CHAPTER 4

Identification of protein/s of *Naja kaouthia* venom with cytotoxic potential

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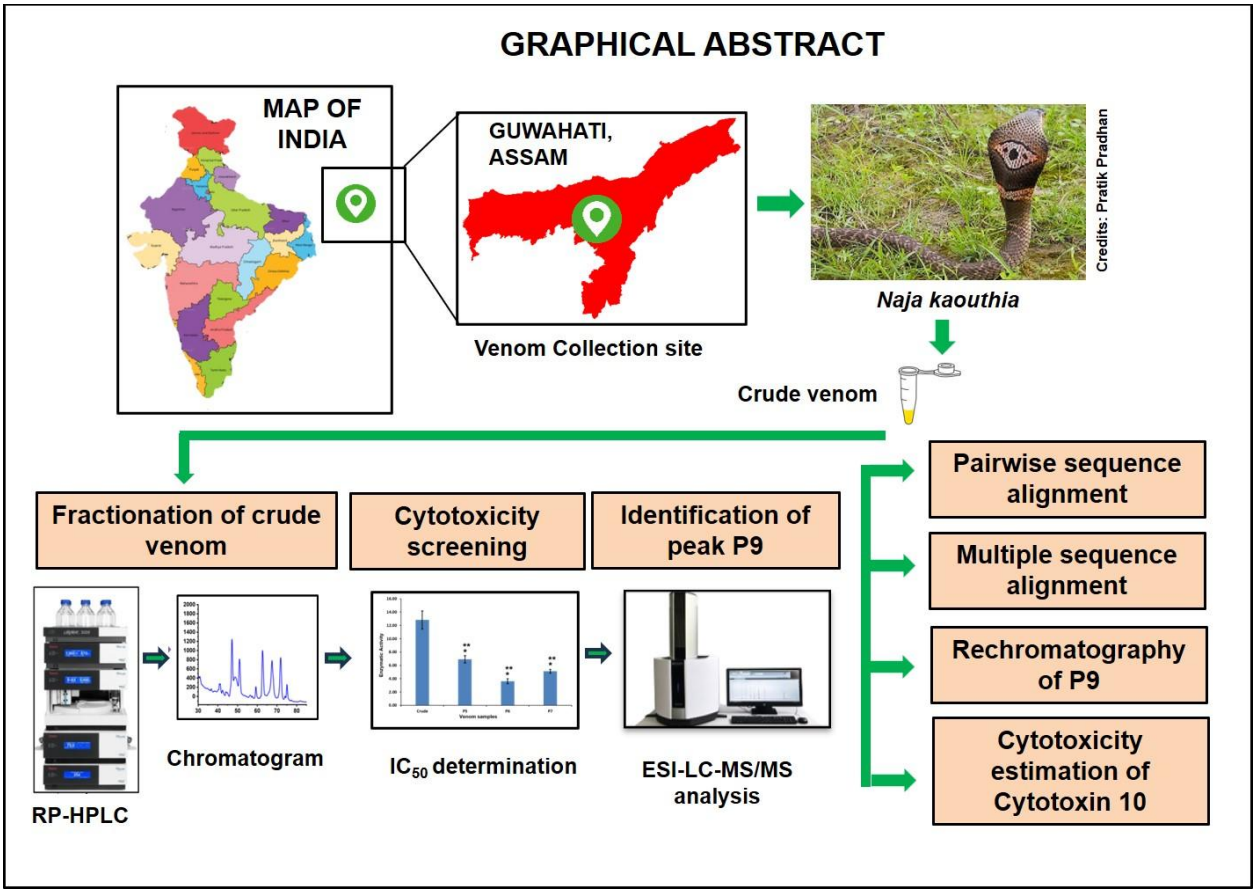

4.1 Introduction

Analysis of existing literature has revealed numerous studies involving snake venoms and their toxic components exhibiting cytotoxicity towards various cancer cell lines, which highlights their potential as an anticancer agent. Snake venom from both Viperidae and Elapidae families have been extensively explored for their anticancer properties in the last few decades [210-212]. Cytotoxic components from various *Naja* species belonging to Elapidae family have been isolated using various chromatography methods and studied for cytotoxicity in *in vitro* culture conditions as well as *in vivo* models [86,212,289-293] . Elapid venoms are primarily composed of low-molecular-mass (<20 kDa) toxins which are either enzymatic or non-enzymatic in nature. The enzymatic component comprises of PLA_2s in abundance whereas, the non-enzymatic

components consist of the three-finger toxin (3FTx), which is the most prominent protein superfamily that contributes to cobra venom-induced pathophysiology and toxicity [280].

Notably, cytotoxins and neurotoxins belonging to the 3FTx superfamily are majorly responsible for lethality and neuromuscular paralysis [294]. Snake venom cytotoxins are composed of approximately 60 amino acid residues and are responsible for hemolysis, cytotoxicity and depolarization of muscles [294,295]. Moreover, the toxins showed a preferential cytotoxicity toward cancer cells, probably mediated by inhibiting protein kinase C activity [291].

In the previous chapter, preliminary investigation of the cytotoxic activity of the crude *N. kaouthia* venom of North-East India origin against breast and lung cancer cell lines suggested the presence of cytotoxic protein/s. Therefore, a better understanding of the potent cytotoxic activity involves purification of the cytotoxic component and identification and demonstration of cytotoxicity against cancer cells by comparing the IC_{50} values of each cell lines. Therefore, in this chapter, the cytotoxin present in *N. kaouthia* venom is isolated and identified (Cytotoxin 10) followed by *in silico* characterization, such as, tertiary structure determination, multiple sequence alignment and phylogenetic tree analysis. This is followed by the determination of time and dose-dependent cytotoxicity of Cytotoxin 10 in breast and lung cancer cell lines.

4.2 Materials

4.2.1 Chemical and reagents

Trifluoro acetic acid, Acetonitrile, Sodium chloride, Sodium bicarbonate, SDS and Trisodium citrate of analytical grade, glycerol, glacial acetic acid, methanol, ethanol, BME and Coomassie brilliant blue R-250 were purchased from Merck (Sigma Aldrich, USA). Bradford Reagent and Precision Plus protein^{TM} standard protein marker were purchased from Bio-Rad, USA. Cell lines MCF-7, MDA-MB-231, A549, NCI-H522 and HEK-293T were procured from NCCS, Pune, India. FBS was purchased from Gibco, USA. DMEM, Trypsin-EDTA, Penicillin-Streptomycin and Mitomycin were purchased from HiMedia, India. MTT, APS, TEMED, acrylamide, bisacrylamide, glycine, tris base, bromophenol blue were purchased from Sigma Aldrich, USA.

4.2.2 Cell culture

MCF-7, MDA-MB-231, A549, NCI-H522 and HEK-293T cell lines were maintained routinely in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C incubator. Culture conditions provided for the experiment were atmosphere air 95% and 5% CO_2. Cells were passaged in accordance with the ATCC guidelines (https://www.atcc.org/resources/culture-guides), with trypsin-EDTA (0.05% trypsin and 0.02% EDTA) and cryopreserved in its respective complete medium with 10% DMSO.

4.2.3 Column

Symmetry™ C18 column (250×4.6 mm, Particle size - 5 µm, Pore size - 300 Å) was obtained from Waters Corporation (Milford, USA).

4.3 Methods

4.3.1 Fractionation of crude *Naja kaouthia* venom using RP-HPLC

Crude *N. kaouthia* venom (2 mg) was subjected to Reverse-Phase High-Performance liquid chromatography (RP-HPLC) using a Symmetry™ C18 column, pre-equilibrated with Milli-Q water containing 0.1% Trifluoro acetic acid (TFA). The crude venom was fractionated using 80% Acetonitrile containing 0.1% TFA. Fractionation was carried out in a RP-HPLC system (Thermo Scientific, USA) at a flow rate of 1 ml/min and monitored at 215 nm with a total run time of 180 min and each fraction was collected manually. The concentration of protein in each of the collected peaks was quantified using the Bradford method [296]

4.3.2 SDS-PAGE

Each of the collected peaks (15 µg) of gel filtration of *N. kaouthia* venom (Assam) was subjected to SDS-PAGE as per the protocol described in section 3.3.1 of Chapter 3.

