Chapter 4

Partial compositional analysis of crude Naja kaouthia venom

4.1 Introduction

Snake venom is a complex mixture of proteins and peptides. They are trophic adaptations which enable them to capture prey or to defend. However, it is reported that venom proteins belongs to a relatively small number of protein families but this limited diversity has undergone accelerated evolution which resulted in exhibiting different activities within a single conserved molecular fold [287, 131]. Venom variation among major and minor taxonomic groups can be between intra species, different age groups in a species, feeding habits and others [262]. Each component in snake venom has its own specificity in exhibiting its pharmacological activity.

In the previous chapter, The SDS PAGE profile of the crude venom under reduced condition shows the presence of low molecular weight proteins at a molecular mass range of ~10kDa. Only a few protein bands were observed at higher molecular weight range. Also, the biochemical and biological characterization of crude *N. kaouthia* venom reveals that it is neurotoxic, anticoagulant, cytotoxic, myotoxic and has edema-inducing activity when tested *in-vitro* and in *in-vivo* conditions. This correlates with the various proteins present in crude venom. *N. kaouthia* from Northeast India has not been characterized and understanding of the venom composition of a particular species is important.

In the present chapter crude *N. kaouthia* venom of Northeast India was analyzed by a combination of reverse phase HPLC (RP-HPLC) and electrospray ionization mass spectrometry (LC-ESI/MS). As most of the snake venom protein families are characterized based on their molecular mass, the present study describes the composition of crude venom from Northeast Indian *N. kaouthia* based on molecular mass.

4.2 Results

4.2.1 Fractionation of crude venom

Crude *N. kaouthia* venom (5mg) was fractionated on a C_{18} reverse phase column HPLC (RP-HPLC). The fractionation was optimized using various gradients of buffer B (80% Acetonitrile + 0.1%TFA) and time intervals. Initially we fractionated the crude venom at 0-100% of buffer B (80% acetonitrile.H_2O+ 0.1%TFA) at time interval of 150mins (Figure 4.1) and a total of 12 distinct protein peaks were eluted. In the elution profile, some of the major crude venom proteins (~70-80% buffer B) eluted was found to be merged with several peaks. The hydrophilic proteins in the crude venom eluted (~30-40% buffer B) were also found to be merged. Therefore, we re-fractionated the crude venom at a gradient of 20-70% buffer B (Figure 4.2) at a time interval of 130mins and15 protein peaks were obtained. However, the elution intensities of the minor protein bands were less. The hydrophilic proteins, which earlier were eluted at ~ 30-40% buffer B, were not observed and hydrophobic proteins eluted approximately at 70% buffer B were not separated.

Finally, the crude venom was fractionated at a gradient of 20-50% buffer B at a time interval of 130mins (Figure 4.3). The crude venom was fractionated to 22 major and minor peaks (Figure 4.3). Protein peaks with percentage area greater than 8 were considered as major peaks. Hence, 5 major protein peaks and 17 minor protein peaks were obtained. All the major peaks were found to elute between ~30-45% of buffer B (Peak no. 7, 10, 13, 15, 17). (Figure 4.3). The comparatively hydrophilic components (Peak no. 1, 2, 3, 4, 5, 6 and 6´) in the crude venom eluted in the gradient ~15-25% of buffer B (Figure 4.3). Proteins with high hydrophobicity values were eluted at a gradient from ~50-75% of buffer B (Peak no. 20, 21 and 22) (Figure 4.3).

For all further fractionation of crude venom and isolation of protein, we used the optimized gradient of 20-50% buffer B. The fractionation result of crude *N. kaouthia* venom has been summarized in table 4.1. Retention time, peak area and concentration of protein present in each peak collected were determined. We injected 5mg of crude *N. kaouthia* venom for fractionation in all the gradients to optimize the elution profile of crude *N. kaouthia* venom

