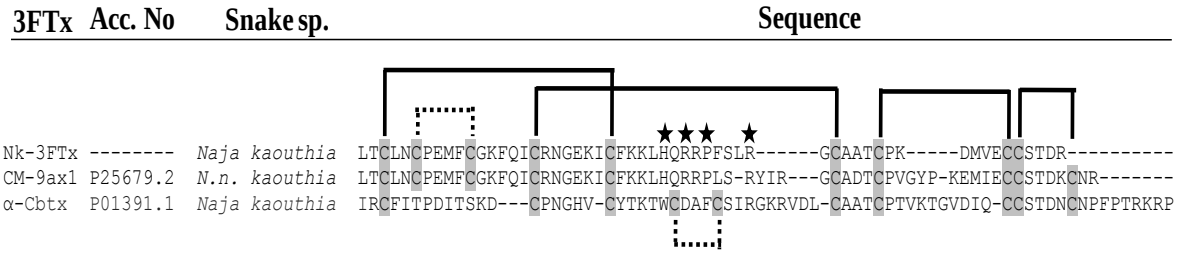

Figure 6.9: Sequence alignment of Nk-3FTx with α-Cbtx (alpha-elapitoxin) from *N. kaouthia*.

Further, Nk-3FTx was compared with reported 3FTxs with calcium channel blocking activity. The sequences for comparison (FS2 and calciceptine) were reported from *D. p. polylepsis* venom [146]. These calcium channel blockers are reported to contain the peptide segment Thr48, Ala49, Met50, and Trp51 which is crucial for binding to calcium channels (Figure 6.10). As the Nk-3FTx was not complete, we compared the sequence using the reference sequence of CM-9a. The peptide segment was found to be substituted with Val48, Gly49, Tyr50 and Pro51 in case of CM-9a (Figure 6.10). However, Thr48 in FS2 and calciceptine was found to get replaced with Lys48 in Nk-3FTx (Figure 6.10).

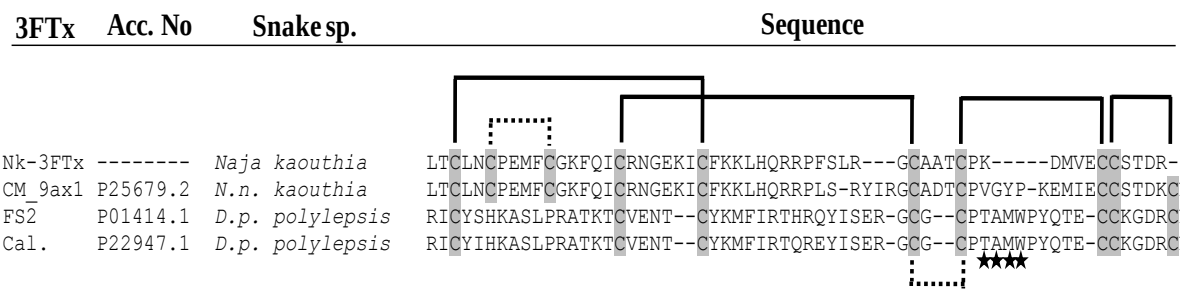

Figure 6.10: Comparison of Nk-3FTx with 3FTxs affecting calcium channel. Nk-3FTx was compared with FS2 (P01414.1) and calciceptine (P22947.1) from *D. p. polylepsis* venom. The starred amino acids (TAMW) are responsible for blocking the calcium channel causing neurotoxicity.

Finally, we compared Nk-3FTx with some toxins reported from other families such as kunitz-type serine protease inhibitors and toxin_2 super family. We compared all the three classes of potassium channel blockers viz. dendrotoxins reported from black mamba venom (Class I), BgK, a sea anemone toxin (Class II) and charybdotoxin from scorpion venom (Class III) [320] (Figure 6.11). All the mentioned toxins are reported to block or impair various voltage gated potassium channels on nerve axon.