4.3.3 Screening of RP-HPLC peaks for cytotoxic activity

The collected RP-HPLC fractions of *N. kaouthia* venom were screened for cytotoxicity using the MTT assay. Aliquots of 100 µl of optimum cells (10,000 cells per well) were

seeded into a 96-well microtiter plates. Following overnight incubation, cells were treated with different concentrations of collected fractions for 24 hours. Cells were then incubated with 10% MTT in the dark for 3-4 hours at 37 °C. NADPH dependent cellular oxidoreductase enzymes in viable cells reduces MTT to purple formazan which was dissolved with MTT dissolving solution containing SDS, 0.2 M HCl and Isopropanol. The absorbance was then recorded at 595 nm using a spectrophotometer (Thermo Scientific, USA). All assays were performed in triplicates. Cell viability percentage was calculated as:

$$\%\ \textbf{Cell viability} = \frac{(\textbf{Absorbance of experimental sample}) - (\textbf{Absorbance of blank sample})}{(\textbf{Absorbance of untreated sample}) - (\textbf{Absorbance of blank sample})} \times 100\%$$

4.3.4 Purification of cytotoxic protein by RP-HPLC

The peak P9 (100 µg) which exhibited the highest cytotoxic effect was subjected to re-chromatography using a Symmetry™ C18 column pre-equilibrated with Milli-Q water containing 0.1% Trifluoro acetic acid (TFA) and fractionated using 80% Acetonitrile containing 0.1% TFA. Fractionation was carried out at a flow rate of 1 ml/min and protein elution was monitored at 215 and 280 nm with a total run time of 180 minutes.

4.3.5 Identification of cytotoxic protein using ESI-LC-MS/MS

In-gel trypsin digestion

The cytotoxic fraction was subjected to 12.5% SDS-PAGE and in-gel trypsin digestion was carried out as per the protocol of Babele et al. along with a few modifications [297]. The bands of interest were cut from the gel using a scalpel followed by destaining of the bands by repeated washes with 200 µl 50% Acetonitrile in 50 mM NH_4CO_3 for 1 hour at 25 ^oC. The excised gel pieces were dried after incubation with Acetonitrile followed by addition of 100 ng Trypsin (Promega, USA) in 50 mM NH_4CO_3. Gel pieces were left for tryptic digestion for overnight at 37^oC followed by extraction of the resulting peptides using 60% Acetonitrile and 0.1% Formic acid and were desalted before LC-MS/MS analysis.

LC-MS/MS conditions

LC-MS/MS was carried out as per the protocol of Babele et al. [298], using an Eksigent nano LC-Ultra® 2D System connected to a Triple TOF 5600 mass spectrometer. Samples underwent offline desalting, loaded onto a C18 column, and then to an analytical micro column. HPLC utilized a water/acetonitrile/formic acid mobile phase (Solution A: 98/2/0.2 %, Solution B: 2/98/0.2%). A 10 µg sample was injected at 5 µl/min. Gradient programming ranged from 10% to 90% B over 34 mins.

Data-dependent acquisition was performed using the Triple TOF 5600 mass spectrometer equipped with an Electrospray ionization (ESI) source. Operational settings included a voltage of 2300 V, 25 psi of curtain gas, 20 psi of nebular gas, and 10 psi of heater gas. MS resolution was set at 30,000 FWHM, covering a mass range of m/z 350-1250. The total cycle time was 2.35 sec. Collision-induced dissociation utilized automatic control for collision energy with a 5eV collision energy spread. Dynamic exclusion time was set at 3 sec. Proteins were identified using ProteinPilot 5.0 and the NCBI *N. kaouthia* database. Parameters included sample type, cysteine alkylation, digestion method, instrument, and search effort. False discovery rate was kept below 1% using a reverse database search strategy.

4.3.6 Sequence analysis

The amino acid sequences of the identified protein were retrieved from the 'BLASTp' server (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) of the NCBI (National Center for Biotechnology Information) database. Multiple sequence alignment of the identified protein was performed in DNAMAN X (Lynnon Biosoft) software.

4.3.7 Phylogenetic tree construction

Phylogenetic tree of the identified Cytotoxin 10 (JK0222) from *N. kaouthia* and other cytotoxins from the same species along with Cytotoxin 10 from other *Naja* sp. was constructed in MEGA v11.0 software using the neighbor-joining algorithm. The bootstrap replication number was considered 1000 and the Poisson substitution model was chosen for the study.

4.3.8 Tertiary structure prediction of Cytotoxin 10

The primary sequence of Cytotoxin 10 was used to predict the tertiary structures by utilizing the SWISS-MODEL Expasy online tool (https://swissmodel.expasy.org/). The obtained template P80245.1 (Cytotoxin 3) was selected for modelling based on Global Model Quality Estimate (GMQE) score and Sequence Identity. The model was visualized using the software Discovery Studio Visualizer (Biovia, USA, version 21). The modelled structure was validated using MolProbity score and Clash score from the website hosted by Duke University, USA (http://molprobity.biochem.duke.edu/). Further accuracy of the prediction was validated using Ramachandran plot from PROCHECK Saves v.6.0 tool (https://saves.mbi.ucla.edu/) to analyse the distribution of peptides in the allowed and disallowed regions of the plot.

4.3.9 Assessment of cytotoxic activity of Cytotoxin 10

Cytotoxicity of Cytotoxin 10 was checked against cancer and normal cell lines using the MTT assay. Twenty-four hours prior to venom treatment, 100 µl aliquots of optimum cells (10,000 cells per well) were seeded into 96-well microtiter plates. Cells were treated with different concentrations of Cytotoxin 10 for 24 and 48 hours. The cytotoxicity was assessed post-treatment with 10% MTT, incubated at 37°C in the dark for 3-4 hours. NADPH dependent cellular oxidoreductase enzymes in viable cells reduces MTT to purple formazan which was dissolved with MTT dissolving solution. The absorbance was finally recorded at 595 nm using ThermoScientific Spectrophotometer. All assays were performed in triplicates. The IC_{50} values for each of the cell lines were calculated for each of the cell lines using the online tool AAT Bioquest Graph™ IC_{50} Calculator (https://www.aatbio.com/tools/ic50-calculator).

4.4 Results

4.4.1 Fractionation and screening of cytotoxic protein

a) Fractionation of crude Naja kaouthia venom using RP-HPLC

To identify the bioactive compound responsible for cytotoxicity in *N. kaouthia* venom, fractionation of the crude was performed. Upon fractionation of crude *N. kaouthia* venom (2 mg) using RP-HPLC system (Thermo Scientific), USA, Symmetry™ C18 column, venom components were separated and a total of 12 peaks according to their

peak distribution were obtained and collected manually (Figure 4.1). The collected peaks were analyzed for cell cytotoxicity against cancer cell lines and compared with normal cell lines in the subsequent sections.

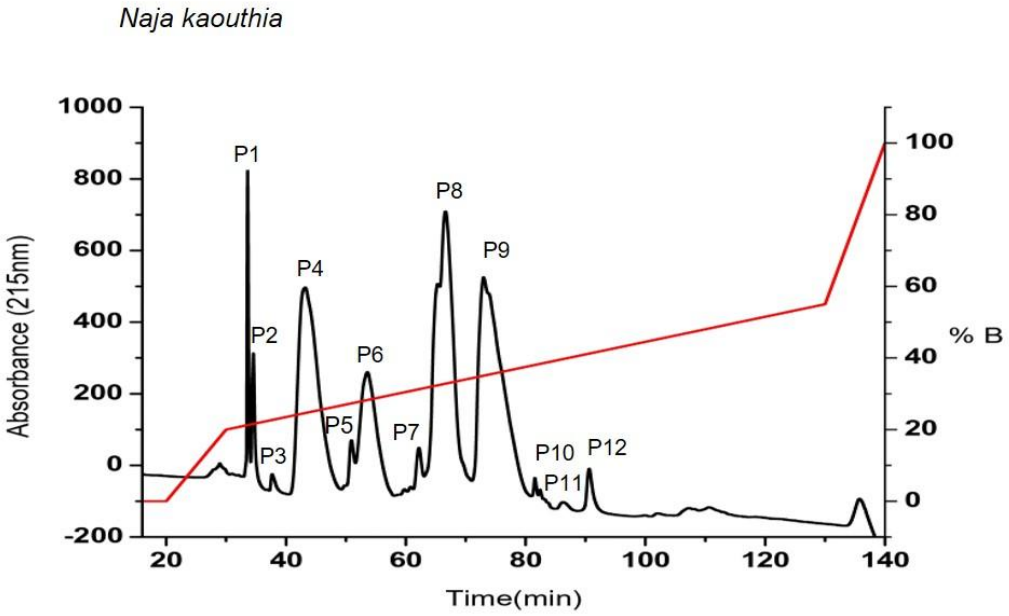

Figure 4.1: Fractionation of crude *Naja kaouthia* venom using Reverse phase high-performance liquid chromatography (RP-HPLC): Crude venom (2 mg) was fractionated in a Symmetry™ C18 column pre-equilibrated with Milli-Q water containing 0.1% Trifluoro acetic acid (TFA) using 80 % Acetonitrile containing 0.1% TFA. Twelve protein fractions according to their peak distribution were collected manually. Elution of the venom protein was monitored at 215 nm and the peaks were collected separately.

b) SDS-PAGE profile of collected RP-HPLC peaks

Combination of RP-HPLC and SDS-PAGE pattern followed by Coomassie brilliant blue staining reveals the abundance of low molecular weight proteins (< 25 kDa) in each of the collected RP-HPLC peaks (Figure 4.2).

