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Technical and Scientific Report

On

A GRANT OF RESEARCH/DEVELOPMENT PROJECTS UNDER R&T SCHEME OF DRDO

(Cost Below 10 lacs)

Title:

Isolation, Characterization, possible use and clinical manifestation of a Neurotoxin from Snake venom (*Naja siamensis*) with special reference to warfare importance neuro- toxin as a model

Submitted to
Defence Research Laboratory
Tezpur
DEFENCE RESEARCH & DEVELOPMENT ORGANISATION
Ministry of Defence, Govt. of India

By
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GRANT OF RESEARCH/DEVELOPMENT
PROJECTS UNDER R&T SCHEME OF DRDO
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1. Title of the research/development:
   Isolation, Characterization, possible use and clinical manifestation of a Neurotoxin from Snake venom (*Naja siamensis*) with special reference to warfare importance neuro- toxin as a model

2. Name of the Investigator(s) : Dr. Robin Doley
   Designation (s) Associate Professor
   Name of the Institution Address : Department of Molecular Biology and Biotechnology, Tezpur University

3. Project sanction No. :DRLT-P1-2010/Task-49 (27/02/2012)

4. Total grant : Rs. 9.8 lakhs

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   (Date of release: 09/07/12)
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5. (i) Duration of the research development proposal : 3 years
   (ii) Date of commencement : February 2012
   (iii) Date of completion : February 2015

6. (i) Deptt. of the Institution where the R&T project will be carried out : Department of Molecular Biology and Biotechnology, Tezpur University

   (ii) Domain Scientist : Dr. P. Chottopadhya, Scientist D, DRL, Tezpur

7. Objectives of the Proposal
   I. Isolation of neurotoxin from the crude venom of Naja siamensis
   II. Biochemical characterization of the isolated neurotoxin
   III. Pharmacological characterization of the isolated neurotoxin
   IV. Prespective in warfare similar type neuro-toxin
   V. Drug and subsidiary clinical manifestation

Objectives

1. Isolation of neurotoxin from the crude venom of *Naja siamensis*
2. Biochemical characterization of the isolated neurotoxin
3. Pharmacological characterization of the isolated neurotoxin
4. Prespective in warfare similar type neuro-toxin
5. Drug and subsidiary clinical manifestation

Objective 1: Isolation of neurotoxin from the crude venom of *Naja siamensis*

1.1 Material and Methods

1.1.1 SDS-PAGE of *Naja siamensis* venom

SDS-PAGE was carried according to the method of Laemmli (Laemmli, 1970). Crude venom or venom fractions were treated with β-mercaptoethanol and loaded into the gel. The electrophoretic run was set at 15 volts until sample enters the stacking gel and 25 volts for the resolving gel. Gel was stained with 0.25% of Coomassie Brilliant Blue R-250 in methanol:water:acetic acid (40:50:10) for 3 h followed by destaining for 4 h using methanol:water:acetic acid (40:50:10). After detaining protein bands were observed were compared with the molecular marker run along with the samples.

1.1.2 Size fractionation of *Naja siamensis* venom by gel filtration chromatography

50 mg of *Naja siamensis* crude venom was dissolved in 2 ml of 20 mM Tris-Cl pH 7.4 and filtered through a 0.2 μm syringe filter. 1 ml of the dissolved sample was loaded into sephadex G-50 gel filtration column. Elution was carried out using HPLC (Waters). Elution was monitored at 215 and 280 nm and the peaks were collected separately.

1.1.3 Reverse phase HPLC

Reverse phase high performance liquid chromatography (RP-HPLC) was carried out using symmetry C18 column (Waters), 3.6*250mm, 300 μ column. The elution was carried out at 0.5 ml/min and the gradient was 20-40% buffer B in 15-45 min. Buffer A was 0.1% TFA and buffer B was 0.1% TFA in 80% Acetonitrile. The elution was monitored at 215 and 280 nm simultaneously. Individual protein peaks were collected separately.

1.2 Results and Discussion

Snake venom is a complex mixture of proteins and polypeptides (Doley and Kini, 2009; Doley et al., 2009b). To check the complexity of *Naja siamensis* venom, the crude venom was run on SDS-PAGE under both reduced and non reduced condition (Figure 1). The SDS-PAGE profile reveals that the majority of the venom contains 7-15 kDa bands indicating that Phospholipase A_2 and three-finger toxin are the major components present in this venom. Three-finger toxin are

responsible for neurotoxin effect of the crude venom. To purify the three-finger toxin present in this venom, it was subjected to size exclusion chromatography using sephadex G-50 column.

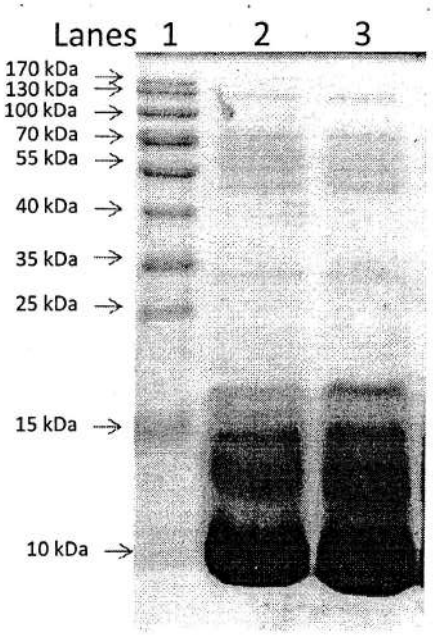

Figure 1: 12.5% SDS-PAGE profile of crude *Naja siamensis* venom. Lane 1: molecular weight marker; lane 2: 30 µg crude venom under reduced condition and lane 3: 60 µg crude venom under reduced condition

50 mg of *Naja siamensis* crude venom was dissolved in 5 ml of 20 mM Tris-Cl pH 7.4 and filtered through a 0.2 µm syringe filter. 1 ml of the dissolved sample was loaded into sephadex G-50 gel filtration column. Elution was carried out using HPLC (Waters). Elution was monitored at 215 and 280 nm and the peaks were collected separately (Figure 2). Fractionation reveals eight major peaks (Fra I-VIII). The total protein content of the pooled peaks were estimated by Lowry's method (Table 1). 86.8% from the total protein loaded into the gel was recovered. The fractions were run on SDS-PAGE to check the purity of fractionation (Figure 3). SDS-PAGE under reduced condition reveals that the Frac V, (8.8%) contain majority of the three-finger toxin (6-10 kDa mol. weight) shown in red box. Hence for further purification of the neurotoxin Fra V was selected.