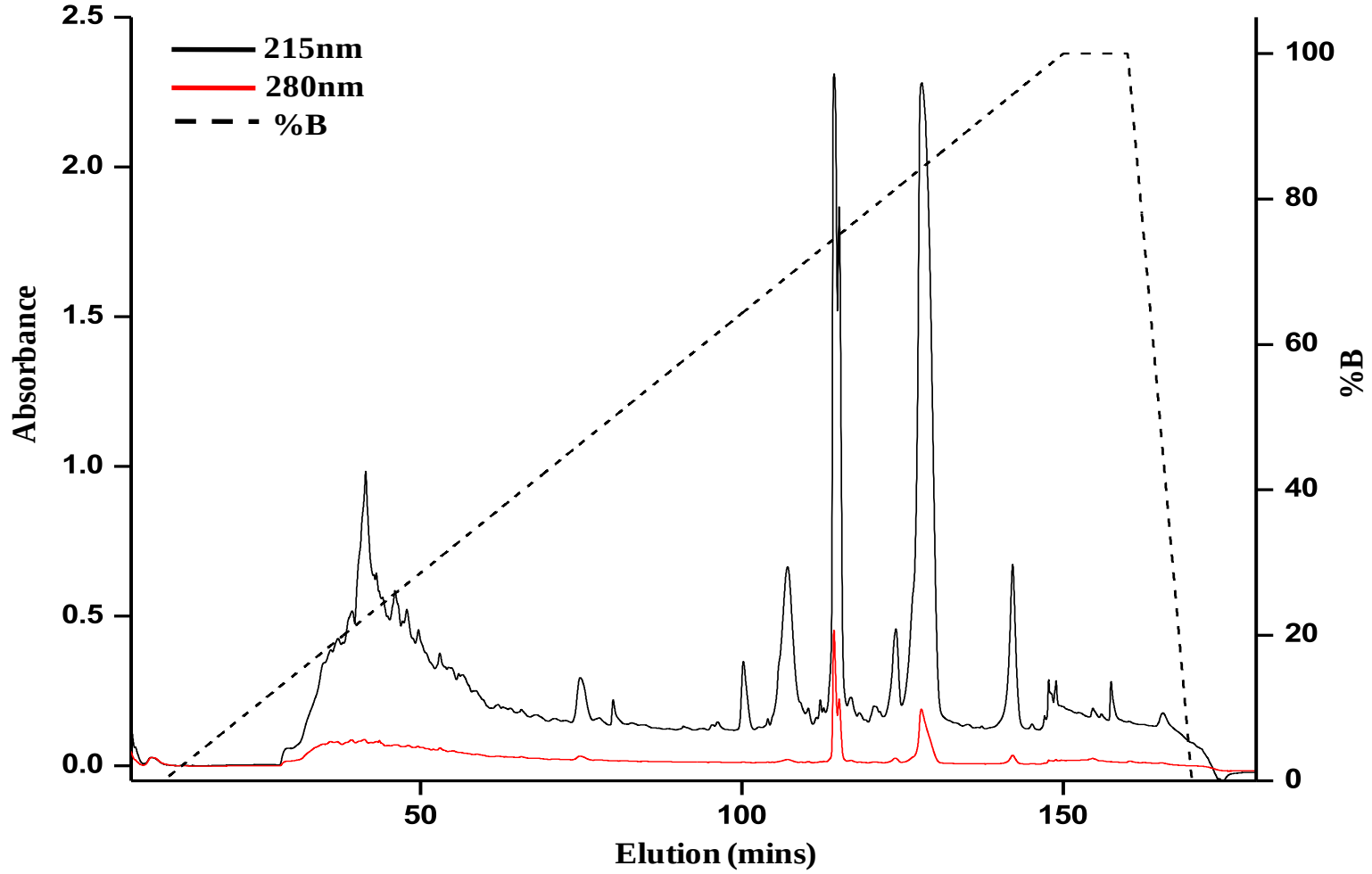

Figure 4.1: Fractionation of crude *N. kaouthia* venom. Elution was carried out using a gradient of 0-100% buffer B (80% Acetonitrile+ 0.1%TFA) at a time interval of 150mins. Absorbance was monitored in 215nm (Black) and 280nm (Red). Flow rate was maintained at 1ml/min.