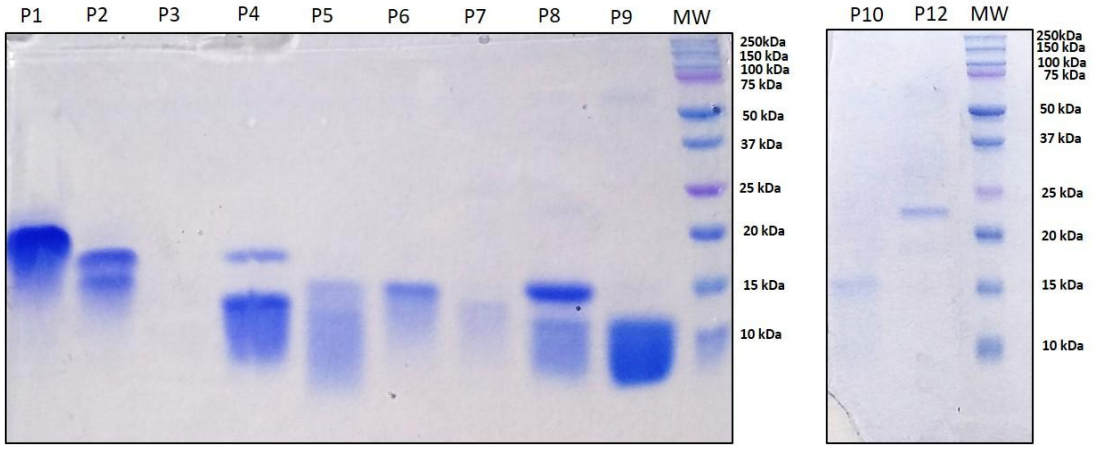

Figure 4.2: SDS-PAGE (12.5%) of RP-HPLC peaks. (M: Page ruler plus protein marker and HPLC peaks P1-P12). 15 µg samples of each peak were separated by 12.5 % SDS-PAGE and subsequently stained with Coomassie brilliant blue.

c) Screening of RP-HPLC peaks for cytotoxicity

The peaks that were obtained using gel filtration were screened for cytotoxicity against breast cancer (MCF-7 and MDA-MB-231), lung cancer (A549 and NCI-H522) and normal kidney (HEK-293T) cell lines using cell viability MTT Assay (Figure 4.3). Out of the 12 peaks, Peak P9 was found to exhibit the most potent effect against the cancer cell lines NCI-H522 and MCF-7 which resulted in only 19% and 23% viability of cells respectively, after treatment with 10 µg/ml for 24 hours. Whereas, for normal cell line HEK-293T, P8 was found to exhibit the most potent effect with ~81% cell death *i.e.*, only 18% cells were viable as compared to P9 which could retain 37% viable cells after the treatment Therefore, P9 was subsequently selected for purification and identification of the cytotoxic protein.

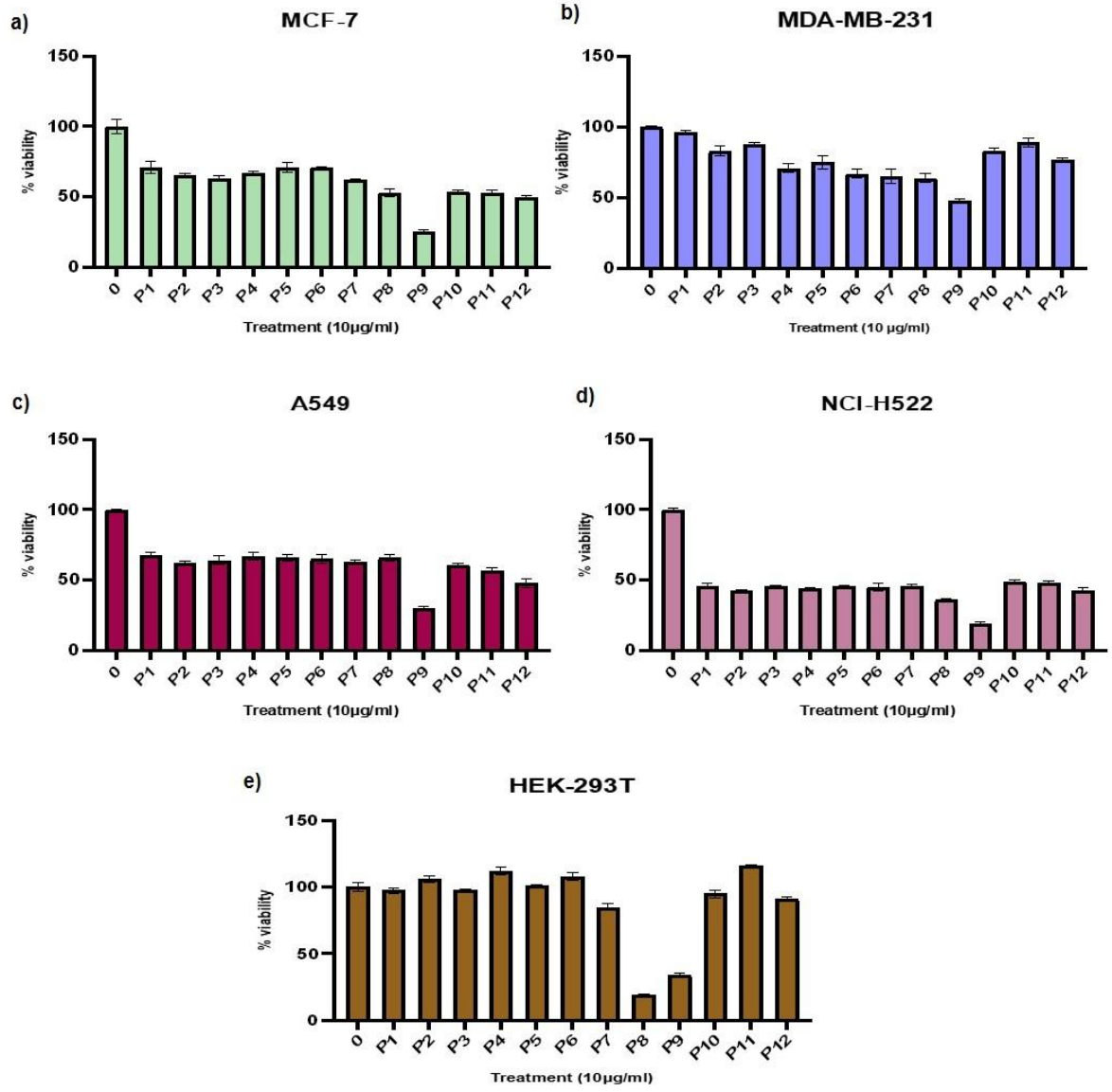

Figure 4.3: Screening of the collected RP-HPLC fractions/peaks for cytotoxic effects against breast cancer a) MCF-7, b) MDA-MB-231, lung cancer c) A549, d) NCI-H522 and normal kidney e) HEK-293T cell lines. Viability of cells is expressed in % upon treatment with 10 µg/ml dose each of RP-HPLC fractions for 24 hours. The cells without treatment were considered as control (100%).

4.4.2 Purification and identification of cytotoxic protein

a) Purification of peak P9

Re-chromatography of P9 revealed a single protein peak RP9 suggesting it contains a single protein and free of contamination (Figure 4.4b). The peak was collected manually for further analysis.