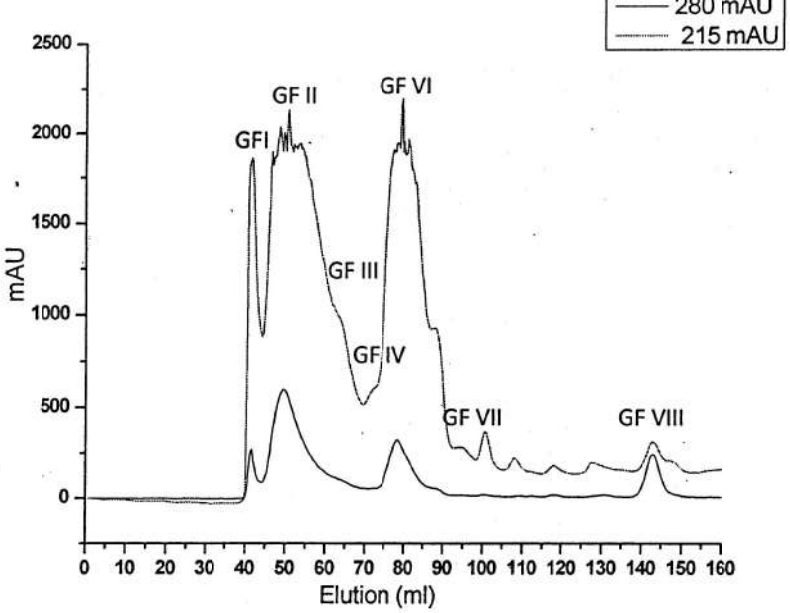

Figure 2. Gel filtration profile of *Naja siamensis* venom on Superdex 30 column.

Table 1: Summary of gel filtration chromatography of *Naja siamensis* venom.

Fraction no	**Volume (ml)**	**Amount of protein (mg)**	**% recovery**
GF I	2.5	2.6	10.4
GF II	1	2.1	8.4
GF III	1.5	1.9	7.6
GF IV	6	4.7	18.8
GF V	3.5	2.2	8.8
GF VI	6.5	5	20.0
GF VII	4	1.9	7.6
GF VIII	3	1.3	5.2
		% total recovery	**86.8**

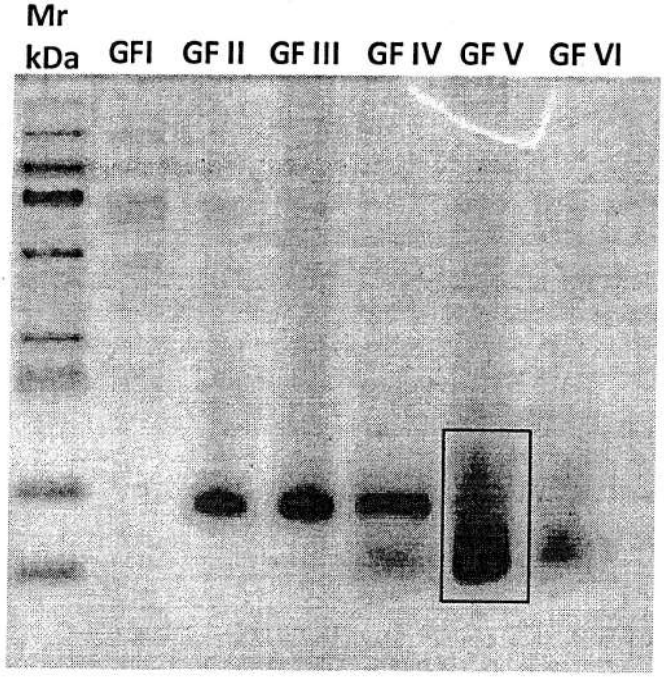

Figure 3: 12.5% SDS-PAGE profile of *Naja siamensis* venom gel filtration fraction under reduced condition. Lane 1: 30 μg crude venom Lane 2: molecular weight marker; lane 3-8: gel filtration fractions.

Fra V was selected for purification of the neurotoxin as the SDS-PAGE reveals the presence of these toxin which varies in molecular weight between 6-7 kDa. Fractionation was carried out as described in the method section. 2 mg of the Fra V was loaded into the column was carried out The elution was carried out at 0.5 ml/min and the gradient was 20-40% buffer B in 15-45 min. Buffer A was 0.1% TFA and buffer B was 0.1% TFA in 80% Acetonitrile. The elution was monitored at 215 and 280 nm simultaneously. Eight individual protein peaks were obtained (RP-I to RP-VIII) during the fractionation (Figure 4). SDS-PAGE of the peaks under reduced condition showed that the peaks are purified to homogeneity (Figure 5). All the peaks were subjected various biochemical and pharmacological test to confirm the identity.

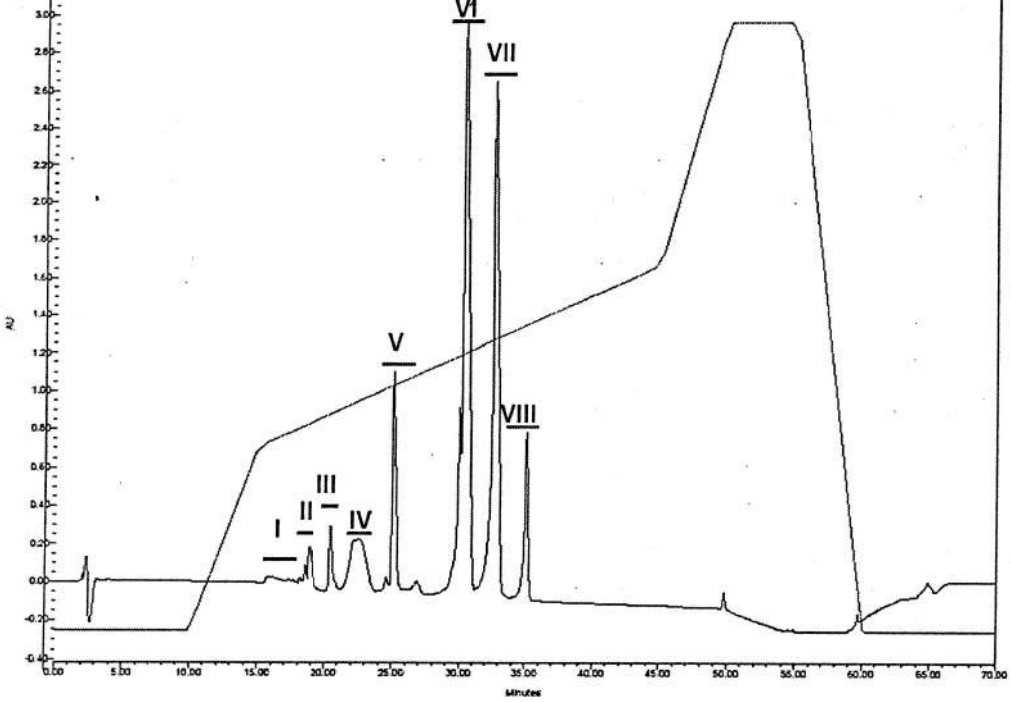

Figure 4: RP-HPLC profile of Fra V on C18 column. I-VIII indicates the peak number.

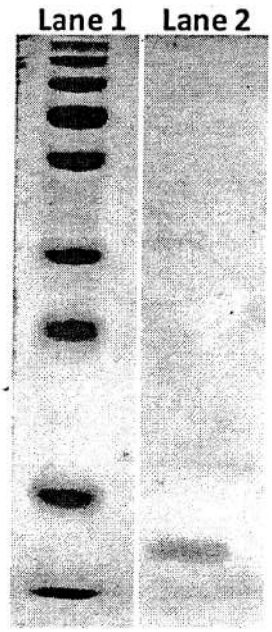

Figure 5: SDS PAGE of RP HPLC fractions of Fra V, **Lane 1:** prestained molecular weight marker (5µl), **Lane 2:** reverse phase fractions RP-V.

