CHAPTER 2

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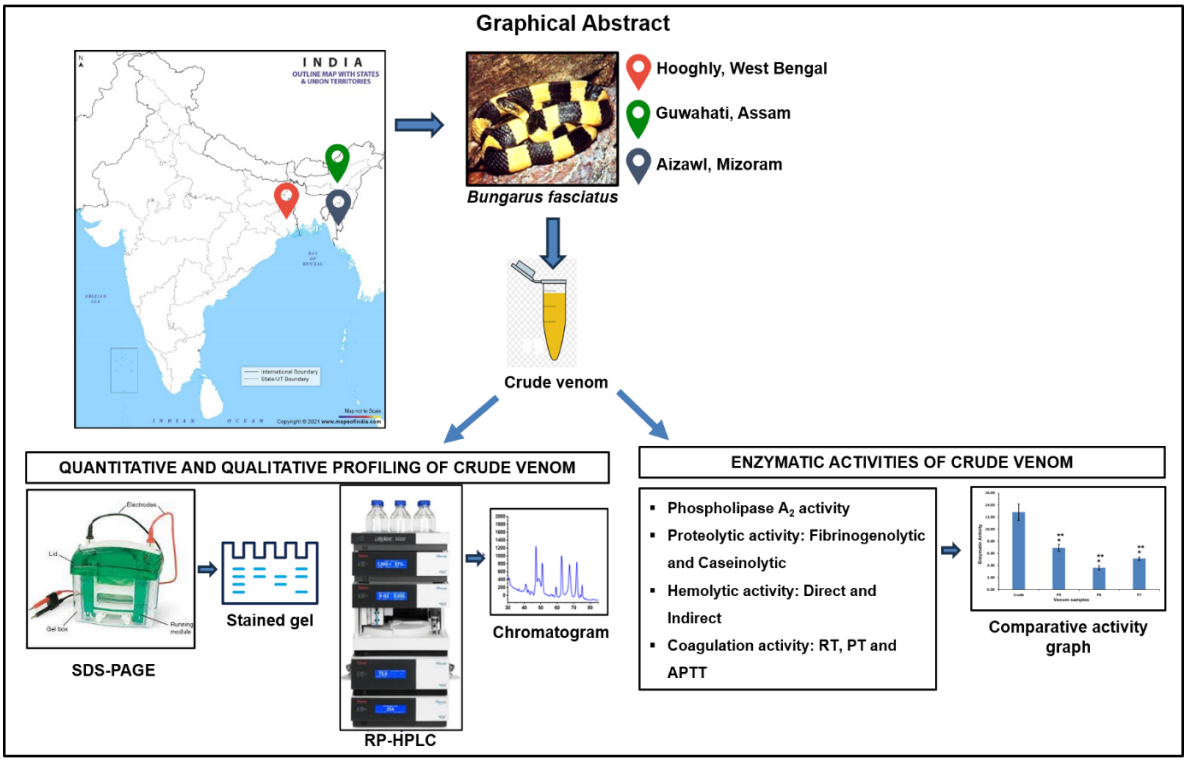

2.1 Introduction

In India, *B fasciatus* is prevalent in the Eastern and North-Eastern regions. According to Suraweera et al., the Eastern states of Bihar, Odisha and Jharkhand fall in the category of 'High burden states' for snakebites with standardized average death of 8.9, 5.9 and 7.1 per 100,000 population rates (between 2010-2014) respectively. In contrast, West Bengal, Assam and other North-Eastern states fall under the 'Low burden states' category where the rate is 2.9, 2.1 and 0.7 respectively [9]. However, the epidemiology of snakebites mostly in North-East India remains poorly highlighted in these survey reports as a large number of snakebite cases go unreported [16,138]. In this chapter, the geographical variation in venom composition was explored by studying the venom

profile of *B. fasciatus* and its associated biochemical activities from 3 different locations, where samples were collected from two nearby suburbs from Hooghly (West Bengal), and one sample each from Aizawl (Mizoram) and Guwahati (Assam) respectively.