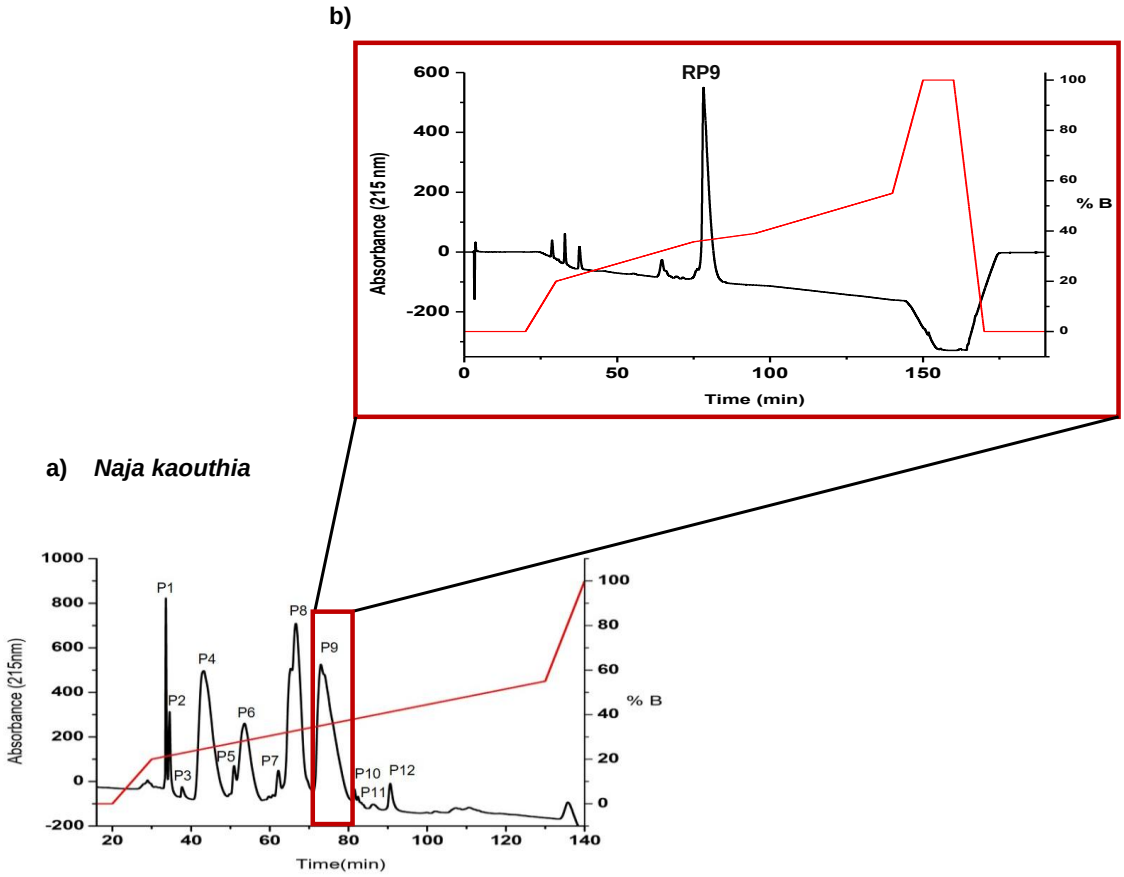

Figure 4.4: Re-chromatography of RP-HPLC fraction P9: a) Crude venom (2 mg) was fractionated in a Symmetry™ C18 column. **b)** Peak P9 was re-chromatographed in a Symmetry™ C18 column using 0.1% TFA and 80 % Acetonitrile buffer in 0.1% TFA (%B). Elution of the venom protein was monitored at 215 nm and the peak was collected separately.

b) Identification using ESI-LC-MS/MS

Following in-gel trypsin digestion and liquid chromatography-tandem mass spectroscopy analysis (LC-MS/MS), 23 peptides were obtained for peak P9. Peptides with z-score less than 2 were not included. Based on NCBI-BLAST search, the protein was identified as Cytotoxin 10 (Accession No. JK0222) which belongs to the three-finger toxin (3FTx) family of snake venom proteins (Table 4.1).

Table 4.1: Summary of peptides/proteins obtained from LC-MS/MS analysis of Peak P9 of *Naja kaouthia* venom (North-East India). (z stands for the number of charges a peptide carries after ionization)

RP-HPLC fraction	Protein name	MS/MS derived sequences	m/z	z	Score	Best NCBI match	Protein family
Peak 9	Cytotoxin 10	LKCNKLVPLFYK	508.30	3	16	*Naja kaouthia*	3FTx
		KCNKLVPLFYK	484.92	3	8	(JK0222)	
		CNKLVPLFYK	427.90	3	10		
		NKLVPLFYK	597.33	2	7		
		LVPLFYKTCPAGK	498.61	3	12		
		VPLFYKTCPAGK	503.61	3	9		
		PLFYKTCPAGK	641.33	2	11		
		TCPAGKNLCYK	437.88	3	10		
		TCPAGKNLCYKMFMVATPK	563.02	4	9		
		NLCYKMFMVATPK	817.89	2	11		
		MFMVATPK	470.73	2	9		
		MFMVATPKVPVKR	398.97	4	12		
		VATPKVPVKR	384.58	3	7		
		ATPKVPVKR	384.58	3	7		
		VPVKRGCIDVCPK	579.24	3	7		
		RGCIDVCPK	368.85	3	11		
		RGCIDVCPKSSLLVK	582.97	3	11		
		GCIDVCPK	474.71	2	7		
		GCIDVCPKSSLLVK	525.94	3	9		
		SSLLVKYVCCNTDRCN	663.64	3	18		
		YVCCNTDR	544.21	2	9		
		YVCCNTDRC	635.75	2	8		
		YVCCNTDRCN	681.25	2	8		