2.1 Material and Methods

2.1.1 N-terminal sequencing

N-terminal sequencing was determined by automated Edman degradation process using PPSQ 31 (Shimadzu, USA) with phenlythiohydantion (PTH) derivative analyzer. Briefly, Lyophilized protein was resuspended in 100µl of denaturant solution (6.0 M guanidinium hydrochloride, 0.13M Tris, 1mM EDTA, pH-8.0) containing 0.07M β-mercaptoethanol and incubated at 37°C for 2hrs. Subsequently blocking of sulfhydral groups was done by adding 4-vinylpyridine and incubated at room temperature. The protein was desalted by RP HPLC after 2hr and subjected to N-terminal sequencing. The retention time of the amino acids were compared with the standard amino acids to identify the amino acid sequence.

2.1.2 Molecular weight determination

Molecular mass of the purified protein was determined by electrospray ionization mass spectrometry (ESI-MS) using an LC Q fleet Ion Trap, Thermo Scientific (USA) mass spectrometry system. 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 Results and Discussion

N-terminal sequencing of the RP-V was performed by Edman degradation using an automated Protein sequencer PPSQ 31A (Shimadzu Asia Pacific) to establish the identity of the purified protein. About 89µg (6545.56 picomoles) of purified protein was dried on PVDF membrane and loaded onto the sequencer. Sequencing was done for 50 cycles and 35 readable amino acid peaks were obtained. The sequence obtained was subjected to BLAST search and found to be homologues to three-finger toxin family of snake venom proteins. The sequences showing 100% similarity to database was manually aligned and the conserved Cys residues are highlighted in red letters (Figure 6). From this it is confirmed that the purified protein is a three-finger toxin which are known to be neurotoxin. Three-finger toxins are low molecular weight protein with a molecular mass of 6-9 kDa. These highly conserved molecules have three beta sheets emerging

from the core hydrophobic region resembling that of three fingers of our hand. Though they have similar structure however they differ in their functional properties (Kini, 2002). The amino acid substitutions in the loops are mainly responsible for the observed functional diversity in this family of snake venom proteins (Doley et al., 2009a; Kini and Doley, 2010). Hence it is important to understand the structural and functional properties of this family of protein.

Snake	Name	Acc. No	Amino acid Sequence	
Naja siamensis	unnamed	Present study	LTXLNXPEMFXGKFQIXRNGEKIXFKKLHQRRPFS----------	
Naja kaouthia	CM-9a	P25679	LTCLNCPEMFCGKFQICRNGEKICFKKLHQRRPLS-RYIRGCADTCPVGYPKEMIECCSTDRCNR	64
Naja naja	unnamed	CAA04578	LTCLNCPEMFCGKFQICRNGEKICFKKLHQRRPFSLRYIRGAATCPETKPRDMVECCSTDRCNR	65
Naja naja	neurotoxin	P60814	LTCLNCPEMFCGKFQICRNGEKICFKKLHQRRPFSLRYIRGAATCPGTKPRDMVECCSTDRCNR	65
Naja atra	neurotoxin	Q9YGI4	LTCLNCPEMFCGKFQICRNGEKICFKKLHQRRPFSLRYIRGAATCPETKPRDMVECCSTDRCNR	65
Naja kaouthia	neurotoxin	P82935	LTCLNCPEMFCGKFQICRNGEKICFKKLHQRRPLSWRYIRGADTCPVGKPYEMIECCSTDKCNR	65
Naja sputatrix	neurotoxin	O42255	LTCLNCPEMFCGKFQTCRNGEKICFKKLQQRRPFSLRYIRGAATCPGTKPRDMVECCSTDRCNR	65

Figure 6: Multiple alignment of the N-terminal sequences of isolated protein from *Naja saimensis* with other three finger toxins. X represents cysteine residues which are not detected Edman degradation protocol.

To determine the mass and purity of the protein, Rp-HPLC purified protein was subjected to electrospray ionization mass spectrometry (Accela LCQ fleet, Thermo scientific). 50%AcCN –0.1%TFA was used as the solvent at a flow rate of 200µl/min. The mass obtained was then analysed by the software Promass for xcalibur. The spectrum shows a series of multiply charged ions, corresponding to a single, homogenous peptide. The reconstructed mass spectrum showed a molecular weight of 7579.5±0.591 Da (Figure 7). This molecular weight is in the rage of three-finger toxins of snake venom proteins.

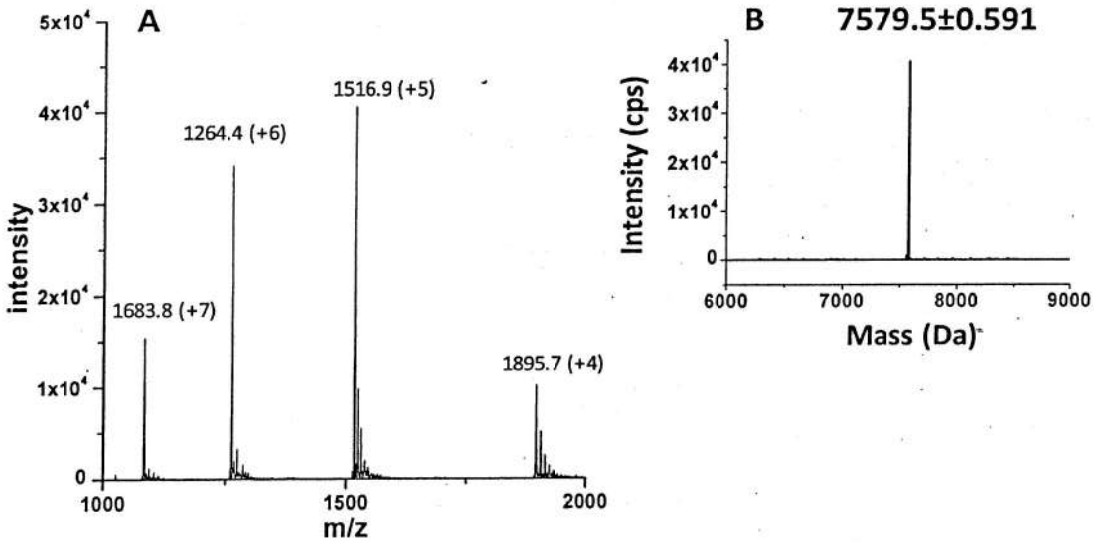

Figure 7: A: ESI-MS profile of the purified RP-HPLC fraction. **B:** Reconstructed mass spectrum of the protein, CPS = counts/s; Da = dalton.

Objective 3: Pharmacological characterization of the isolated neurotoxin

3.1 Materials and Methods

Animals

Swiss albino mice of both sexes were supplied by central animal facility, University of Mysore. The animals were housed at 25± 3°C on a 12 hr light/dark cycle with access to food and water and were used for *in-vivo* studies. The studies followed reviewed procedures and approved guidelines from Animal Ethical Committee Protocol (University of Mysore, Mysore, India, Proposal no.UOM/IAEC/25/2011).

3.1.1 Phospholipase A_2 activity:

Phopsholipase A_2 activity of the crude venom as well as the various fractions obtained after gel filtration was assayed according to the method of Joubert and Taaljard 1980 with slight modification (Doley and Mukherjee, 2003) using egg yolk as source of phospholipid. One unit of PLA_2 activity is defined as amount of protein with decrease of 0.01 absorbance at 740 nm after 10 mins.