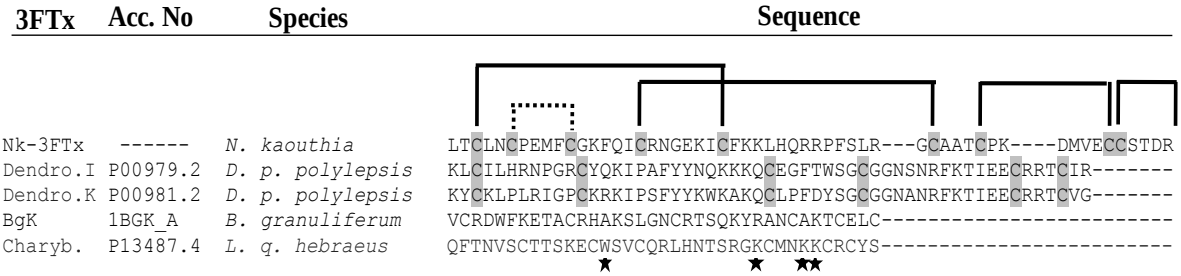

Figure 6.11: Sequence alignment of Nk-3FTx with reported potassium channel blockers from different species. The starred amino acid residues are the crucial amino acid residues in charybdotoxin for potassium channel binding.

The comparative analysis with dendrotoxin I, K and BgK confirms that Nk-3FTx do not posses any similar amino acid at specific position responsible for binding to voltage gated potassium channels. However, charybdotoxin from scorpion venom belongs to toxin_2 superfamily; was found to be similar with the N-terminal sequence of Nk-3FTx in carrying the crucial Lys27 residue, which is responsible for binding to potassium channel. Other important residues such as Trp14, Lys31 and Ly32 in charybdotoxin were replaced with Phe14, Arg31 and Arg32 in Nk-3FTx.

6.2.4 Neurotoxicity studies

Treatment of the sciatic nerve with Nk-3FTx was found to decrease the compound action potential (CAP) and nerve conduction velocity (NCV) (Figure 6.12). The control nerve was found to elicit normal amplitude of 9mV at the distal electrode (Figure 6.10A); however, with increasing concentration of Nk-3FTx, the CAP decreases in a dose dependent manner.

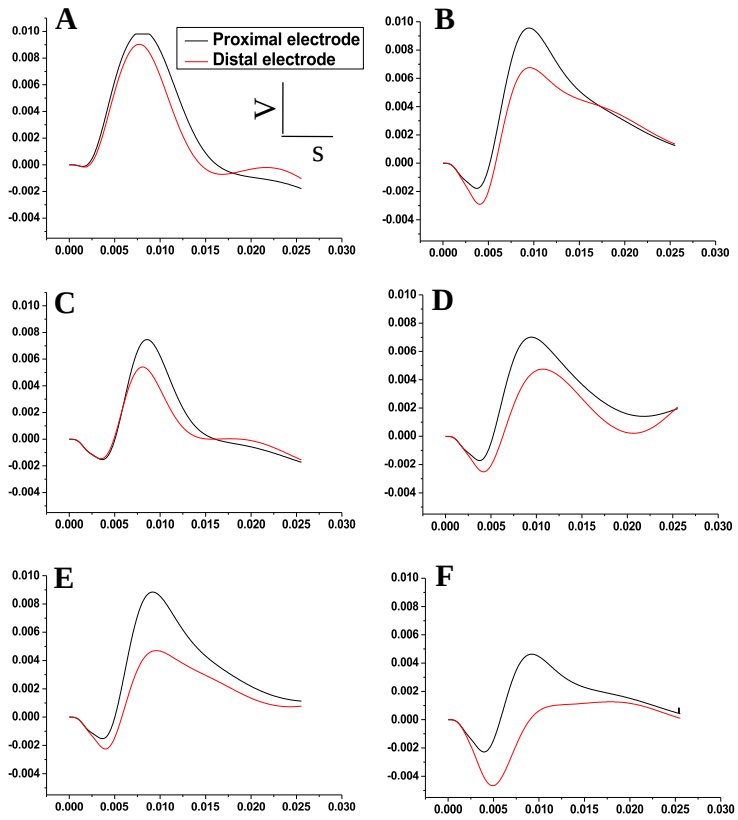

Figure 6.12: Effect of Nk-3FTx on isolated toad sciatic nerve. A-F: Representative plot of CAP of isolated sciatic nerve pre-treated with various concentration of Nk-3FTx (**A:** Nerve treated with Ringer's solution as control; **B:** 0.132nmol/L of Nk-3FTx; **C:** 1.319nmol/L of Nk-3FTx; **D:** 13.194nmol/L of Nk-3FTx; **E:** 131.194nmol/L of Nk-3FTx; **F:** 1319.4nmol/L of Nk-3FTx). CAP of sciatic nerve in absence of Nk-3FTx was considered as control. The values are ±SD of 3 independent experiments.