c) Sequence alignment

The 23 peptide sequences derived from LC-MS/MS analysis of Peak P9 were assembled to obtain the complete sequence of the protein. Identical residues of the peptides have been shown in similar color (Figure 4.5).

```
Peptide 1: LKCNKLVPLFYK
Peptide 2:  KCNKLVPLFYK
Peptide 3:   CNKLVPLFYK
Peptide 4:    NKLVPLFYK
Peptide 5:      LVPLFYKTCPAGK
Peptide 6:       VPLFYKTCPAGK
Peptide 7:        PLFYKTCPAGK
Peptide 8:             TCPAGKNLCYK
Peptide 9:             TCPAGKNLCYKMFMVATPK
Peptide 10:                  NLCYKMFMVATPK
Peptide 11:                      MFMVATPK
Peptide 12:                      MFMVATPKVPVKR
Peptide 13:                         VATPKVPVKR
Peptide 14:                          ATPKVPVKR
Peptide 15:                              VPVKRGCIDVCPK
Peptide 16:                                  RGCIDVCPK
Peptide 17:                                  RGCIDVCPKSSLLVK
Peptide 18:                                   GCIDVCPK
Peptide 19:                                   GCIDVCPKSSLLVK
Peptide 20:                                           SSLLVKYVCCNTDRCN
Peptide 21:                                                 YVCCNTDR
Peptide 22:                                                 YVCCNTDRC
Peptide 23:                                                 YVCCNTDRCN
Assembled :LKCNKLVPLFYKTCPAGKNLCYKMFMVATPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN
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Figure 4.5: Assembled sequence of identified peptides obtained from the LC-MS/MS analysis. Identical residues of the peptides are shown in similar color.

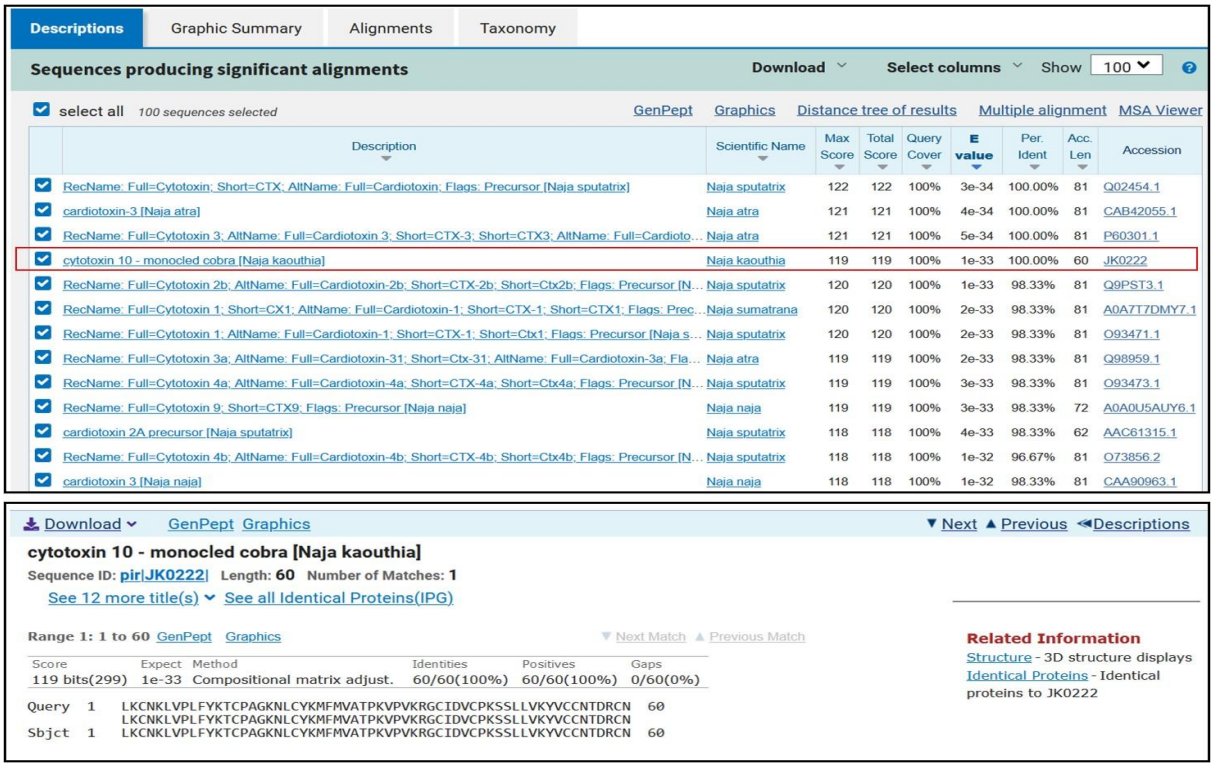


Figure 4.6: Results of NCBI-BLASTp: The assembled sequence of the identified peptides exhibited 100% sequence identity with Cytotoxin 10 (JK0222) from *Naja kaouthia* venom.

Pairwise Sequence Alignment

Pairwise Sequence Alignment of Cytotoxin 10 from *N. kaouthia* venom revealed Proline-30 at the phospholipid-binding site (conserved at the 30th position of amino acid sequence) revealing the Cytotoxin 10 of *N. kaouthia* venom from North-East India origin to be a P-type cytotoxin. The obtained peptide sequence showed 100% query coverage (Figure 4.7).

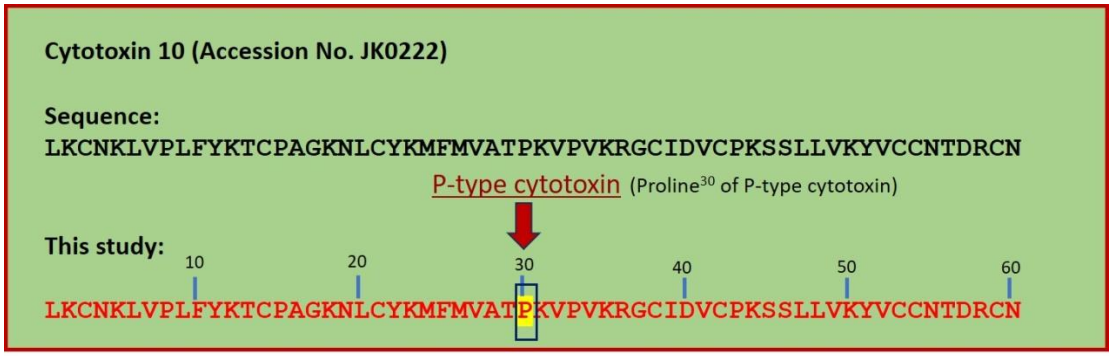


Figure 4.7: Pairwise sequence alignment of Cytotoxin 10 from *Naja kaouthia* venom with their homologous protein using NCBI BLASTp.

Multiple sequence alignment

The peptide sequences were assembled and aligned with other known Cytotoxins from *N. kaouthia* venom and with Cytotoxin 10 from other *Naja sp*. (Figure 4.8). The alignment was performed in DNAMAN X (Lynnon Biosoft). Cytotoxins from snake venoms (~6.5-7.0 kDa) belong to the non-enzymatic 3FTx superfamily and are highly basic (pI >10) consisting of 60-70 amino acid residues [299]. They have been previously classified as P-type (containing Pro-30 residue) and S-type (containing Ser-28 residue). Multiple sequence alignment of the identified Cytotoxin 10 with other known cytotoxins from *N. kaouthia* venom (Cytotoxin 1, 2, 3, 4 and 5) and Cytotoxin 10 from other *Naja* species (*N. atra, N. annulifera and N. naja*) revealed that Cytotoxin 10 from *N. kaouthia*, *N. atra* and *N. naja*, and Cytotoxin 4 and 5 from *N. kaouthia* belong to the P-type cytotoxins, whereas Cytotoxin 1, 2 and 3 from *N. kaouthia* and Cytotoxin 10 from *N. annulifera* belong to the S-type cytotoxins. Cytotoxin 10 was found to contain 8 conserved cysteine residues which forms 4 disulfide bonds. Thus, Cytotoxin 10 identified in this study belonged to the P-type cytotoxins as the Pro-30 residue was conserved. The percent identity matrix revealed that Cytotoxin 10 shared an average 90.93% identity with other cytotoxins in terms of amino acid residues.

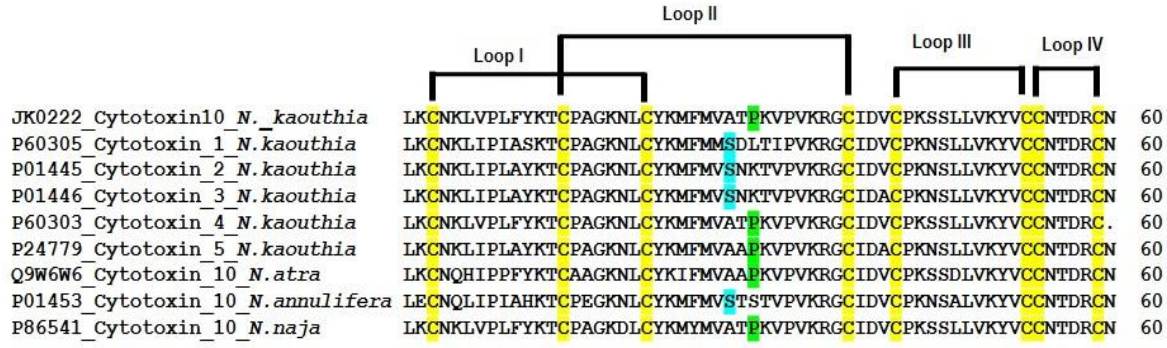


Figure 4.8: Multiple Sequence Alignment of Cytotoxin 10 with other known Cytotoxins from *Naja kaouthia* venom and Cytotoxin 10 from other *Naja* species. The amino acid sequences of Cytotoxins were retrieved from NCBI database and the alignment was performed in DNAMAN X (Lynnon Biosoft) software. The Pro-30 (P-type) cytotoxins are visualized in light green background and Ser-28 (S-type) cytotoxins are visualized in light blue background and the four conserved disulphide bonds can be observed in four loops.

d) Phylogenetic Tree analysis

The Phylogenetic Tree analysis revealed that Cytotoxin 10 from *N. kaouthia* is evolutionary most closely related to Cytotoxin 4 from the same species, followed by Cytotoxin 10 from *N. naja* and *N. atra*, and Cytotoxin 5 from *N. kaouthia* respectively. All of these cytotoxins belong to the P-type cytotoxins. Moreover, the P-type cytotoxins are distantly related to the S-type cytotoxins, such as, Cytotoxin 1,2 and 3 from *N. kaouthia* and Cytotoxin 10 from *N. annulifera* (Figure 4.9)

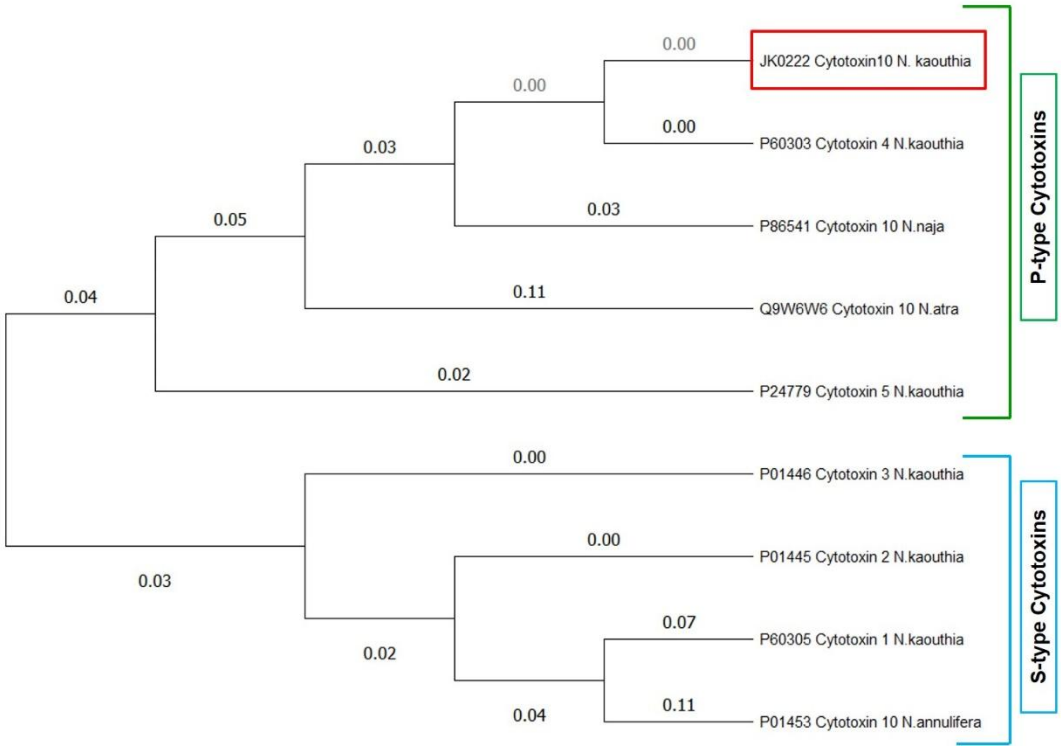


Figure 4.9: Phylogenetic tree analysis: The P-type and S-type cytotoxins from different *Naja species* form distinct clades and are highlighted in green and blue brackets respectively. Cytotoxin 10 (JK0222) from *Naja kaouthia* venom (Assam) is highlighted in red box. The phylogenetic tree is prepared using the MEGA v11.0 software using the neighbour-joining method.

e) SDS-PAGE profile of Cytotoxin 10

Combination of RP-HPLC and SDS-PAGE pattern reveals abundance of low molecular weight proteins (<20 kDa) (Figure 4.10). Protein bands were observed in both the non-