3.1.2 Anticoagulant activity

The anticoagulant activity of the crude venom along with the venom fractions obtained after gel filtration of crude venom were assayed according to the method of Joseph *et. al.* (Joseph et al., 2002). The venom sample with fresh goat plasma was incubated with 20mM Tris-Cl pH 7.4 and 100mM $CaCl_2$ and the clotting time was recorded. Normal clotting time was determined by incubating the plasma with buffer and $CaCl_2$.

3.1.3 Recalcification time

Recalcification time of goat PPP was measured using coagulation analyzer. Various amount of venom in 50µl of 20mM Tris Cl, pH 7.4 was pre-incubated with 150µl of goat PPP at 37°C for 2min and 75µl of 50mM $CaCl_2$ was added to initiate the clot formation. The clotting time with buffer was considered as normal clotting time. The results are as mean ±SD of three experiments.

3.1.4 Prothrombin time (PT) test

Prothrombin time was measured using PT reagent (UNIPLASTIN) obtained from Tulip Diagnostics (India). Various amount of venom in 50µl of 20mM TrisCl, pH 7.4 was pre-incubated with 100µl of goat PPP at 37°C for 2min and 100µl of PT reagent was added to initiate the clot formation. The clotting time with buffer was considered as normal clotting time. The results are mean ±SD of three experiments.

3.1.5 Activated partial thrombin time (APTT) test

Activated partial thrombin time was determined using APTT reagent (LIQUICELIN-E) obtained from Tulip Diagnostics (India) on a coagulation analyzer. Various amount of venom in 50µl 20mM Tris Cl, pH 7.4 was incubated with 100µl of goat PPP and 40µl of APTT reagent for 3min at 37°C. The clot formation was initiated by adding 50µl of 25mM $CaCl_2$. The clot formation time with buffer was considered as normal clotting time. The results are mean ±SD of three experiments.

3.1.6 Hemolytic activity

The haemolytic activity of the crude venom as well as the gel filtration fractions were carried out according to the method describe by Jiang and Co-workers (Jiang et al., 1989). 10% RBC was incubated with different venom fractions along with 0.9% NaCl as buffer at 37^0C for 1hr in water bath. Distilled water with 10% RBC was taken as positive control. The reaction mixtures were centrifuged at 10,000rpm for 10mins and OD was measured at 540 nm in thermo scientific spectrophotometer.

3.1.7 Indirect haemolytic activity

The percentage of indirect haemolytic activity of the venom fractions and crude venom were determined according to the method of Mukherjee and others (Mukherjee et al., 1998). 10% of RBC along with the venom fractions were incubated with 0.9% NaCl and egg yolk as the phospholipid source at 37^0C for 1hr in water bath. 10% RBC in water was taken as the positive control. The reaction mixtures were then centrifuged at 10,000 rpm for 10 mins and OD was measured at 540nm in thermo scientific spectrophotometer.

3.1.8 Bactericidal activity

The bactericidal activity of the crude venom and venom fractions were carried out on both gram positive (*Bacillus sp.*) and gram negative bacteria (*E. coli.*) were spread on fresh LBA (luria

bertani agar) plates and holes were punched on each plate. 50ug/ml of ampicillin was used as the positive control and 20mM Tris -Cl buffer as the negative control. The venom fractions were put in the respective wells followed by the incubation of the plates at 37^0C for 4 hrs.

3.1.9 Cytotoxicity

Cytotoxicity of the purified protein was determined using HEK 293 (Human embryonic kidney cells) cells. Exponentially growing cells (10^6 cells /ml) were incubated with various concentrations of purified protein for 24hrs at 37°C with 5% CO2 in a CO2 incubator (Eppendorf, USA)). Cell count was done by vital staining with trypan blue dye and finally counting on a haemocytometer. MTT assay was performed to observe the cytotoxic effect of protein. For MTT assay, 20µl of MTT (5mg/ml) was incubated for 3.5hrs prior to the end of 24hrs of incubation with the protein at 37°C in a biosafety cabinet. The formazan granules formed by viable cells are dissolved in 150µl MTT solvent and agitated for 15mins. Absorbance at 570nm was estimated using a MultiSkan Go spectrophotometer (ThermoScientific, USA). The cells were observed under an Axio Vert.A1 (Zeiss, USA), inverted microscope for any changes in the morphology and viability.

3.2 Results and Discussion

Some of the biological activities of the purified protein is summarized in table 2. The purified protein did not exhibit the tested biological activities. However when the protein was injected to experimental mice it showed neurotoxic symptoms like difficulty in breathing, paralysis and constant drinking of water.

Table 2: Summary of some biological activities of the purified three-finger toxin

Activity	Crude venom	Purified three-finger toxin
LD_{50}	0.148 mg/kg	ND
PLA_2 activity	7.9±0.24$^{\Psi}$	Nil
Direct hemolytic (100µg venom)	1.4±0.51%	Nil
Indirect hemolytic (1µg venom)	23.0±3%	Nil
Caseinolytic activity (100µg venom)	0.14±0.02*	ND
Creatine kinase (CK) (15µg i.m. injection)	6.6±0.2 U/l	Nil
Lactate dehydrogenase (LDH) (15µg i.m. injection)	26.3±2.3U/l	Nil
Minimum edema dose (MED)	11.2±0.18 µg	Nil
Antibacterial activity	Nil	Nil
Neurotoxic symptoms	Present	Present

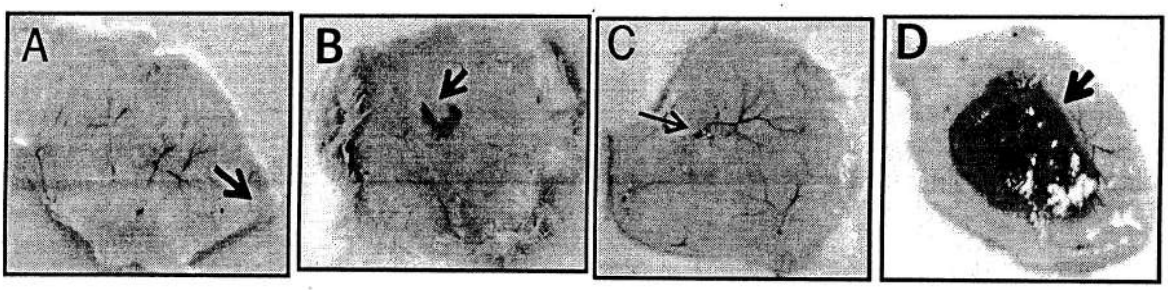

Figure 8: Haemorraghic activity. A: Control (30µl of saline), **B:** Crude venom (15µg), **C:** Saw scaled viper venom (3µg) (Positive control), **D:** Purified protein (20µg). The arrow indicates site of injection.

Moreover the purified protein did not exhibit any PLA_2 activity. Hence the purified protein is devoid of any contamination and does not form any complexes with PLA_2 enzymes (Doley and Kini ,2009). Some of the snake venom proteins are known to affect the coagulation cascade of prey/victim leading to excessive bleeding or formation of thrombus for immediate immobilization. Three finger toxin with anticoagulant activity has been reported from cobra venom (Banerjee et al., 2005). To check the effect of the purified protein on blood coagulation recalcifications, prothrombin and activated partial thrombin time was tested using goat plasma. The purified protein did not show significant delay in the clotting time as compared to the crude venom and gel filtration peak when tested for recalcification time (Figure 9). Similarly the PT was also unaffected (Figure 10). However there was slight increase in the APTT as compared to the crude venom (Figure 11).