The three locations were chosen based on the assumption that differences in terrain and elevation from sea level may affect the intra-species venom composition of *B. fasciatus.* The Hooghly district (latitude 22^{o}39' N and longitude 88^{o}30' E) of West Bengal is a plains area, about 200 m above sea level, and has three soil groups viz. Gangetic alluvium, alluvium deposits and red soil region [139,140]. The second location of Guwahati, Kamrup metropolitan district, Assam (latitude 26^{o}20' N and longitude 91^{o}75' E) is at a displacement of 491 km from Hooghly (West Bengal) and 286 km from Aizawl district (Mizoram). The city consists of low-lying plains with an average elevation of 50-55 m above sea level, however, it is also surrounded by hills on three sides with a maximum elevation of 300 m [141]. Lastly, Aizawl (Mizoram) (latitude 23^{o}58' N and longitude 92^{o}16' E) is located at a displacement of 463 km from Hooghly district (West Bengal) and the elevation is about 1,132 m from sea level and it consists of a hilly to mountainous terrain [142]. Hence, the three locations encompass low-lying plains to moderately hilly terrain and finally to mountainous terrain.

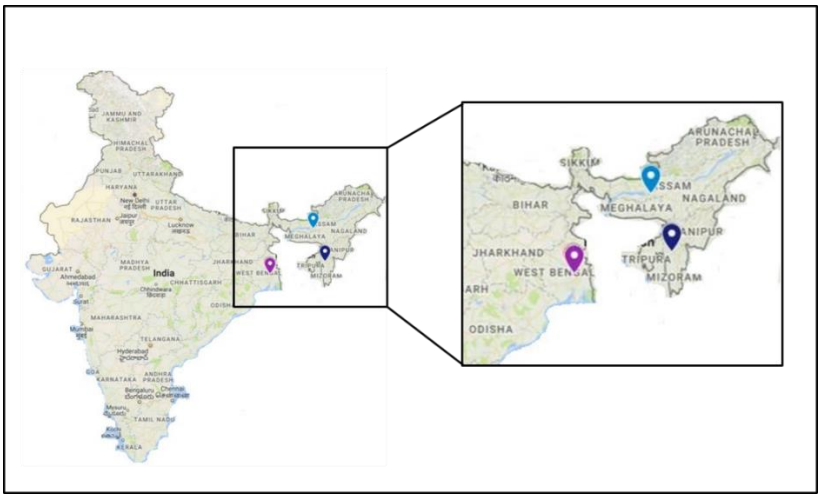

Figure 2.1: *B. fasciatus* venom collection locations of Hooghly (West Bengal ; in purple color), Aizawl (Mizoram ; in dark blue), and Guwahati (Assam; in light blue).

2.2 Materials

2.2.1 Snake venoms

Dr. Dayal Bandhu Majumdar, Calcutta National Medical College and Hospital, Kolkata (West Bengal) graciously provided us with freeze-dried venom of *B. fasciatus* from Eastern India. Venom was extracted from two separate snakes found in neighbouring villages of district Hooghly (named Hooghly1 and Hooghly2 for this study) of West Bengal, and permission to collect the venom was provided by the Additional Principal Chief Conservator of Forests (Wildlife) and ex-officio Additional Chief Wildlife Warden, West Bengal (Permission No. 5141/WL/4R-6/2017, on 27th November 2017). *B. fasciatus* venom from Aizawl (Mizoram) was a generous gift to us by Dr. H. T. Lalremsanga, Mizoram University. The venom was collected after obtaining necessary permissions from the Chief Wildlife Warden, Mizoram (Permision No. A.33011/5/2011-CWLW/305, on 18th July 2016). *B. fasciatus* venom from Guwahati (Assam) was collected after necessary approval was obtained from the Principal Chief Conservator of Forests (Wildlife) and Chief Wildlife Warden, Assam (Permission No. 450, on 1st October 2011). The venoms were lyophilized immediately after milking. Russell's viper (*Daboia russelii*) and Common krait (*B. caeruleus*) venom in lyophilized form were obtained from the Irula Snake Catchers' Industrial Co-operative Society (Tamil Nadu, India). The lyophilized venoms were kept at -20 ^{o}C for subsequent experiments.