The amplitude of sciatic nerve was decreased in a dose-dependant manner. At 1319.4nmol/L of Nk-3FTx, the amplitude at proximal electrode was observed below 5mV (Figure 6.13A). In the similar concentration of Nk-3FTx, the amplitude at distal

electrode was significantly reduced to 0.87mV (Figure 6.13A). The amplitude at proximal and distal electrode helped in determining nerve conduction velocity (NCV) on the sciatic nerve. The NCV on the sciatic nerve got decreased at increasing concentration of Nk-3FTx. The NCV at the control nerve was calculated at 32.1ms (Figure 6.13B). The nerve treated with 1319.4nmol/L of Nk-3FTx was found to record a NCV of 16.77ms (Figure 6.13B).

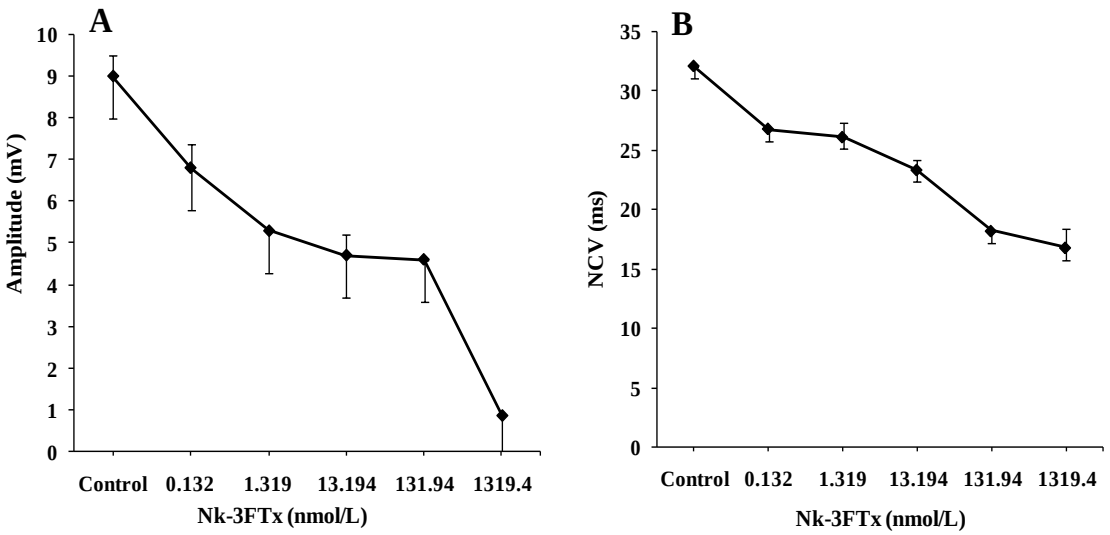

Figure 6.13: Effect of Nk-3FTx on amplitude and NCV of isolated toad sciatic nerve. A: Dose dependent effect of Nk-3FTx on amplitude of distal electrode on sciatic nerve. **B:** Dose dependent effect of Nk-3FTx on NCV (nerve conduction velocity) of sciatic nerve. Amplitude of CAP and NCV of sciatic nerve in absence of Nk-3FTx were considered as control. The values are ±SD of 3 independent experiments.

To understand the effect of Nk-3FTx on voltage-gated sodium and potassium channels on the sciatic nerve; specific channel blockers bupivacaine hydrochloride dihydrate (BH) and quinine hydrochloride dihydrate (QH) were used. Both the channel blockers were tested on dissected sciatic nerve at various concentrations (0.132nmol/L to 1319.4nmol/L) to determine the concentration at which amplitude of CAP is approximately 50%. At 13.194nmol/L concentration both channel blockers, BH and QH inhibited the CAP to ~50% as compared to nerve treated only with Ringer's solution (Figure 6.14A&B).