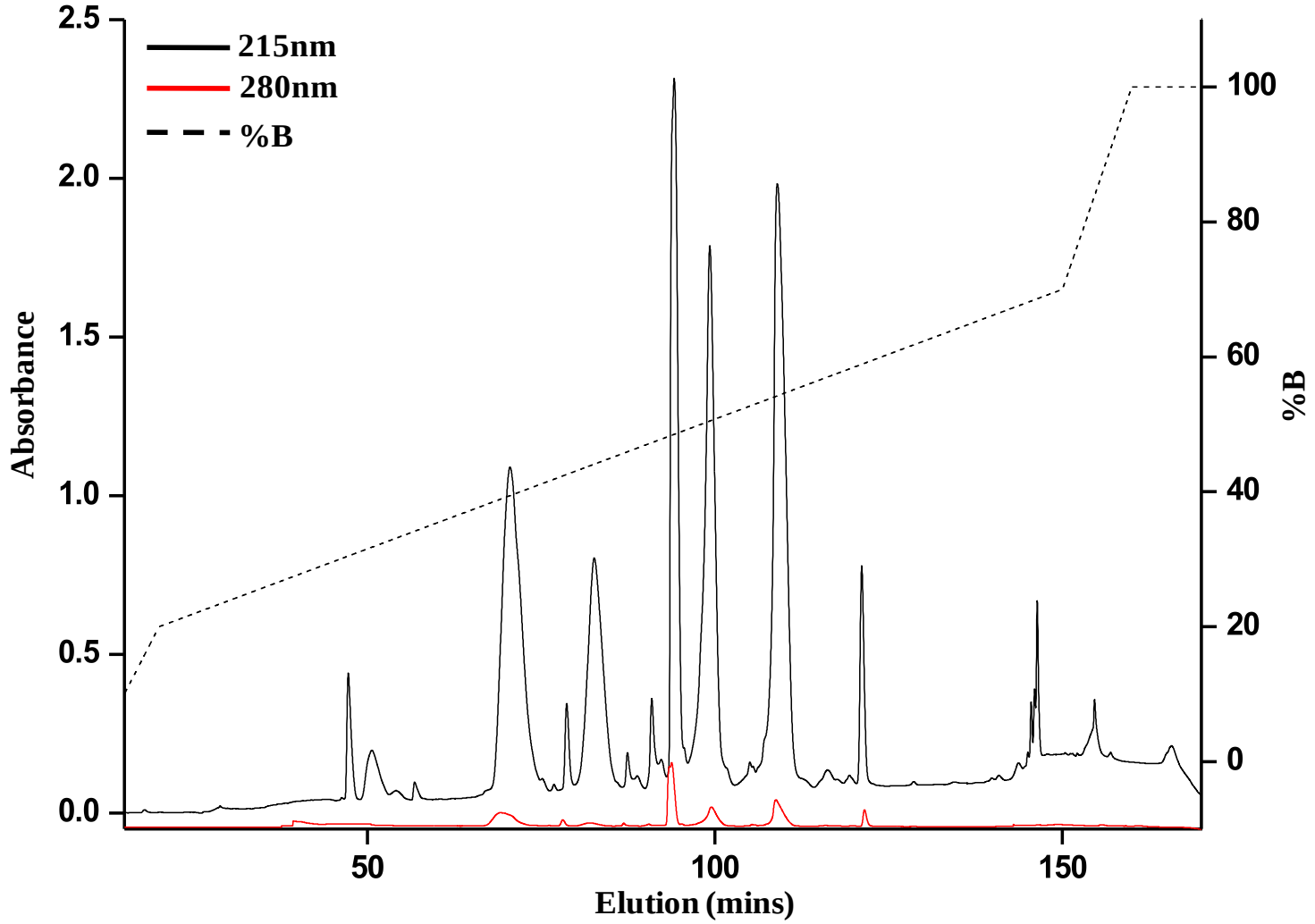

Figure 4.2: Fractionation of crude *N. kaouthia* venom. Elution was carried out using a gradient of 20-70% buffer B (80% Acetonitrile+ 0.1%TFA) at a time interval of 130mins. Absorbance was monitored in 215nm (Black) and 280nm (Red). Flow rate was maintained at 1ml/min.