2.2.2 Chemicals and reagents

Glycerol, tris base, tricine, sodium lauryl sulphate, bromophenol blue, methanol and glacial acetic acid, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), acrylamide, bisacrylamide, ethanol, sodium thiosulfate, silver nitrate, and 2-mercaptoethanol (BME) were procured from Sigma Aldrich, USA, PageRuler™ plus prestained protein ladder were purchased from Thermo Scientific, USA. Trifluoroacetic acid (TFA) and acetonitrile (MeCN) of HPLC grade were procured from Sigma Aldrich, USA. Bovine serum fibrinogen, casein and trichloroacetic acid (TCA) were purchased from Merck (Sigma Aldrich, USA), Trisodium citrate and sodium chloride of analytical grade were purchased from Merck (Sigma Aldrich, USA). Uniplastin and Liquicelin-E were purchased from Tulip Diagnostics Pvt. Ltd., India. $sPLA_2$ assay kit was procured from Cayman Chemical Company, USA.

2.2.3 Columns

For examination of crude venom using RP-HPLC, Acclaim™ 300 C18 column (150 × 2.1 mm, 3 µm, 300Å) was purchased from Thermo Fisher Scientific, USA.

2.3 Methods

2.3.1 Protein estimation

Total protein content of the venoms was estimated utilizing a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) by selecting the Protein A20 option and 1 mg/ml extinction coefficient. The lyophilized snake venoms dissolved in purified (Milli-Q) water (2 mg/ml) was used for the estimation, and only Milli-Q water was taken as the blank. Two microlitre (2 µl) of the dissolved venom sample was placed in the detector and concentration of the protein content was measured. The percentage of total protein in the crude venom samples were calculated using the following formula:

$$\text{Percentage of protein in crude venom (\%)} = \frac{\text{Estimated protein concentration (mg/ml)}}{\text{Amount of crude venom dissolved (mg)}} \times 100$$

2.3.2 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

The samples of *B. fasciatus* venom were profiled in a 10% Tris-tricine gel so that the small molecular weight proteins could be visualized properly. The 10% resolving gel (final volume 6 ml) was prepared by adding 1.8 ml ethylene glycol, 3M Tris-Cl (1.5 ml), 40% bis-acrylamide (1.5 ml), distilled water (1.2 ml), 40% APS (6 µl) and TEMED (9 µl). The 4% stacking gel (final volume 3 ml) was made by mixing 3M Tris-Cl (0.75 ml), 30% bisacrylamide (0.51 ml), distilled water (1.74 ml), 40% APS (3 µl) and TEMED (12 µl). Each of the venom (5 µg) was mixed with 5x loading dye (5% BME, 0.001% bromophenol blue) and placed in a water bath for 3 min set to 100 ^{o}C. After loading the samples onto the gel, running buffer (0.1 M Tris, 0.1 M tricine, 4% SDS) was introduced to the electrophoresis tank (Mini-Protean Tetra Cell, Bio-Rad Laboratories, USA). The stacking gel was subjected to electrophoresis at 110 V while the resolving gel underwent electrophoresis at 120 V, and subsequently silver staining was carried out for visualization [143]. The image of the gel was documented using an imaging system (ChemiDoc XRS+, BioRad Laboratories, USA).