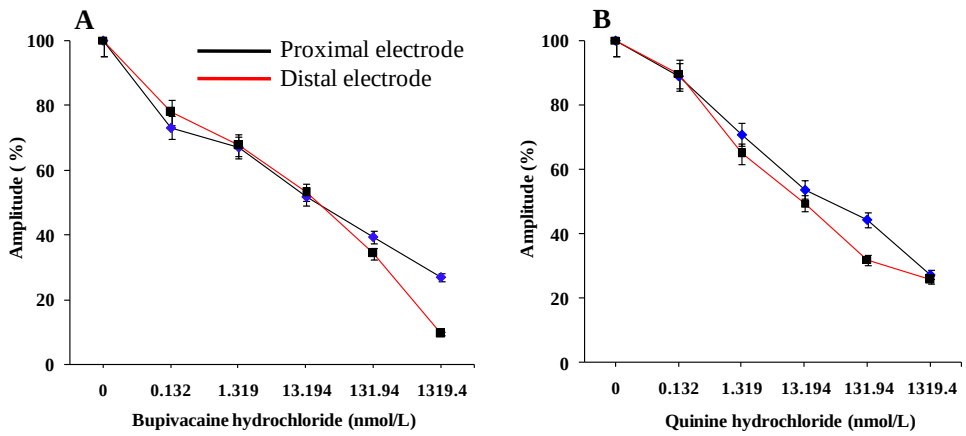

Figure 6.14: Standardization of sodium and potassium channel blockers on sciatic nerve. The dotted line corresponds to the concentration at which ~50% decrease in amplitude was observed. Amplitude of the nerve only treated with Ringer's solution was considered as 100%. Values shown are mean ±SD of three individual experiments.

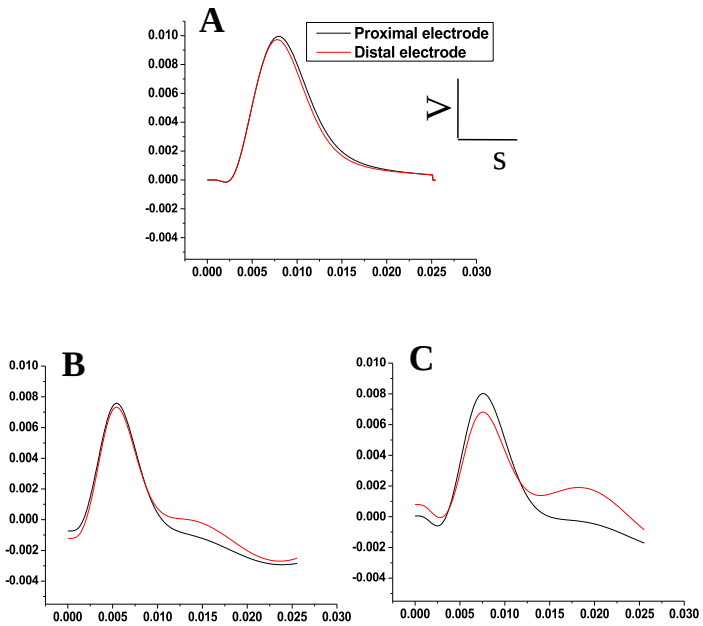

Figure 6.15: Determination of channel targeting by Nk-3FTx using sodium channel blocker. A-C: Representative plot of CAP of sciatic nerve pre-treated with specific channel blockers and Nk-3FTx (**A:** Nerve treated with Ringer's solution as control; **B:** 13.194nmol/L of bupivacaine hydrochloride (sodium channel blocker); **C:** 13.194nmol/L of Nk-3FTx. The experiment was performed in a sequential manner.