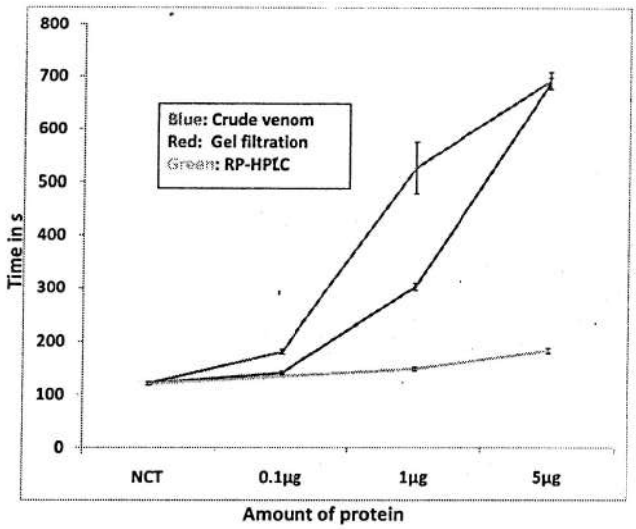

Figure 9: Effect of crude venom, gel filtration peak and RP-HPLC purified protein on recalcification time of goat plasma. Results are mean ±SD of three independent experiments.

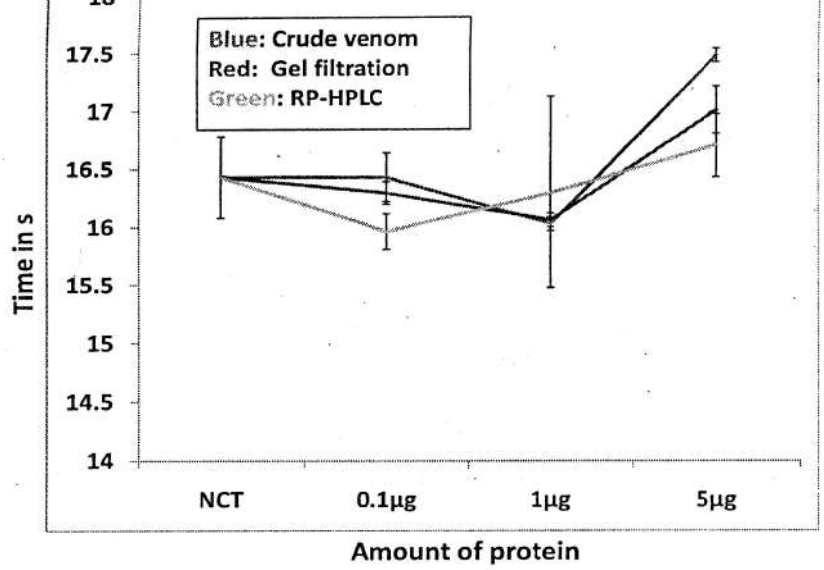

Figure 10: Effect of crude venom, gel filtration peak and RP-HPLC purified protein on prothrombin time of goat plasma. Results are mean ±SD of three independent experiments.

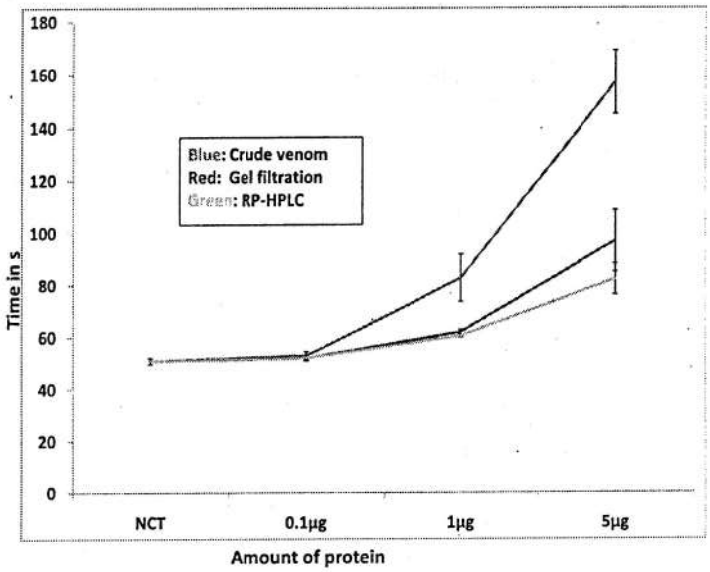

Figure 11: Effect of crude venom, gel filtration peak and RP-HPLC purified protein on APTT of goat plasma. Results are mean ±SD of three independent experiments.

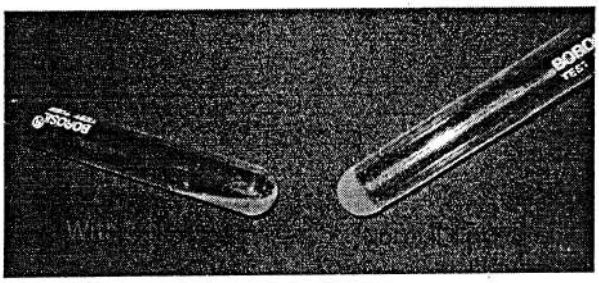

Figure 12: Representative of test tube showing coagulated and non-coagulated goat plasma.

Three finger toxins are well known cytotoxin, damaging the cardic cells, RBC and others. To check if the purified protein damages the RBC, proteins were incubated with washed RBC for 1 hr at 37 °C. Even after 1 hour release of heamoglobin from the RBC was not detected. The bacterial membrane damaging activity was also studied using *E. coli*. The well diffusion method did not result into any antibacterial activity as compared to the ampicillin (Figure 13). Moreover it did not show any cytotoxic activity on human cell lines (Figure 14). Hence the purified three finger is a non-cytotoxic protein which can be explored for therapeutic application.

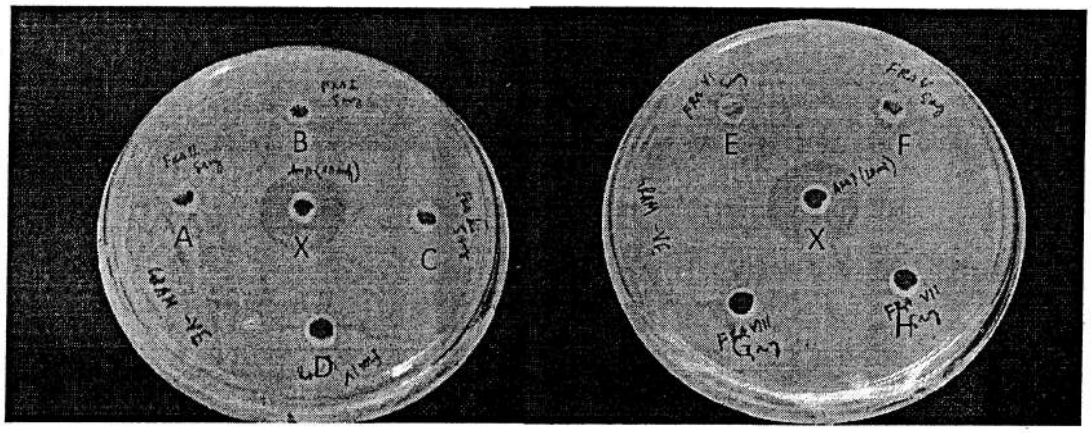

Figure 13: Bacterial plate showing zone of clearance. Ampicillin (100 µg/ml) was used as positive control (central hole). The peripheral holes are the gel filtration fractions (RP-I-RP-VIII)