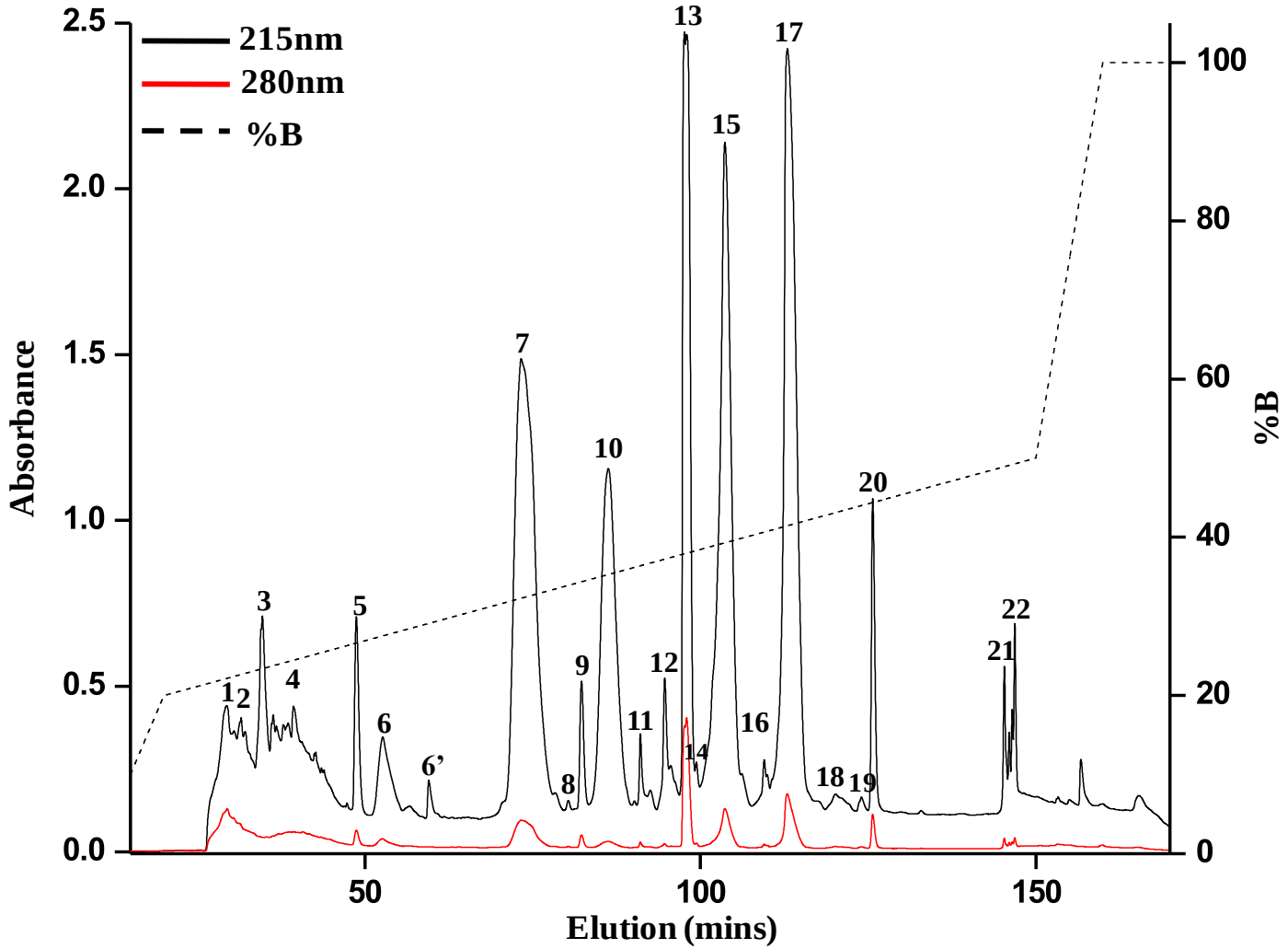

Figure 4.3: Fractionation of crude *N. kaouthia* venom. Elution was carried out using a gradient of 20-50% buffer B (80% Acetonitrile+ 0.1%TFA) at a time interval of 130mins. Absorbance was monitored in 215nm (Black) and 280nm (Red). Flow rate was maintained at 1ml/min. Peaks are numbered according to retention time.

Table 4.1 Summary of fractionation. The retention time and peak area was obtained and calculated using Empower 2 data software. Concentrations of various eluted fractions were calculated by considering amount (µg) of total venom injected and percentage area of the eluted peaks.

Sl No	RT (in mins)	% Area	Conc. (µg/µl)
1	46.362	2.1	0.0017
2	48.742	0.18	0.00902
3	58.089	2.17	0.11573
4	69.589	1.29	0.02201
5	76.886	1.27	0.0155
6	78.43	1.04	0.54111
7	80.923	10.82	1.21474
8	87.954	0.31	0.0035
9	90.978	1.05	0.03251
10	93.564	8.26	0.33955
11	93.99	1.1	0.0551
12	96.224	1.06	0.40308
13	99.7	17.08	0.85417
14	106.086	0.32	0.016
15	108.627	9.2	0.46007
16	109.132	2.3	0.03651
17	118.641	12.97	0.62563
18	121.866	0.19	0.0095
19	123.049	0.16	0.00354
20	145.039	1.81	0.04051
21	145.913	0.79	0.014
22	146.791	1.73	0.08652
Amount recovered (in mg)			**4.90**

***RT: Retention time.**

The percentage recovery of fractionation was 98.60% (4.9mg) based on the peak area. This reveals the presence of complex mixture of proteins in the venom.

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

Various proteins and peptides present in crude *N. kaouthia* venom of Northeast India have been identified based on their molecular mass. The method followed was a

combination of fractionating crude venom using RP-HPLC and identifying various proteins in the peaks by ESI/MS.