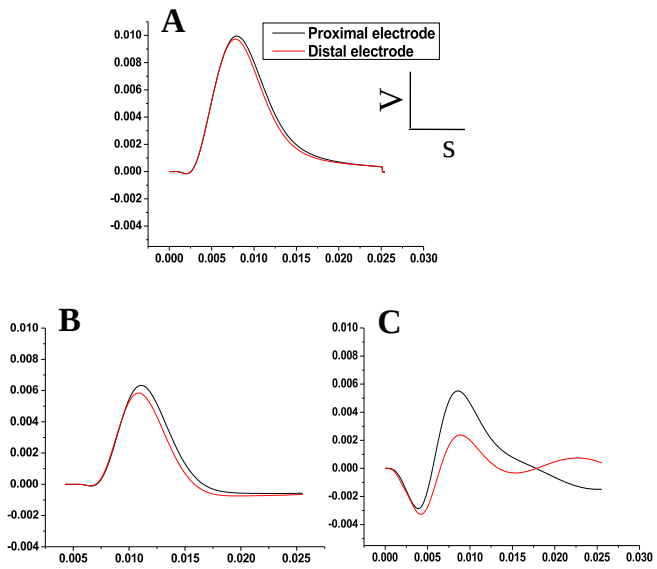

Figure 6.16: Determination of channel targeting by Nk-3FTx using potassium channel blockers. A-C: Representative plot of CAP of sciatic nerve pre-treated with specific channel blockers and Nk-3FTx (**A:** Nerve treated with Ringer's solution as control; **B:** 13.194nmol/L of quinine hydrochloride dihydrate (potassium channel blocker); **C:** 13.194nmol/L of Nk-3FTx). The values are ±SD of 3 independent experiments.

The consecutive treatment of nerve by BH and Nk-3FTx at a concentration of 13.194nmol/L did not decrease the amplitude of CAP on sciatic nerve (Figure 6.15C). However, nerve treated with QH and Nk-3FTx at similar concentration was found to decrease the amplitude of CAP up to 2.3mV (Figure 6.16C).

In the experiment with sodium channel blocker (BH) with Nk-3FTx, the amplitude or the ionic current did not reduce significantly. However, in case of potassium channel blocker (QH), the amplitude at distal electrode reduced significantly, inoic drop of ionic current. The repolarization phase is associated with the flow of K^+ ions. Delay in repolarization will form a flattened line suggesting lower ionic content. The amplitude of the experiment was determined which is represented by a bar diagram to understand the significance level of Nk-3FTx in potassium and sodium channel blocking (Figure 6.17).

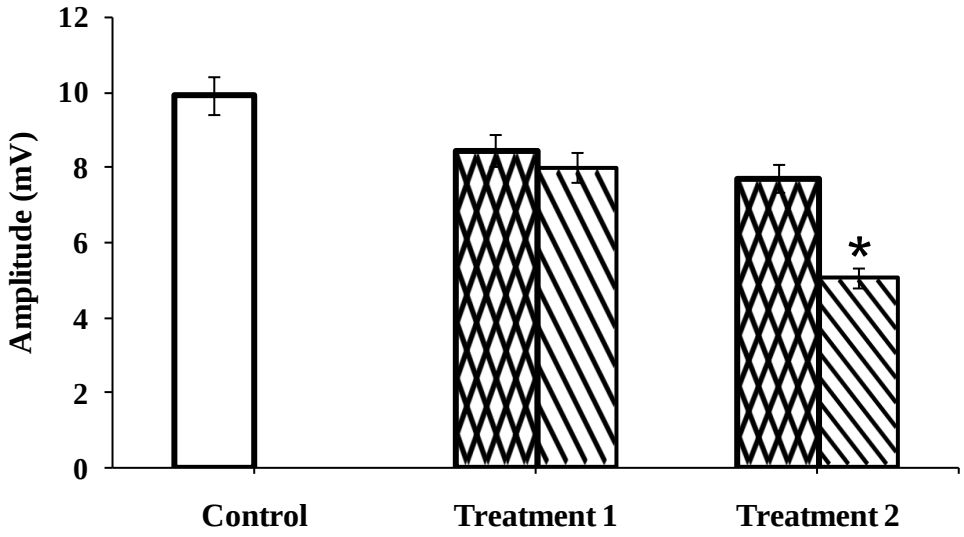

Figure 6.17: Bar diagram to show the effect of channel blockers and Nk-3FTx on CAP of sciatic nerve. CAP of sciatic nerve in absence of Nk-3FTx was considered as control. Treatment 1- nerve treated with BH and Nk-3FTx. Treatment 2- nerve treated with QH and Nk-3FTx. The values are ±SD of 3 independent experiments. The "*" indicates the *p*-value, * $p < 0.01$. The values shown are ±SD of 3 independent experiments.