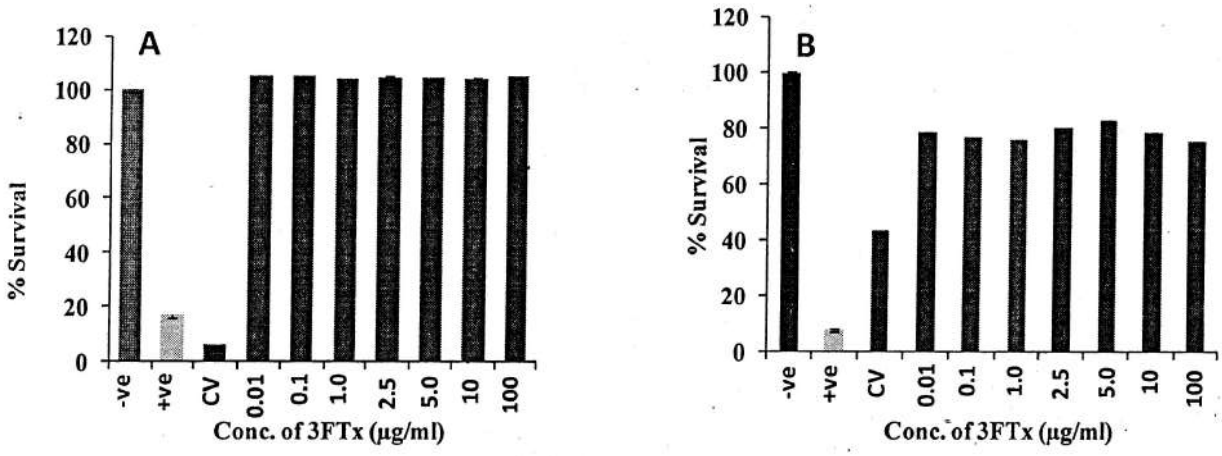

Figure 14: Effect of purified 3FTx on HEK (A) and L6 (B) Cell lines. Cells (10^6/ml) were treated with various concentration of 3FTx, crude *Naja kaouthia* venom (100 ng/ml), $AgNO_3$ (300mM) (+ve) and only media (-ve). After treatment, MTT assay was done to assess the number of viable cells. The experiment was performed in triplicates and values shown are ±SD.

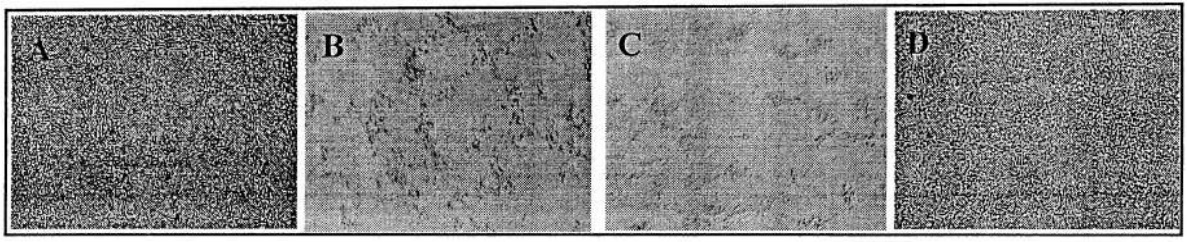

Figure 15: Representative of cell images showing **A)** Cells treated with 0.9% NaCl (control) **B)** 300mM AgNO3 (positive control) **C)** Treatment of cells with 100ng/ml of crude venom **D)** Effect of purified protein (100ng/ml).

Objective 4: Perspective in warfare similar type neuro-toxin

4.1 Material and methods

4.1.1 Preparation of Sciatic nerve from common Asian toad (*Duttaphrynus melanostictus*)

The study was carried out with toad as an animal model. Efforts were done to minimize both animal sufferings and animal number. Common Asian toads *(Duttaphrynus melanostictus)* weighing 30-45gm of either sex were decapitated and then pithed. The sciatic nerve (length 3-5cm; 0.5-1mm diameter) was dissected from lumber plexus to the knee in Ringer solution. During dissection care was taken so that the nerve must not get touched by fingers, cut muscle, frog skin or with any metal instruments. The nerve was kept moist all the time during the procedure. Further the 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 in moist condition using Ringers solution.

4.1.2 Recording of CAP and calculation of NCV from sciatic nerve

Determination of compound action potential (CAP) and nerve conduction velocity (NCV) was studied in dose dependant manner. The dissected sciatic nerve was treated with various concentrations of purified (0.0001 µg/ml to 10µg/ml) for 2 mins. The treated nerve where placed in the nerve chamber and the CAP was measured (AD Instruments (Powerlabs, Sydney, Australia). After recording the CAP of purified protein treated nerve, it was washed extensively with ringer's solution and checked again for any changes in CAP. Nerve with only ringer's solution was considered as control and compared with treated CAP for any significant changes. The experiment was performed in triplicates and standard deviation was determined. NCV of the sciatic nerve is related to the nerve diameter of a given type. It is deduced from the latency of the stimulated peak by the following formulae:

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

4.2 Results and Discussion

Snakes capture their prey by immediate immobilization by targeting their neuro-mascular system. Snake venom protein families like PLA_2 and 3FTx are mainly responsible for this actvitiy. Several proteins from this familes have been reported to exhibit neurotoxicity in victims/prey (Abe et al., 1977; Bhat et al., 1991; Chang et al., 1973; Doley et al. 2009b; Krizaj et al., 1997; Nirthanan and Gwee, 2004). Neurotoxic three-finger toxins interfere with cholinergic transmission at various post-synaptic sites in the peripheral and central nervous systems. Based on their receptor selectivity, they can be broadly classified as curaremimetic or α-neurotoxins, κ-neurotoxins and muscarinic toxins that target muscle nAChR, neuronal nAChR and various subtypes of muscarinic receptors, respectively (Kini and Doley ,2010). There is a group of 3FTxs, which include calciseptine and FS2, that specifically block L-type calcium channels (Albrand et al., 1995; de Weille et al., 1991). These polypeptides are structurally similar to short-chain neurotoxins, in number of amino acid residues and conserved disulphide bridges. They bind to the 1,4-dihydropyridine binding site of the L-type calcium channels and physically block the calcium currents (Yasuda et al., 1993). In the present study we investigated the effect of purified protein on compound action potential (CAP) and nerve conduction velocity (NCV) of frog sciatic nerve. CAP describes the excitability of the bundles of nerves present in the sciatic nerve in response to external stimuli. The opening and closing of the ion channels present in the nerve is responsible for propagation of the action potential. Any blockage in the ion channel would lead to inhibition of the NCV. The sciatic nerve when treated with purified protein lead to decrease in NCV (Figure 16). With increase in concentration of the protein the NCV was found to decrease dose dependently (Figure 17).