The principle followed by ESI-LC/MS has been shown in Figure 4.4. In the process, various proteins peaks eluted by HPLC are applied with high voltage and are electrically charged and sprayed, resulting into fine charged droplets (Figure 4.4). These droplets follow the Columbs repulsion principle and all charges move to the surface of the droplet. Finally vaporization of the droplets occurs with vaporization of ions. The resulted ions were checked for mass in a spectrometer (Figure 4.4).

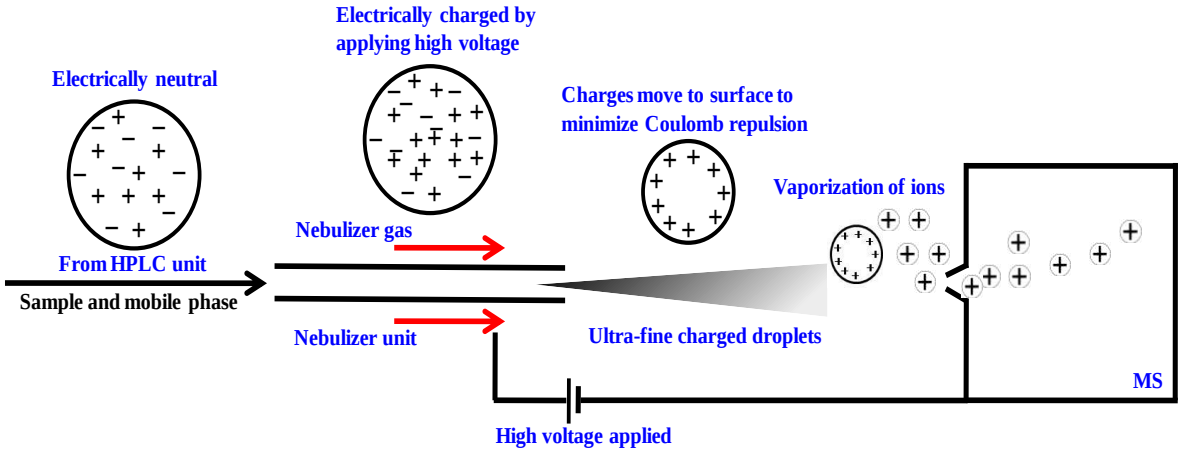

Figure 4.4: Schematic representation of ESI-LC/MS. (www.shimadzu.com)

Each fractionated protein peak from RP-HPLC resulted in a total of 11 MS spectra of different intensities (Figure 4.5). The raw data of peaks at different intensities of variable time intervals were analyzed for mass using Promas for Xcaliber. Mass of the various proteins, their retention time and intensity etc. are listed in table 4.2. The molecular mass obtained for different proteins in the crude venom were assigned to respective protein families (Table 4.2) based on their molecular mass. Molecular mass range from 6-7.5kDa were categorized under 3FTx family, proteins with molecular mass range from 13-14kDa were categorized under PLA_2 enzymes, proteins with a molecular mass from 12-13kDa were categorized in Ohanin/vespryn, thaicobrin protein groups and protein from 18-19kDa molecular mass range was categorized in C-type lectin like protein family [92, 117, 157, 288].