reduced and reduced samples of P9 (Cytotoxin 10). Proteins including PLA_2 and 3FTxs represent the lower molecular weight range proteins. 3FTx represents the most abundant venom proteins in cobra venoms with effects including neurotoxicity, cardiotoxicity, cytotoxicity, anti-coagulation and platelet aggregation inhibition.

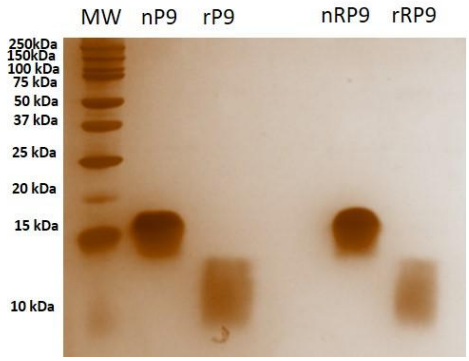


Figure 4.10: SDS-PAGE (12.5%) of RP-HPLC peak P9 and re-chromatography peak of P9 (RP9). (M: Page ruler plus protein marker. 5 µg samples of each peak were separated by 12.5 % SDS-PAGE and subsequently stained with silver nitrate. nP9: non reduced P9, rP9: reduced P9, nRP9: non reduced RP9, rRP9: reduced RP9.

4.4.3 *In silico* characterization of Cytotoxin 10

Tertiary structure modelling

Tertiary structure of Cytotoxin 10 (JK0222) was predicted using the Swiss-Model EXPASY tool using the template P80245.1. Tertiary structure modelling of Cytotoxin 10 was visualized as the ribbon-structure model which revealed the three characteristic β-loops that extends from a globular hydrophobic core consisting of eight conserved cysteine residues cross-linked by four disulphide bridges. The alpha-Helix has been represented in turquoise color, beta-sheets in white color and coils in light green color (Figure 4.11a). The conserved P-30 residue is present at the tip of the second β-stranded loop.

Structure validation

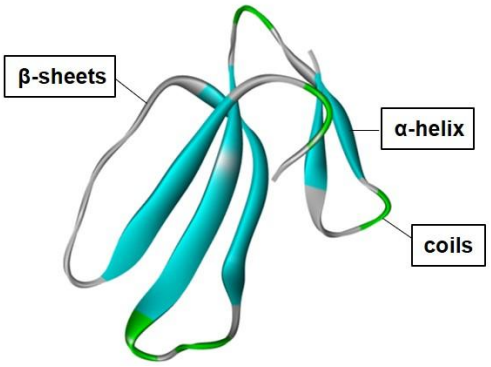
The tertiary structure model of Cytotoxin 10 (JK0222) was validated using the Ramachandran plot where 90.2% of the amino acid residues were located in the allowed region with additional allowed region consisting of 9.8 % residues. However, there were no residues in the disallowed region (Figure 4.11b). Moreover, the Clash score for the

model was 0, and the MolProbity Score (which is based on the overall bond angles, bond length, torsional angles and clash score) was 0.50 for the model. The sequence identity score with the template P80245.1 was calculated to be 91.67%.

a)

Cytotoxin 10 (JK0222)

Identity with template (P80245): 91.67%



b)

Identified enzyme	Accession number	Template name	Template ID	Sequence Identity	Residues in most favoured region	Residues in additional allowed region
Cytotoxin 10	Q9DGH9	Cytotoxin 3	P80245.1	91.67%	90.2%	9.8%

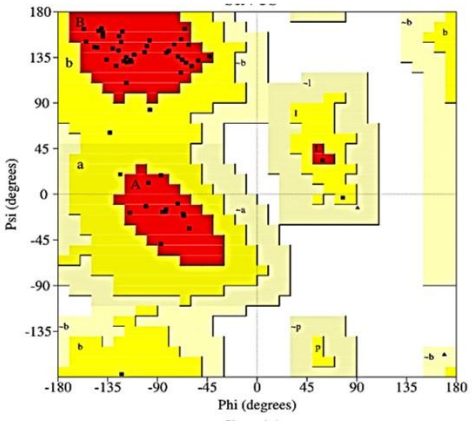


Figure 4.11: *In silico* study of Cytotoxin 10: a) Tertiary structure (ribbon model) of Cytotoxin 10 (JK0222) predicted using Swiss-Model EXPASY tool. The model was predicted using the template P80245.1. The alpha-Helix is represented in turquoise color, beta-sheets in white color and coils in light green color. **b) Structure validation- Ramachandran plot of Cytotoxin 10 (JK0222)** showing residues in most favoured region and additional allowed region.

It has been observed that the predicted ribbon model of Cytotoxin 10, which is a 3FTx from *N. kaouthia* venom, exhibits similarity of structure when compared to 3FTxs from other *Naja* sp. venoms, such as, Cytotoxin II from *N. kaouthia*, and Cytotoxin I and Cytotoxin II from *N. oxiana* venoms (Figure 4.12). Hence, there is a possibility that Cytotoxin 10 may share some functional similarities with the other structurally similar cytotoxins, which needs to be further investigated.

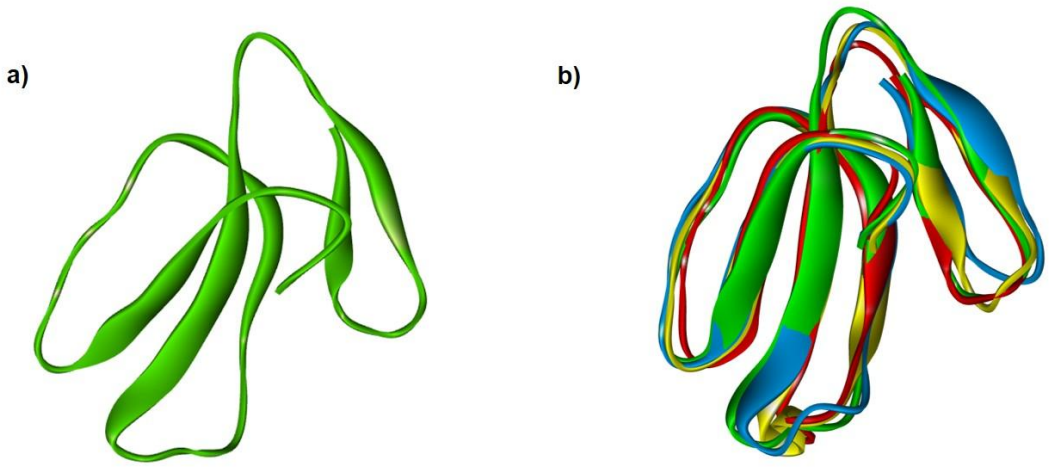


Figure 4.12: Comparative analysis of predicted structure of Cytotoxin 10. a) Ribbon model of Cytotoxin 10 (Green) from *Naja kaouthia* venom. b) Overlay of ribbon models of Cytotoxin 10 exhibiting structural similarity with the NMR-structure of 3FTxs from other *Naja* sp. venoms. Yellow = Cytotoxin II from *N. kaouthia* (PDB ID: 7O2K), Red = Cytotoxin I from *N. oxiana* (PDB ID: 5NPN), and Blue = Cytotoxin II from *N. oxiana* (PDB ID: 1CCQ).

4.4.4 Cytotoxic effect of Cytotoxin 10

The cytotoxic efficacy of Cytotoxin 10 was determined against all the experimental cell lines used in this study for concentrations ranging from 0-30 µg/ml for two time points *i.e.*, 24 and 48 hours. The treated cells showed an increase in cell death in dose and time-dependent manner. Treatment up to a maximum dose of 30 µg/ml of Cytotoxin 10 for 24 hour resulted in cell death of approximately 81% in MCF-7 cells, 84% in MDA-MB-231 cells, 89% in A549 cells and 76% in NCI-H522 cells (Figure 4.13).

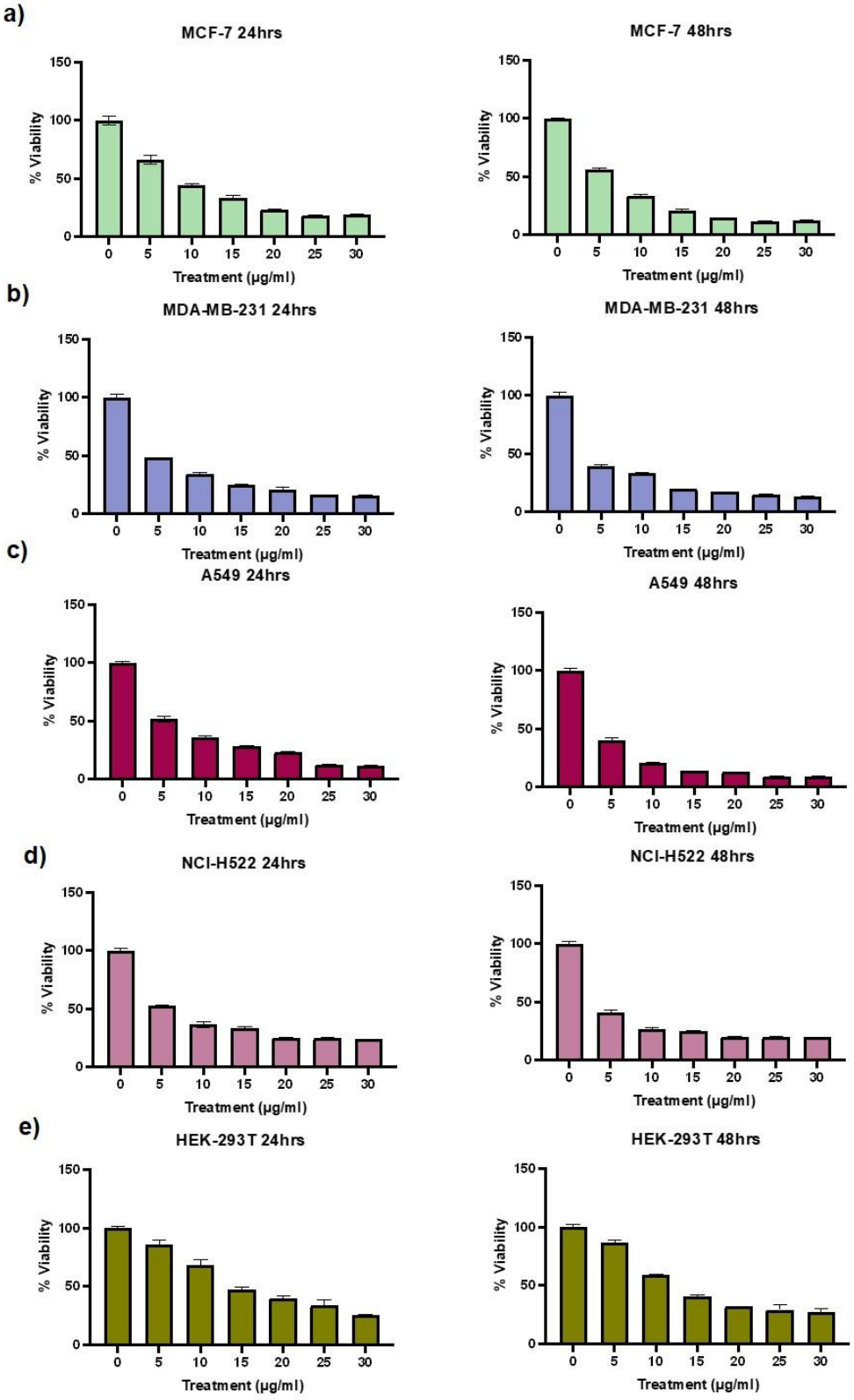


Figure 4.13: Cell viability in response to treatment of cancer and normal cell lines with Cytotoxin 10 protein of *Naja kaouthia* venom quantified by MTT Assay. Dose-dependent effect on **a)** MCF-7**, b)** MDA-MB-231, **c)** A549, **d)** NCI-H522, and **e)** HEK-293T cells treated with Cytotoxin 10 for 24 hours. The cells without treatment were considered as control (100%).

Half maximal inhibitory concentration (IC_{50}) of cell lines treated with Cytotoxin 10 was calculated for each of the cell lines.

Determination of IC_{50} value

The IC_{50} values for Cytotoxin 10 treatment for 24 hours against MCF-7, MDA-MB-231, A549, NCI-H522, and HEK-293T were calculated to be 8.71, 6.04, 6.44, 6.60 and 15.50 µg/ml respectively (Table 4.2). However, there was a drop in IC_{50} values after 48 hours treatment for each of the cell lines MCF-7, MDA-MB-231, A549, NCI-H522 and HEK-293T to 6.50, 5.39, 4.83, 5.19 and 13.00 µg/ml respectively indicating the dose and time dependent cytotoxic effect of the venom protein (Table 4.2).

Table 4.2: Calculated IC_{50} values of *Naja kaouthia* crude venom and purified Cytotoxin 10 against all experimental cell lines.

Tissue origin	Cell line	Cytotoxin 10 (µg/ml) 24 hr	Cytotoxin 10 (µg/ml) 48 hr	Crude venom (µg/ml)
Breast (cancer)	MCF-7	8.71	6.50	10.08
	MDA-MB-231	6.04	5.39	14.87