6.3 Discussion

The complex mixture of snake venom contains a large number of pharmacologically active proteins and peptides which influences various physiological processes. Especially the elapid venom is a source of such toxins which target specifically the cardiovascular and nervous systems [217]. 3FTxs are target specific and is responsible for variety of pharmacological effects in the prey or victim [299].

Nk-3FTx was found to be devoid of PLA_2, indirect hemolytic, direct hemolytic activity and caseinolytic activity when tested *in-vitro*. However, previously it was reported that PLA_2 and low molecular weight proteins (6-8kDa) are held together by non-covalent interactions [321]. Hence, Nk-3FTx, a 3FTx (7579.5 ±0.591Da) was found to be independent of such interactions with higher molecular weight proteins such as PLA_2. The crude venom was found to have minimal direct and caseinolytic activity at higher concentrations which was previously discussed in section 3.2. However, Nk-3FTx was found to be devoid of any such activities even when tested at a similar amount. The *in-vivo* tests of edema inducing activity and hemorrhagic activity in experimental mice also correlated with the *in-vitro* assay in regard with

Nk-3FTx. Some three finger cytotoxins which are basic proteins with ~9 to 12 lysine and arginine residues are reported with hemolytic, cytotoxic and depolarization effect on myofibril membrane activities where mostly these residues are exposed to the surface of the toxin molecule and responsible for interaction [274, 322]. In the partial sequence of Nk-3FTx, we were able to determine 9 lysine and arginine residues, however Nk-3FTx was found to be devoid of hemolytic and hemorrhagic activities *in-vitro* and *in-vivo*. In the case of Nk-3FTx, these positively charged amino acid residues might not be exposed to the surface to interact and exhibit its activity.

Cytotoxic 3FTxs are reported to interact with lipid or biological membranes by a combination of electrostatic and hydrophobic forces with lipid and biological membranes which is due to the presence of positively charged residues at their surface [323]. It was previously shown that the CTs fuse with phospholipid vesicles [324, 325] which in turn induces separation in mixtures of anionic and zwitter ionic phophotidylcholines [326]. This fusion finally modifies the thermotropic characteristics of phospholipid membranes [327]. Nk-3FTx did not exhibit any cytotoxic effect on HEK 293 cell lines even when treated for 24hrs. Cell death in L6 cell line was observed at higher concentration of Nk-3FTx. However, the toxicity towards L6 cell lines was found negligible and cannot be accounted for cytotoxicity when compared with crude venom cytotoxic effects. In was reported that most cytotoxic 3FTx (CTXs A2, A3, and A4) have a spatial distribution of positively charged or continuous hydrophobic domains (~40% of the total molecular surface) [175]. These domains of cytotoxins cause distinct changes in their endocytotic pathway through sulfate glycoconjugate mediated processes [175, 328]. However, Nk-3FTx was devoid of such patches of hydrophobic amino acid residues. Also, interaction studies of CTs have divided them into P-type and S-type CTs i.e. presence of Pro31(30) or Ser29(28) residues respectively [329]. In Nk-3FTx these residues are replaced with Glu30, Arg31 and Leu28, His29. Hence, it can be concluded that Nk-3FTx do not belong to the subdivisions cytotoxins. However, studies reveals that distribution of non-polar residues on CTs depends not only on the amino acid composition but also the spatial structure and arrangement of the loops (especially loop I and loop II) within CTs for embedding into a membrane [330].