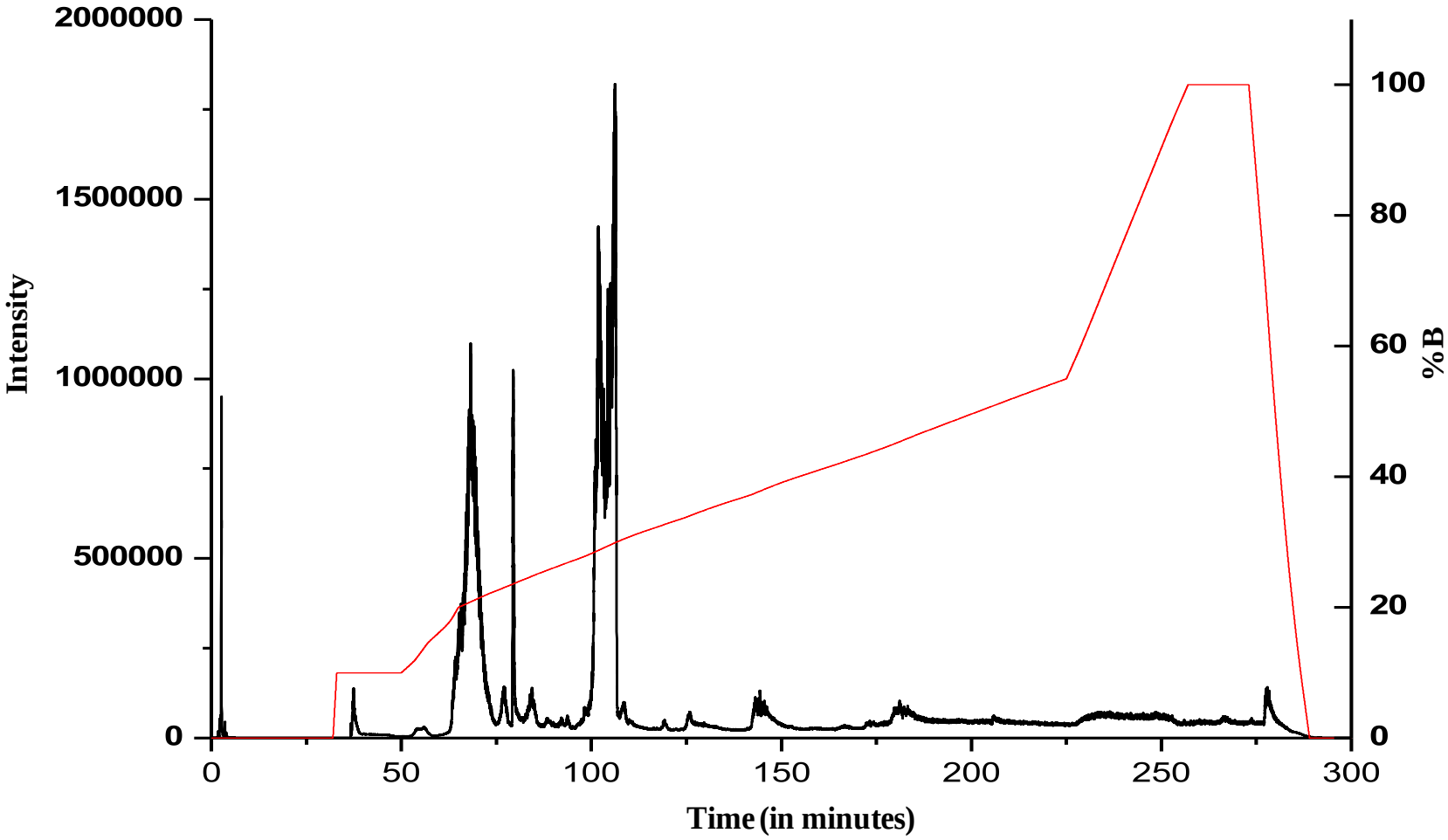

Figure 4.5: Fractionation of crude *N. kaouthia* venom coupled to ESI-MS. Total 11 peaks of protein was determined by fractionating crude venom using RP-HPLC. The molecular mass of the proteins present in each peak was determined by coupled ESI/MS.

Table 4.2: Molecular mass of various proteins in crude *N. kaouthia* venom as identified by ESI-MS

Retention time (min)	Mass (Da)	Intensity	Score	Delta mass	% relative	% total	Assigned proteins / protein family
36.63 to 37.57	6969.9	4.10E+04	13.93	0	100	68.53	3FTx
	7007	7.09E+03	8.15	37.1	17.31	11.86	
	6979.5	5.60E+03	4.1	9.6	13.67	9.37	
54.19 to 56.09	6888.4	3.73E+03	11.06	0	100	29.82	3FTx
	6853	3.06E+03	12.45	-35.4	82.05	24.47	
	6881.1	1.86E+03	6.16	-7.3	50.03	14.92	
	6978.9	1.51E+03	8.44	90.5	40.63	12.12	
63.27 to 70.96	7824.6	1.06E+05	16.64	0	100	50.79	3FTx
	7579.4	4.70E+04	13.04	-243.4	44.21	22.46	
	7615.3	1.15E+04	8.32	-209.3	10.77	5.47	
74.00 to 75.54	7110.3	2.47E+04	14.36	0	100	62.67	3FTx
	7824.7	3.43E+03	9.67	714.4	13.92	8.72	
81.17 to 82.41	7361.9	2.32E+04	14.9	0	100	51.77	3FTx
	7398.7	3.07E+03	6.06	36.8	13.25	6.86	
86.04 to 86.65	6755.6	9.79E+03	12.76	0	100	28.57	3FTx
89.09 to 89.69	18178.2	3.29E+03	2.94	15462.5	44.88	13.82	C-type lectins
	13480.7	3.18E+03	9.71	10765	43.38	13.36	PLA_2
90.61 to 91.15	7615.7	9.06E+03	8.57	0	100	38.98	3FTx
97.20 to 98.81	13319.9	7.54E+04	5.92	0	100	35.45	PLA_2
	13264.6	6.76E+04	6.77	-55.3	89.62	31.77	
	6739.8	2.10E+04	12.27	-6580.1	27.88	9.88	3FTx
98.88 to 99.22	6739.6	1.19E+05	15.61	0	100	64.73	3FTx
	6775.2	2.54E+04	1.43	35.6	21.35	13.82	
100.38 to 104.03	6740.6	2.49E+05	14.77	0	100	46.86	3FTx
	6839.5	2.19E+05	14.85	98.9	88.14	41.31	
	6775.4	2.13E+04	9.5	34.8	8.55	4	
	6789.6	4.77E+03	3.7	-5252.1	99.79	23.12	
120.45 to 123.14	13057.4	7.11E+03	8.87	0	100	22.52	PLA_2
	12901.4	5.10E+03	3.62	-156	71.68	16.14	Ohanin/vespryn,
	13095.8	1.12E+03	5.59	38.4	15.8	3.56	PLA_2
	12938.9	1.07E+03	2.85	-118.5	14.99	3.37	Ohanin/vespryn, thaicobrin
	12581.9	1.04E+03	4.52	-475.5	14.6	3.29	
136.88 to 141.21	12472.6	9.32E+03	5.51	0	100	36.43	Ohanin/vespryn, thaicobrin