Lung (cancer)	A549	6.44	4.83	30.12
	NCI-H522	6.60	5.19	15.30
Kidney (normal)	HEK-293T	15.50	13.00	13.00

Cytotoxin 10 showed the least cytotoxic effect towards HEK-293T as observed from the approximately two-fold higher IC_{50} value compared to IC_{50} values for breast and lung cancer cells, suggesting the preferential cytotoxic activity against the cancer cell lines (Table 4.2).

4.5 Discussion

Crude *N. kaouthia* venom was subjected to RP-HPLC which led to the separation of the venom components and a total of 12 peaks according to their peak distribution were obtained. The peaks were subjected to SDS-PAGE followed by Coomassie staining and it was observed that the peaks formed one or more bands or a smear within the molecular weight range of 10-25 kDa (Figure 4.2) which was comparable to the SDS-PAGE profile of the crude venom, as observed in Chapter 3 (Figure 3.1) where an intense thick smear

was observed in the molecular weight range (<10-15 kDa). The collected peaks were further screened for cytotoxicity against cancer cell lines and compared to the cytotoxicity against normal cell lines. Peak P9 exhibited the maximum cytotoxic activity towards the experimental cancer cell lines and purification of the peak revealed a single sharp peak suggesting that the peak contains a single protein and is devoid of contamination. The molecular weight of Peak P9 was observed to be ~10-15 kDa from SDS-PAGE profile. Further, P9 was selected for identification using ESI-LC-MS/MS analysis. Peptide sequences derived from LC-MS/MS profiling identified the protein as Cytotoxin 10. Cytotoxins from cobra venom are polypeptides consisting of ~60–70 amino acid residues which are held by four disulfide bridges of cysteine residues, developing a structure that resembles a three fingers-like projections [300-302]. Cytotoxins belong to the 3FTx superfamily of snake venom proteins, thus named based on their structural resemblance to the three open fingers of the hand. The structure of cytotoxins give rise to its diverse pharmacological actions which includes induction of cytolytic activity by generating pores on cell membranes and breaking down the membrane lipid bilayer which eventually initiates cell death [295,303].

The tertiary structure of Cytotoxin 10 was predicted using Swiss-Model EXPASY tool which revealed that it is a 3FTx. The modelled structure exhibited the characteristic three β-stranded loops arising from the hydrophobic core consisting of four disulphide bridges. Further, accuracy of the structure prediction was validated using the Ramachandran plot from PROCHECK Saves v.6.0 tool which revealed distribution of 90.2% peptides in most favoured region and 9.8% peptides in additional allowed region of the plot and the sequence identity score with the template P80245.1 was calculated to be 91.67%. It has been previously highlighted by Ma et al. that the loops of the cytotoxins have a hierarchy-based cytolytic activity [304]. They demonstrated that the first and second loop were mainly responsible for the lytic activity exhibited by chimeric cytotoxins from *N. sputatrix* venom. It was observed that the hydrophobicity of loop I is the highest, followed by loop II and loop III. Hence, during the interaction with lipid bilayer, loop I is the first to be incorporated followed by loop II and loop III.

The pairwise sequence alignment of Cytotoxin 10 from *N. kaouthia* venom revealed conserved Proline-30 residue at the phospholipid-binding site confirming the Cytotoxin

10 to be a P-type cytotoxin. However, some of the previously reported cytotoxins from *N. kaouthia* venom belonged to the S-type cytotoxin. For instance, Cytotoxin 3 from *N. kaouthia* (Thailand origin) is reported to be a S-type cytotoxin [290]. Further, multiple sequence alignment of Cytotoxin 10 with other known cytotoxins from *N. kaouthia* venom suggested that Cytotoxin 1 (P60305), 2 (P01445) and 3 (P01446) belong to the S-type cytotoxins, whereas, Cytotoxin 4 (P60303) and 5 (P24779) belong to the P-type cytotoxins. However, multiple sequence alignment of Cytotoxin 10 from *N. kaouthia* with Cytotoxin 10 from other *Naja* species revealed that Cytotoxin 10 from *N. kaouthia*, *N. atra* (Q9W6W6) and *N. naja* (P86541) belong to the P-type cytotoxins, whereas, Cytotoxin 10 from *N. annulifera* (P01453) belong to the S-type cytotoxins.

Previous reports suggested that the Proline-30 (Pro30) residue in P-type cytotoxin is responsible for binding to phospholipid bilayer of membranes which is followed by insertion into the membranes, and compared to the S-type cytotoxins, they have a stronger interaction with membranes [305]. However, recently Dubovskii and Utkin have suggested that special residues in the three loops such as Pro9 in loop I, Ser28/Pro30 in loop II and Ser45-Ser46/Asn45-Ser46 in loop III is responsible for the membrane activity. Based on the presence or absence of these special residues, they suggested a classification system for cytotoxins where these are classified into 8 groups. Group 1 has the least activity having Pro9 in loop I, Ser28 in loop II and Ser45-Ser46 in loop III, and Group 8 with the highest membrane activity lacks all these special residues. Based on this classification system, the Cytotoxin 10 identified in this study from *N. kaouthia* venom which has Pro9 in loop I, Pro30 in loop II and Ser45-Ser46 in loop III belongs to Group 3, which includes other cytotoxin members like P01462, P01463, P01464, P01465 and P01466. The presence of Pro resides in loop I and II, and Ser-Ser residues in loop III may help in membrane interaction leading to internalisation of Cytotoxin 10 in cancer cells. According to this classification, Cytotoxin 10 should have a higher membrane-activity based cytotoxicity compared to the cytotoxins of Group 1 (P01458, P01461, P01457, P01460, P01455 and P01456) and Group 2 [306].