Snake venom enzymatic components such as PLA_2, LAAO, SVMP, nucleases and nucleotidases have remarkable effects in blood coagulation process [4]. Additionally,

there are three structurally different non-enzymatic proteins; disintegrins, snaclecs and 3FTxs which are reported to disrupt hemostasis [4]. Initially, a short chain neurotoxin analogue, "Mambin" from the venom of *Dendroaspis jamesonii* venom was reported with a RGD motif similar to disintigrins at the tip of one of the loop [154]. This small peptide fragment was found to inhibit platelet aggregation and integrin $\alpha_{IIb}\beta_3$-mediated platelet adhesion [331]. Another short chain neurotoxin γ-bungarotoxin from the venom of *Bungarus multicinctus* was reported to contain RGD motif in its molecular structure which acts as a weak antagonist of platelet aggregation [332]. However, in the partial amino acid sequence of Nk-3FTx, such functional motif for anticoagulant effect was found to be absent. The unidentified peptide fragments in Nk-3FTx also are not expected to contain the respective anticoagulant sequence as Nk-3FTx was not found to significantly reduce the coagulation time as observed in the *in-vitro* coagulation assays. In 2005, two synergistically acting anticoagulant 3FTxs were reported from the venom of *Hemachatus hemachatus* (African Ringhal's cobra) [150]. Hemextin A was found to exhibit mild anticoagulant activity, however its anticoagulant potency was found to be enhanced when hemextin B interacts and forms a complex (hemextin AB complex) [138, 150]. Morever hemextin B alone was not found to exhibit any anticoagulant property [150]. However, Nk-3FTx was found to be monomeric protein from our previous studies as explained in section 5.2.2 and 5.2.3.

The behavioral study of experimental animals upon crude venom injection suggests the neurotoxic potency of the crude *N. kaouthia* venom (section 3.1.5). In the present work, we tried to understand the influence of Nk-3FTx on mice behavioral reactions and symptomatology of intoxication. This has added for further systematic investigation of *in-vitro* biological activity. The behavioral study of experimental animal was performed to shed light on the nature of biological target of Nk-3FTx. The experimental animal was observed for paralysis and difficulties in breathing after 2.5-4hrs post injection. This decrease in locomotion and difficulties in breathing can be attributed to functional inhibition of nerve-muscle neurotransmission [333]. The *in-vitro* activity of a nontoxic antagonist of α7 and muscle-type nAChRs can be correlated with this particular activity of Nk-3FTx [164]. A recent work on a weak neurotoxin from *N. kaouthia* venom which affects the haemodynamic parameters in rats and mice shows the biological activity of the weak toxins *in-vivo* [334].

Nk-3FTx was found to be a non-conventional 3FTx from its partial amino acid sequence with maximum similarity with CM-9a. However, CM-9a was not found contain any of the functionally invariant neurotoxic amino acids [191]. But the behavioral changes in the experimental mice by Nk-3FTx suggest its neurotoxic property. Previously, a Weak neurotoxin from *N. kaouthia* was at micromolar concentration was found to irreversibly inhibit both muscle-type and neuronal α7 nAChR [164]. Other reports of weak toxin such as NNA2 from *N. naja atra* which was found to inhibit acetylcholine-induced muscle contractions [335], γ-bungarotoxin from *Bungarus multicinctus* binding to muscarinic acetylcholine receptors *in-vitro* [336], Wntx-5 from *N. sputatrix* and candoxin from *Bungarus candidus* inhibiting muscle-type and neuronal α7 nAChR were determined [135, 194]. However, variation in expression level and presence of unique toxins in these venoms cannot be denied. Snake venom 3FTxs are structurally similar but functionally diverse group of proteins [85, 314, 315] which binds to their targets through specific amino acid residues [85, 157]. The functional diversity in 3FTx toxin family is attributed to accelerated non-synonymous substitutions of nucleotides in the protein coding region especially in the loops [131]. In case of CM-9a, no structure function relationship had been carried out hence functional residues for its toxicity could not be obtained.

However, A weak tryptophan-containing neurotoxin was found to compete with α-bungarotoxin to bind with for binding to AChR [192] which later was found to interact with both nicotinic and muscirinic acetylcholine receptor [337]. Other comparisons of Nk-3FTx with various reported 3FTxs with multiple functional attributes did not offer any homology. A scorpion toxin, charybdotoxin was found to be similar with the N-terminal sequence of Nk-3FTx. However, charybdotoxin belongs to toxin_2 family protein with a different molecular structure [338]. Although, Nk-3FTx was found to carry some of the crucial amino acid such as Lys27 similar to charybdotoxin [339]; mere similarity in carrying crucial amino acid in structurally different proteins cannot be conclusive. Hence, further biological activity of Nk-3FTx was tested.