3FTxs: three finger toxins, PLA_2: phopholipase A_2, Proteins/ protein family were assigned based on their molecular mass.

4.2.3 Relative abundance of various proteins

The observation with regard to various proteins assigned in different protein families were summarized and approximate relative abundance of proteins in crude *N. kaouthia* venom of Northeast India was determined considering the total protein as 100%. (Figure 4.6).

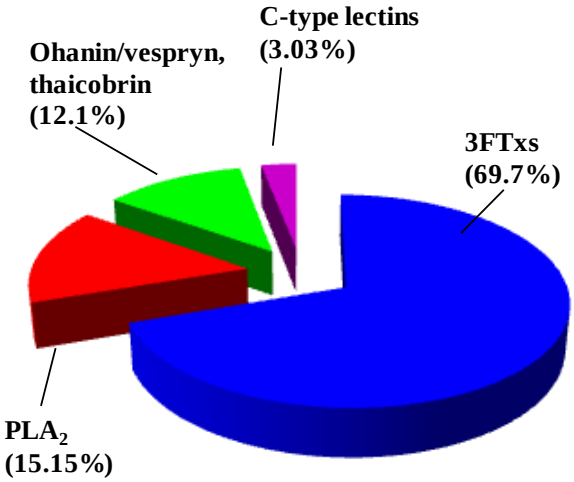

Figure 4.6. Relative abundance of various protein present in *N. kaouthia* of Northeast India

In our study we found 69.7% of proteins belong to 3FTx family, 15.5% proteins were PLA_2 enzymes, 12.1% proteins were either ohanin/vespryn or thaicobrin and 3.03% proteins were C-type lectin like proteins (Figure 4.6). However, there might be also other proteins such as L-amino acid oxidases (LAAO) and proteases which might not had been detected to due ionization problem associated with high molecular mass.

4.3 Discussion

Snakebite is a serious medical issue and per year large number of envenomation reports are recorded worldwide especially in Asia, Africa and Latin America [289, 290]. Asia is the worst affected region in world with maximum number of snakebite and fatality rate [14]. The notorious species mostly responsible for these bites are the cobras, kraits and vipers. At present, 29 different terrestrial species of genus *Naja* have been identified [291]. There are 11 species of genus *Naja* is found in Asia and 18 are found in Africa [291, 292]. *N. kaouthia* is one of the species which is widespread and endemic throughout South East Asia [44, 293]. In India, it is prevalent in northeast, West Bengal, Orissa and some parts in Uttar Pradesh. *N. kaouthia* of eastern India have been studied and a few proteins like PLA_2, neurotoxins, cardiotoxins have been reported [88, 220, 221, 241]. However, detailed proteomic study has not been carried out in terms of cobras found in India and it is important to understand the composition, variation and presence of unique toxins. Also, understanding venom composition of a particular species will lead to improvement of efficiency of commercial antivenom to treat the *Naja kaouthia* envenomation in a particular locality.