2.3.3 Reversed phase- High Performance Liquid Chromatography (RP-HPLC)

The venom proteins were fractionated to understand their compositional variations by subjecting the crude venoms to RP-HPLC. Injection of the samples (50 µg) into the RP-HPLC (UltiMate™ 3000 UHPLC, Thermo Fisher Scientific, USA) was performed utilizing a 2 ml syringe and then fractionation of the samples was carried out in a C18 column (150 x 2.1 mm, 3 µm, 300Å) equilibrated previously with 0.1% TFA. The proteins were eluted using two buffers: Buffer I (Purified water, 0.1% TFA) and Buffer II (80% MeCN, 0.1% TFA) with a 40-50% MeCN gradient continued for 20-80 min. The flow rate was 0.2 ml/min for a total duration of 120 min. Elution of the venom peaks were monitored at a wavelength of 215 nm and the resolved venom peaks were manually collected for further experiments.

2.3.4 Phospholipase A_2 activity

Turbidometric method

The PLA_2 activity of the crude venom of *B. fasciatus* was evaluated using the turbidometric methodology outlined by Joubert and Taljaard, further refined by Doley and Mukherjee [144,145]. In brief, crude *B. fasciatus* venoms 1.0 µg (final concentration 5 µg/ml) from different locations were added to assay buffer (20 mM Tris, pH 7.4) and made up to a total volume of 60 µl, followed by the addition of 140 µl egg yolk solution substrate (one chicken egg yolk mixed with 0.9% NaCl (250 ml)), with optical density (O.D) pre-adjusted to 1.0 at 740 nm, and then the decrease in absorbance was documented continuously for 10 min at 15 s time-gap at 740 nm in a multiplate reader (MultiSkan GO, Thermo Fisher Scientific, USA). Assay buffer was taken as the blank and bee venom (0.05 µg) was taken as the positive control. A unit of PLA_2 activity (µg/min/mg) is the reduction in absorbance by 0.01 O.D at 740 nm for 10 min.

Colorimetric method

The PLA_2 activity of the crude venoms were also evaluated by the colorimetric technique using a $sPLA_2$ assay kit following the instruction manual. In brief, a venom dose of 1.0 µg (final concentration 8.88 µg/ml) was dissolved in 7.5 µl of assay buffer (0.3 mM Triton X-100, 10 mM $CaCl_2$, 100 mM KCl in 25 mM Tris-Cl, pH 7.5) and 5 µl of 10 mM DTNB. Next, 100 µl substrate (Diheptanoyl Thio-PC, 1.66 mM) was added to the

buffer. Assay buffer mixed with DTNB and substrate was taken as the blank, and bee venom (0.05 µg) was considered as the positive control. The absorbance change was recorded at 414 nm for 10 min in a multiplate reader (MultiSkan GO, Thermo Fisher Scientific, USA). A unit of PLA_2 activity is denoted by the specific activity (µmol/min/ml/µg), which is the µmol of the substrate hydrolysed by one µg of the sample per min at 25 ^{o}C.

2.3.5 Proteolytic activity

Fibrinogenolytic activity

Fibrinogen is a large, soluble glycoprotein which helps in blood clot formation and has two sets of Aα, Bβ and γ-chains connected by disulphide bonds, and an outer D-domain, which is connected to a centrally coiled E-domain [146]. The *B. fasciatus* venoms' proteolytic activity was evaluated by taking fibrinogen as a substrate according to the methodology outlined by Ouyang and Teng [147]. Briefly, fibrinogen (2mg/ml) of bovine origin was dissolved in 50 mM Tris (pH 7.5) and 150 mM sodium chloride for 4 h at 37 ^{o}C. Following incubation, the venom samples (10 µg) were added to the fibrinogen solution for different time intervals (1 h and 24 h) at 37 ^{o}C. For negative control, fibrinogen without the addition of any sample was considered. The samples were analysed using 10% Glycine gel under reducing conditions to observe any potential change in fibrinogen bands followed by Coomassie staining and subsequent destaining using Water, Methanol and Acetic acid at 50:40:10 ratio for visualization.