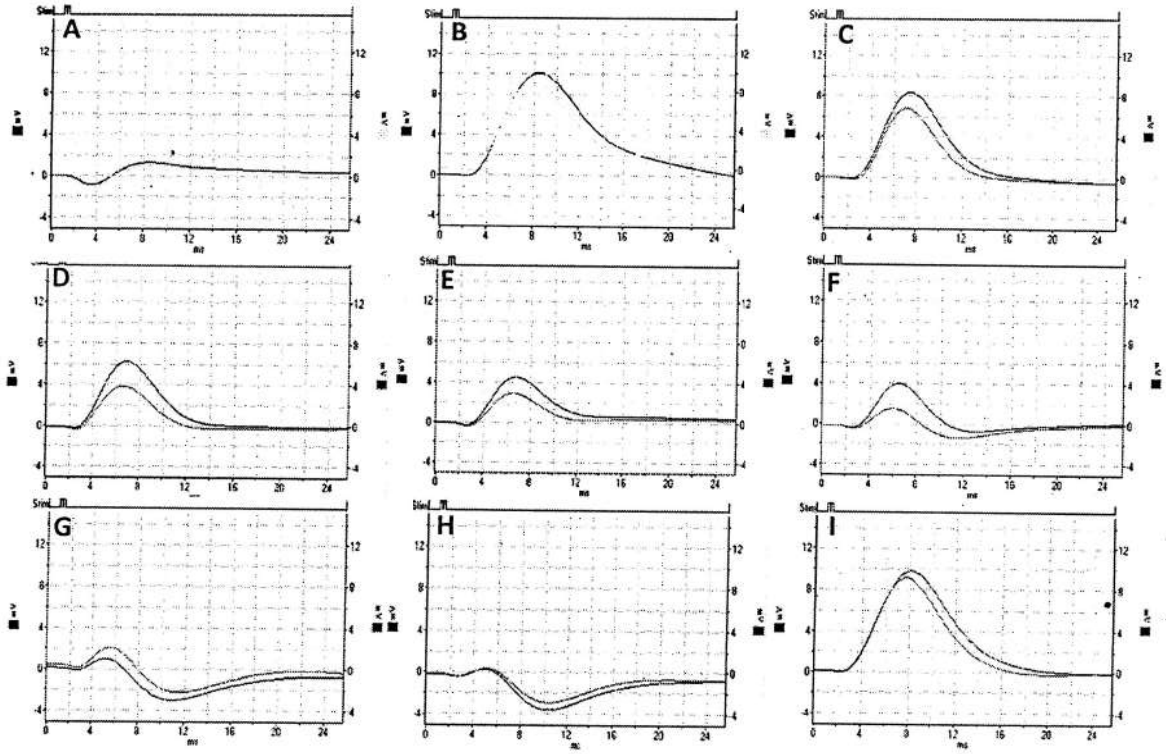

Figure 16: Effect of purified protein on compound action potential (CAP) of toad sciatic nerve. A: Potential difference in the empty nerve chamber, B: Nerve only treated with ringer's solution served as control, C-H: Treatment of nerve with purified protein (0.1µg/ml to 10mg/ml), I: Recording of CAP after vigorous washing of the nerve with ringer's solution. The experiment was repeated three times and the figures are representative of single reading.

However when the treated nerve was washed extensively with Ringer's solution the nerve regained its NCV. The purified protein might be blocking the ion channels but the binding is found to be reversible. When the nerve was treated with crude venom and washed the NCV was not bound to be reversed. The exact mechanism how the purified protein binds to the ion channel and selectivity of the protein for the ion channels needs to be confirmed. The reversibility of the protein binding to the ion channel and inhibiting the NCV can be further characterized for use in war fare as neuro protectant.

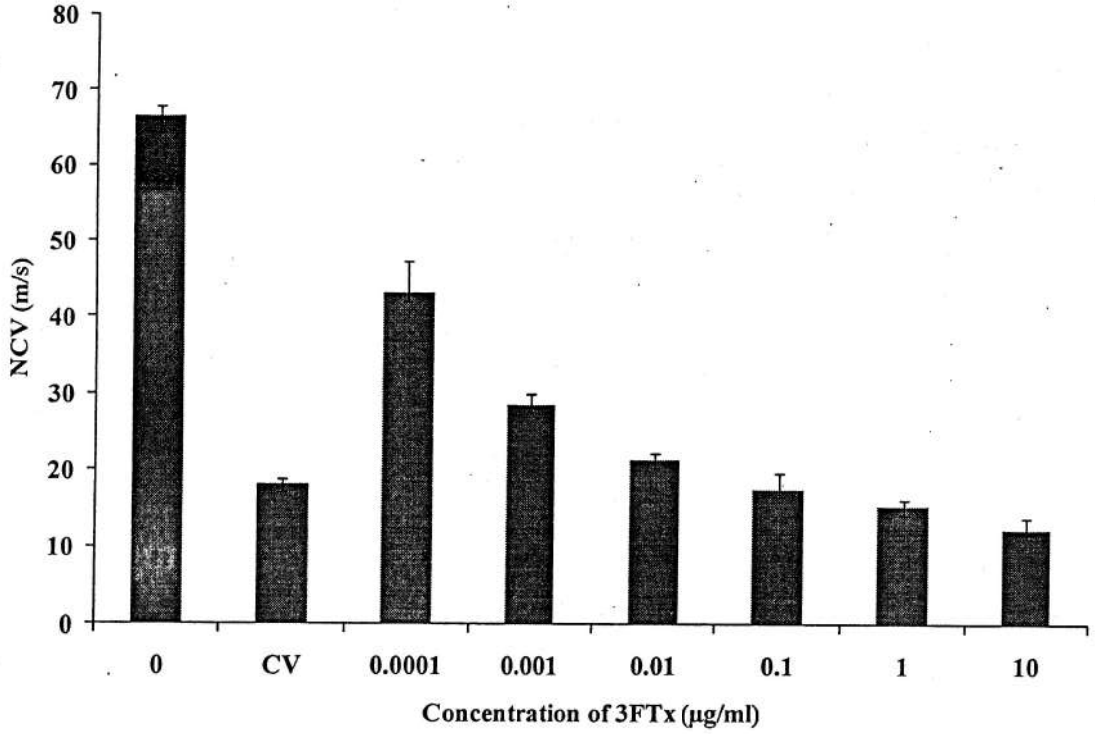

Figure 17: Effect of purified 3FTx on nerve conduction velocity (NCV). Isolated toad sciatic nerve was treated with various concentrations of 3FTx (0.0001-10µg/ml) and crude *Naja kaouthia* (CV) (1µg/ml) venom. Nerve only with Ringer's solution was considered as control. Average values with ±SD are shown from three observations.

Objective 5: Drug and subsidiary clinical manifestation

Will be carried out in future without any financial assistance.

Conclusion

A protein with a molecular weight of 7579.5 Da was purified and its N-terminal sequence up to 35 amino acid residues was determined. The Cys residue pattern in this protein and the molecular weight confirms that the purified protein belongs to the family of snake venom three-finger toxin. Three finger toxins are small molecules with a molecular weight range of 6-8 kDa with 4-5 disulphide bridges. The disulphide bridges of this protein forms the core hydrophobic region and the three beta sheets emerge out of it to form the loops. The loops of these proteins are involved in binding to the targets and exerting the pharmacological affects.. This purified protein showed maximum homology to weak neurotoxin CM-9a reported from *Naja kaouthia* of Thailand. Pharmacological characterization reveals that the protein is devoid of any anticoagulant activity and is a non-cytotoxic protein. The biological activity assayed in this study reveals that the purified protein might be contributing towards the neurotoxicity of the crude venom. Interesting the protein was found to be inhibiting the ion channels by reversibly binding to it. The reversible binding to the ion-channels can be further explored for its use in war-fares.