Snake venom varies in composition species to species and also within a species. The reasons for variation of venom composition can be due to various geographical location, feeding habits, age, post translational modifications etc. [218, 288, 294]. Seasonal variation and sex-based venom variation are also documented [262].

Elapid venoms have a major percentage of PLA_2 and 3FTxs [84, 295, 296]. However, their percentage in the whole venom in different species and within a species may vary. Recently, Laustsen et al., reported the venomics of Thailand cobra *(N. kaouthia)* by RP-HPLC, SDS-PAGE and MALDI-TOF. They documented the percentages of various proteins present [67]. In their study they determined 3FTxs as the major protein family consisting a total of 77.5% (24.3% cytotoxins and 24.3% were neurotoxins) and PLA_2 consisting about 13.5%. However, such compositional study had not been carried out with respect to Indian *N. kaouthia.* As *N. kaouthia* is endemic to South East Asia, comparison of fractionation profile of *N. kaouthia* crude venom from Thailand on C_{18} column was found to be similar with *N. kaouthia* of northeast India. Also, comparing the

relative abundance of toxins present in *N. kaouthia* of northeast India and Thailand, it was found to be closely related in PLA_2 and 3FTxs percentage. In prior analysis (section 3.1.3) of crude *N. kaouthia* venom from northeast India by SDS-PAGE under reduced and non reduced condition deciphered presence of higher molecular weight proteins at a range of 50-70kDa as well as major percentage of low molecular weight proteins (20kDa to below 10kDa). Comparison of SDS-PAGE profile with crude venom ESI/MS results, it was found that both correlated well, however the larger molecular mass proteins could not be detected in ESI/MS which might be due to difficulties in ionization. The SDS-PAGE of crude venom revealed protein bands at 25kDa, 35kDa, 50kDa and 70kDa under both reduced and non-reduced conditions for which molecular mass could not be determined on ESI/MS. The relative abundance of various proteins of *N. kaouthia* of northeast India confirms that PLA_2 and 3FTx family are the major proteins. The percentage of PLA_2 and 3FTxs in *N. kaouthia* of Thailand origin were found to be 13.5% and 77.5% respectively [67] which is closely related with the results obtained for venom composition of northeast Indian *N. kaouthia*. However, the Thailand *N. kaouthia* venom also showed nerve growth factors (0.3%), ohanins/vespryns (0.6%), C-type lectins (0.5%), nucleotidase (0.4%), phophodiesterase (0.4%), metalloproteinase (2.4%), LAAO (0.4%), cobra venom factors (0.1%) and cytidyltransferase (0.3%) [67]. Nucleosides such as adenoside, guanosine and inosine were detected too [67]. In the venom of northeast Indian *N. kaouthia,* ohanin/vespryn and C-type lectins were found to be 12.1% and 3.03% respectively. The present compositional study for northeast Indian *N. kaouthia* is only based on molecular mass; detailed studies will be required to decipher the various proteins present.

The present study reveals the partial composition of the crude venom, which can be correlated with various pharmacological effects, tested *in-vitro* and *in-vivo* in our previous studies as described in chapter 3. This kind of study is important because of venom variation in and within the species at different geographical location [68]. A recent study by Tan et al., (2015), where they have analyzed *N. kaouthia* venom from Malaysia, Thailand and Vietnam using reverse-phase HPLC, SDS-PAGE and tandem mass spectrometry [68]. In their studies they had determined 13 toxin families with 3FTxs (63-77%) being the most abundant and most varied isoforms (11-18) in all the three

snakes [68]. Cobras from Thailand and Vietnam were found to contain the highest content of neurotoxins, followed by cobra from Malaysia. However, both Malaysian and Vietnamese cobra constitute the major percentage of cytotoxins (up to 45% each) which was found less in Thailand cobra (up to 27%) [68]. All the cobras also showed different lethal potencies reflecting their proteomic findings [68]. This study represents the importance of detailed proteomic studies in and within species where variation in venom composition can be in closely settled geographical locations. Further, this compositional analysis will aid in development of species specific antidotes which will reduce risk in use of commercially available polyvalent antivenom.

Even the *in-vitro* and *in-vivo* biochemical and biological activities by crude *N. kaouthia* venom with its derived partial composition, it was found to be similar and is correlated. The presence of higher percentage of PLA_2 and 3FTxs in crude *N. kaouthia* venom can be related with the significant PLA_2 activity, myotoxicity, edema inducing activity, anticoagulant activity, cytotoxicity and neurotoxic property which the venom exhibit on the prey or the victim. This is the first report of proteomic characterization based on molecular mass of various proteins in crude *N. kaouthia* venom of Indian origin.