Caseinolytic activity

Preparation of standard tyrosine curve

A stock solution of tyrosine (200 µg/ml) prepared in distilled water was heated till the tyrosine dissolved and then different concentrations of tyrosine (0.1 to 100 µg/ µl) were taken and protein estimation was done by Lowry's method [148]. The values were plotted on a graph to obtain the standard curve.

Preparation of casein solution

Casein solution (1%) was prepared by dissolving 100 µg casein in 10 ml of 20 mM Tris (pH 7.4), and then stirred at 25 ^{o}C for 0.5 h, followed by moderate heating at 37 ^{o}C for 20 min. Stirring the solution was continued for another 20 min after which it was kept at

4 ^{o}C for overnight. The next day, the solution was subjected to centrifugation at 1000 rpm for 15 min followed by the collection and filtration of the supernatant using a syringe filter (0.2 µm).

Digestion of casein and estimation of released tyrosine

The caseinolytic activity of the crude venoms from *B. fasciatus* were estimated utilizing the methodology of Ouyang and Tang, which was further modified by Mukherjee et al. [147,149]. In brief, the crude venoms (20 µg) were mixed with casein solution (1%) at 37 ^{o}C for 1.5 h after which 10% ice-cold TCA was added to stop the reaction and then incubated for another 30 min at 4 ^{o}C. Next, the digested proteins were collected by centrifugation at 5000 rpm for 10 min. Lowry's method was utilized to estimate the tyrosine content of the supernatant [148] and the absorbance was captured at 660 nm utilizing a multiplate reader (Thermo Scientific, USA). The enzyme activity and specific activity was determined using the following calculation:

$$Enzyme\ activity = \frac{Amount\ of\ tyrosine\ released\ (\mu g) \times Total\ volume\ of\ assay\ mixture\ (ml)}{Volume\ of\ enzyme\ \ (ml) \times Incubation\ time\ (min)\ \times Volume\ in\ colorimetric\ test\ (ml)}$$

$$Specific\ activity = \frac{Enzyme\ activity}{Concentration\ of\ protein\ (mg)}$$

2.3.6 Hemolytic activity

Separation of Platelet-poor plasma (PPP) and Red Blood Cells (RBCs) from whole blood

Fresh blood (45 ml) from a sacrificed goat was obtained from a local butcher shop, and was mixed with 3.8% sodium citrate (5 ml) and then subjected to centrifugation at 5000 rpm for 20 min at 4 ^{o}C. The platelet-poor plasma (PPP) was pipetted and stored at -20 ^{o}C and the pellet containing RBCs was washed with 0.9% NaCl 3-4 times at 5000 rpm for 15 min and then re-suspended in 0.9% NaCl for further use.

Direct hemolytic activity

From 10% RBC suspension, 150 µl was taken and mixed with *B. fasciatus* venom samples of increasing concentrations (0.01, 0.1, 1.0 and 10.0 µg/ml) and the volume of the reaction mixture was adjusted to 2 ml. The mixture was kept for 1 h at 37 ^{o}C and then

subjected to centrifugation at 10,000 rpm for 10 min. This was followed by collection of the supernatant and estimating its absorbance in a multiplate reader at 540 nm. For negative control, RBC suspension mixed with 0.9% NaCl was taken, and RBC mixed with distilled water was taken as the positive control. Hemolysis caused by the positive control was considered 100%.

Indirect hemolytic activity

The methodology for determining indirect hemolytic activity was similar to that of direct hemolytic activity. In the 10% RBC solution, 20 µl of egg yolk substrate (one chicken egg yolk mixed with 250 ml of 0.9% NaCl) was added followed by the addition of different doses (0.01, 0.1, 1.0 and 10.0) of *B. fasciatus* venom samples, and then the reaction volume was adjusted to 2 ml. RBC suspension mixed with 0.9% NaCl and 20 µl of egg yolk solution was taken as the negative control, and RBC mixed with distilled water and 20 µl of egg yolk solution was taken as the positive control. The further steps were exactly the same as that of direct hemolytic activity.