When CAP was recorded after treatment of sciatic nerve, changes in the recordings of distal electrodes were observed which suggests an effect on any of the voltage-gated ion channels on nerve axon. When an equimolar concentration of sodium channel blocker and Nk-3FTx was treated to the sciatic nerve, the waveform

obtained did not deviate from the nerve treated with only sodium channel blocker. However, when the equimolar concentration of potassium channel blocker and Nk-3FTx was treated to sciatic nerve, it reduced the amplitude significantly and also the repolarization was delayed. This might be due to binding of Nk-3FTx to potassium channels for the observed inhibition of CAP. Blocking of these channels would affect the nerve depolarization and repolarization causing delay in the signal transmission which can be correlated to neurotoxic activity [340]. Opening of Na^+ channel leads to depolarization whereas opening of K^+ channel leads to repolarization of the nerve during nerve transmission [341–343]. Potassium channels are abundant in all types of cells where they contribute to the regulation of membrane potential [320]. These potassium channels operate in response to changes in voltage, intracellular calcium ions or by specific ligands [320]. Venoms from various sources such as bees, scorpions, sea anemones and snakes modulate these ion channels. It was proposed that toxins find its binding site in external vestibute of the potassium channel and blocking the outer entry of potassium current [344]. The electrostatic interaction of negatively charged residues of the channel and positively charged residues of the toxin are reported to be crucial in this interaction [320, 344]. Sequence analysis of Nk-3FTx reveals presence of together 9 Lys and Arg residues in its partial primary structure. This higher number of positively charged amino acid residues might be involved in interaction with potassium ion channel and hence blocking the ion current. However, location of these residues on Nk-3FTx needs to be deciphered to understand if any interaction persists between the potassium ion channel and Nk-3FTx.

The potassium channels are reported as α4β4 complexes where α-subunits are integral membrane protein which contains the ion pore. The β-subunits are peripheral proteins which modulates the α-subunit rate of inactivation [345–347]. Snake venom dendrotoxins (DTXs) from *Dendroaspis p. polylepsis* venom are reportedly accounted for voltage gated potassium channel blocking. Among several DTXs, dendrotoxin-I (DTX-I) from the venom of black mamba, *Dendroaspis polylepis polylepis* [348], a-dendrotoxin (a-DTX) from the venom of green mamba, *Dendroaspis angusticeps* [349] and dendrotoxin-K (DTX-K) from the venom of *Dendroaspis polylepis* [348] are found to specifically target neuronal voltage-sensitive potassium channels with high affinities. DTXs are called as the class I toxins. For DTX-I, it was reported that the modification of Lys5, Lys19, or Lys29 led to decrease in binding

affinity to potassium ion channel by a factor of 20, 11, or 1300, respectively. However, in Nk-3FTx, the amino acid residues were replaced with Asp5, Asp19 and Leu29. The class II toxins which targets the voltage gated potassium channels are isolated from sea anemones, BgK from *Bunodosoma granulifera* [350] and ShK from *Stichodactyla helianthus* [351] .

Charybdotoxin, the third class of potassium channel blockers are extensively studied toxins, isolated from the venom of the Israel scorpion, *Leiurus quinguestriatus hebraeus* [339]. This toxin was reported to inhibit several types of potassium channels including Ca^{2+} activated channels from many vertebrate tissues, aplysia neurons, red blood cells, and voltage-dependent potassium channels from lymophocytes [339]. The inhibition of voltage-gated potassium channels by charybdotoxin is through a dyad consisting of positively charged Lys residue at 27th or 23th position [352] and hydrophobic residue (Tyr36) at the C-terminal end remain exposed [352]. Mutant cycle analysis along with *in-silico* studies have shown that mutation of Lys31 and Lys32 position of ChTx decreased binding affinity to wild type IKCa1 channel and Kv 1.3 potassium channel by ~47 fold and ~350 fold [353]. In Nk-3FTx, the Lys residue is also found at 27th position and Phe (34th position) at C-terminus of the protein is present. Though there is a substitution of Tyr to Phe and the position is shifted however, it might be involved in interaction with the channel for inhibiting the CAP. However, further analysis needs to be carried out to elucidate the molecular mechanism of interaction.