The high average percent identity (90.93%) shared among the cytotoxins indicate the evolutionary closeness among the cytotoxins. This is further validated using the phylogenetic tree analysis where the P-type (including Cytotoxin 10 from *N. kaouthia*) and S-type cytotoxins from different *Naja sp.* form distinct clades indicating their

evolutionary closeness. Thus, it can be inferred from the phylogenetic tree that considerable genetic changes may have accumulated in the P-type and the S-type cytotoxins due to which they form evolutionary distinct clades.

Studies on the evolution of 3FTxs (which includes cytotoxins) have indicated that the three-finger projections of 3FTx have not evolved exclusively in snake venoms as various other proteins and polypeptides, not related to venom are also included in 3FTxs. Hence, it is likely that venom 3FTxs evolved from non-toxic proteins. These toxins have evolved *via* gene duplication and increased rate of non-synonymous nucleotide replacements in the exons of 3FTx genes [307]. These mutations were presumed to be responsible for the alteration in the molecular structure of the toxins due to which it could bind to different receptors. Thus, adaptive Darwinian evolution have led to structural and functional diversification in 3FTxs [308,309]. However, these point mutations were not sufficient enough to describe the diverse pharmacological manifestations of 3FTxs, which suggest that some other mechanisms possibly also played significant role in their evolution. Firstly, in α-neurotoxins and κ-neurotoxins, a fifth disulphide bridge is inserted the second loop which allows the toxin to bind to α7-nAChRs [310]. Secondly, 3FTxs in Colubrids, such as denmotoxin, have 4 exons compared to the three exons in Elapid and Viperid 3FTx genes. The additional exon (exon-2) codes for a longer N-terminal segment although its functional significance is not clearly known [311,312]. Thirdly, in 3FTx genes of Viperids, rapid changes may undertake through a phenomenon called ASSET (Accelerated segment switch in exons to alter targeting) which may lead to structural and functional diversification in 3FTxs [313]. Thus, 3FTxs have evolved through several mechanisms forming a functionally diverse superfamily with a conserved structural scaffold.

Elapids, mostly cobras, consist of cytotoxins in abundance and are known for their cytotoxicity against various cells, e.g. red blood cells (RBCs), cardiac cells, spleen cells, lymphocytes, tumor and cancer cells [74,314-316]. Cytotoxins from *N. oxiana*, *N. kaouthia*, and *N. haje* demonstrated cytotoxicity in adenocarcinoma cells, human lung cancer (A549) and leukemic (HL60) cell lines [74]. A lethal cardiotoxic–cytotoxic protein (Mol wt. 6.76 kDa) purified from *N. kaouthia* venom showed dose dependent cytotoxic effect on human leukemic U937 and K562 cells [241]. Similar cytotoxic and

antioxidant protein from *N. naja* venom (Mol wt. 6.7 kDa) was reported against murine Ehrlich ascites carcinoma [317]. The cytotoxin also inhibited human leukemic U937 cell growth by inducing apoptosis and arresting cell cycle [292]. Cytotoxin-II isolated from the venom of Caspian cobra was reported to exert anticancer activity *via* induction of apoptosis in MCF-7 cell line [293]. A cytotoxic protein (13 kDa) isolated from *Bungarus fasciatus* snake venom showed cytotoxicity in both *in vitro* (leukemic cell line U937) and *in vivo* (EAC induced BALB/c mice) by inducing apoptotic cell death. The protein also have been reported to downregulated PI3K/Akt and MAP-kinase pathways [318].

In this study, breast cancer (MCF-7 and MDA-MB-231), lung cancer (A549 and NCI-H522) and normal kidney (HEK-293T) cell lines were utilized for *in vitro* examination of the cytotoxic effect of purified protein Cytotoxin 10 and to determine the IC_{50} values against each of the cell lines accordingly. Decrease in % viability of cells was observed when cells were treated with Cytotoxin 10. IC_{50} values for 24 hours treatment against MCF-7, MDA-MB-231, A549, NCI-H522, and HEK-293T were calculated to be 8.71, 6.04, 6.44, 6.60 and 15.50 µg/ml respectively. However, drop in IC_{50} values was observed after 48 hours treatment for MCF-7, MDA-MB-231, A549, NCI-H522 and HEK-293T to 6.5, 5.39, 4.83, 5.19 and 13.00 µg/ml respectively indicating the dose and time dependent cytotoxic effect of the venom protein. Cytotoxin 10 exhibited the maximum cytotoxic effect against lung cancer A549 cell line with IC_{50} value of 4.83 µg/ml. Interestingly, higher IC_{50} value was observed against HEK-293T cell line (13.00 µg/ml) suggesting its low cytotoxicity against normal cells. The preferential cytotoxic activity of Cytotoxin 10 against the cancer cell lines could be important for its potential use as an anti-cancer drug.

This study suggests that *N. kaouthia* venom, a medically important cobra species in North-East India known for causing severe local necrosis, exerts potent cytotoxicity when investigated *in vitro* in breast and lung cancer cell lines. Therefore, it is crucial to understand the mechanism of cell death induced by Cytotoxin 10 as it could help in unfolding the valuable prospect of cytotoxin-derived anticancer therapeutics.