Major findings of the research project and future prospects

1. A three finger toxin with a molecular weight of 7579.5 Da was purified from the venom of *Naja siamensis*.
2. This purified three finger toxin showed mild anticoagulant activity.
3. It is a non-cytotoxic protein as confirmed from cell line studies.
4. It inhibited the compound action potential (CAP) of toad sciatic nerve in a dose a dependent manner suggesting it is a neurotoxin
5. The inhibition of CAP was reversible.
6. This neurotoxin can be used as a protective agent in warfare against biotoxin targeting neurons.
7. Potential to develop as drug in future could also be investigated.

Manuscript under preparation

1. **Isolation and characterization of 3FTx toxin, a reversible CAP inhibiting peptide from cobra venom.**

29/4/15

[Signature(s) of the Investigator(s)]

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GRANT OF RESEARCH/DEVELOPMENT
PROJECTS UNDER R&T SCHEME OF DRDO
(Cost Below 10 lacs)

1. Title of the research/development:
   Isolation, Characterization, possible use and clinical manifestation of a Neurotoxin from Snake venom (*Naja siamensis*) with special reference to warfare importance neuro-toxin as a model

2. Name of the Investigator(s) : Dr. Robin Doley
   Designation (s) Associate Professor
   Name of the Institution Address : Department of Molecular Biology and Biotechnology, Tezpur University

3. Project sanction No. :DRLT-P1-2010/Task-49 (27/02/2012)

4. Total grant : Rs. 9.8 lakhs

   (i) First year release : Rs. 7.3 lakhs
   (Date of release: 09/07/12)
   (ii) Second year release : Rs. 1.3 lakhs
   (Date of release: 10/06/13)
   (iii) Third year release : Rs. 1.2 lakhs
   (Date of release: 18/09/14)

5. (i) Duration of the research development proposal : 3 years
   (ii) Date of commencement : February 2012
   (iii) Date of completion : February 2015

6. (i) Deptt. of the Institution where the R&T project will be carried out : Department of Molecular Biology and Biotechnology, Tezpur University

   (ii) Domain Scientist : Dr. P. Chottopadhya, Scientist D, DRL, Tezpur

7. Objectives of the Proposal

   I. Isolation of neurotoxin from the crude venom of Naja siamensis

   II. Biochemical characterization of the isolated neurotoxin

   III. Pharmacological characterization of the isolated neurotoxin

   IV. Prespective in warfare similar type neuro-toxin

   V. Drug and subsidiary clinical manifestation

Appendix-A (Details of the grant received)

1. Project sanction No. : DRLT-P1-2010/Task-49 date: 27/02/2012
2. Total amount sanctioned : Rs. 9,8 lakhs
3. Total amount of grant received : Rs. 9.8 lakhs
4. Details of the grant received under various heads

Sl No.	Head	1st yr (Rs.)	2nd yr (Rs.)	3rd yr (Rs.)	Total (Rs.)
1	Staff Salary	Nil	Nil	Nil	0
2	Equipment	4,00,000.00	0	0	4,00,000.00
3	Consumable stores and chemicals etc	3,00,000.00	1,00,000.00	1,00,000.00	5,00,000.00
4	Misc. Expenditure Contingency	20,000.00	20,000.00	10,000.00	50,000.00
5	Overhead	10,000.00	10,000.00	10,000.00	30,000.00
	Total	**7,30,000.00**	**1,30,000.00**	**1,20,000.00**	**9,80,000.00**

28/4/15

(PROJECT INVESTIGATOR)

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(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Annexure I

Details of equipment acquired with actual cost:

S. No.	Items	Make	Amount in Rupees	Status
1	Coagulometer with accessories	Tulip Diagnostics (p)	89,000.00	Installed
2	HPLC columns	Phenomenex	1,33,346.00	Installed
3	Analytical balance	Wenser	70,370.00	Installed
4	pH meter	Merck	25,538.00	Installed
		Total	**3,18,254.00**	

*Balance of Rs. 81,746.00 was re-appropriate to consumable head (letter enclosed)

(PROJECT INVESTIGATOR)

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25/11/15

FINANCE OFFICER
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure II

Financial Year wise

Statement of Expenditure
(February 2012 to 31st March 2013)

Sl No.	Head	Grant received for 1st year	Amount Utilized	Balance
1	Staff Salary	Nil	Nil	Nil
2	Equipment	4,00,000.00	3,18,254.00	81,746.00
3	Consumable stores chemicals	3,00,000.00	2,99,704.00	296.00
4	Misc. Expenditure (Contingency & Overhead)	30,000.00	19291.00	10,709.00
	Total	**7,30,000.00**	**6,37,249.00**	**92,751.00**

Statement of Expenditure
(1st April 2013 to 31st March 2014)

Sl No.	Head	Balance carried forward from 1st year	Grant received for 2nd year	Total amount	Amount Utilized during 2nd year	Balance
1	Staff Salary	Nil	Nil	Nil		
2	Equipment	81,746.00*	0	0	0	0
3	Consumable stores chemicals	296.00	1,00,000.00	81,746.00+1,00,296.00 =1,82,042.00	1,01,296.00	80,746.00
4	Misc. Expenditure (Contingency & Overhead)	10,709.00	30,000.00	40,709.00	25,132.00	15,577.00
	Total	**92,751.00**	**1,30,000.00**	**2,22,751.00**	**1,26,428.00**	**96,323.00**

*The balance amount of Rs. 81,746.00 under equipment head was reappropriated to Consumable and store chemicals (Letter enclosed)

Statement of Expenditure
(1st April 2014 to 31st March 2015)

Sl No.	Head	Balance carried forward from 2nd year	Grant received for 3rd year	Total amount	Amount Utilized during 3rd year	Balance
1	Staff Salary	Nil	Nil	Nil	Nil	Nil
2	Equipment	0	0	0	0	0
3	Consumable stores chemicals	80,746.00	1,00,000.00	1,80,746.00	1,80,741.00	5.00
4	Misc. Expenditure (Contingency & Overhead)	15,577.00	20,000.00	35,577.00	35,576.00	1.00
	Total	**96,323.00**	**1,20,000.00**	**2,16,323.00**	**2,16,317.00**	**6.00**

(PROJECT INVESTIGATOR)

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Annexure III

Utilization Certificate

(for the project duration from February 2012 to 31st March,2015)

1. Title of the Project/Scheme: Isolation, Characterization, possible use and clinical manifestation of a Neurotoxin from Snake venom (*Naja siamensis*) with special reference to warfare importance neuro- toxin as a model

2. Name of the Organization: **Dept. of Mol. Bio & Biotech, Tezpur University**

3. Principal Investigator: **Dr. Robin Doley.**

4. Sanction order No. & date of sanctioning the project: **DRLT-P1-2010/Task-49 date: 27/02/2012**

5. Amount received from DRDO during the financial year *(2012-2013, 2013-2014 and 2014-2015)*: **9.8 Lakhs**

6. Actual expenditure (excluding commitments) incurred during the project duration (2012-15): **9.79994 Lakhs**

7. Balance amount available at the end of the project: Rs. **6.00**

Certified that the amount of **Rs. 9.79994 lakhs** mentioned against col. 6 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of **Rs. 6.0** remained unutilized at the end of the year.

Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

(PROJECT INVESTIGATOR)

Dr. Robin Doley
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