2.3.7 Coagulation activity

Recalcification Time

Recalcification time (RT) was determined for the crude venoms of *B. fasciatus* following the methodology outlined by Sharma et al. [150]. In brief, increasing concentrations of crude venoms (0.01, 0.1, 1.0 and 10.0 µg/ml) were mixed with 25 µl of Tris-Cl (20 mM, pH 7.4) and added to 50 µl of PPP followed by incubation for 120 s at 37 ^{o}C. The coagulation of PPP was started by adding 25 µl of $CaCl_2$ (50 mM) and the alteration in absorbance was recorded every 15 s for 15 min at 405 nm using a multiplate reader. The time taken by PPP with 20 mM Tris-Cl for coagulation was taken as the normal clotting time (NCT).

Prothrombin Time

Prothrombin time (PT) was determined for the crude venoms of *B. fasciatus* following the methodology of Sharma et al. [150]. In brief, increasing concentrations of crude venom (0.01, 0.1, 1.0 and 10.0 µg/ml) were added to PPP (50 µl) for 120s at 37 ^{o}C after which Uniplastin (50 µl) was added for starting clot formation. The alteration in

absorbance was recorded every 2 s for 2 min at 405 nm using a multiplate reader. The time taken by PPP with Uniplastin for coagulation was considered as the NCT.

Activated Partial Thromboplastin Time

Activated partial thromboplastin time (APTT) was determined for the crude venom of *B. fasciatus* venoms following the methodology outlined by Sharma et al. [150]. In brief, increasing concentrations of crude venom (0.01, 0.1, 1.0 and 10.0 µg/ml) were added with 50 µl PPP after which Liquicelin-E (50 µl) was added and incubated for 180 s at 37 ^{o}C. To initiate the clot formation, 50 µl of $CaCl_2$ (25 mM) was added to the mixture. The alteration in absorbance was recorded every 2 s for 300 s at 405 nm using a multiplate reader. The time taken by PPP with Liquicelin-E and 25 mM of $CaCl_2$ for coagulation was considered as the NCT.

2.3.8 Statistical analysis

The statistical significance of the values obtained for the *B. fasciatus* venom samples was tested by One-Way ANOVA using the software Microcal Origin version 6.0. The significance level is designated by their p-value ($^{\#}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$) after evaluation.

2.4 Results

2.4.1 Quantitative and qualitative analysis of crude venom profile

2.4.1.1 Protein estimation of crude venoms

The crude venoms (2 mg dry weight) of *B. fasciatus* from different locations dissolved in Purified (Milli-Q) water (2 mg/ml) have been found to have minor variations in total protein content compared to their respective dry weights. The highest protein content was present in the sample from Hooghly2, West Bengal (81.60 %) and the lowest was present in the sample from Aizawl, Mizoram (74.55 %) (Table 1). The remaining non-proteinaceous portion of crude venom mostly comprises metal ions, lipids, carbohydrates, citrate, amines, nucleosides, and inorganic anions [113,114].

Table 2.1: Estimation of protein concentration of crude *B. fasciatus* venoms

Location of sample collection	Protein concentration (mg/ml)	Percentage of dry weight of venom (%)
Hooghly1, West Bengal	1.557±0.18	77.85
Hooghly2, West Bengal	1.632 ±0.20	81.60
Aizawl, Mizoram	1.491±0.31	74.55
Guwahati, Assam	1.581±0.11	79.05

2.4.1.2 Gel electrophoresis of crude B. fasciatus venoms

From the gel electrophoresis analysis of *B. fasciatus* venoms using 10% Tris-tricine gel, it has been observed that several protein bands of different intensity were observed in the venom samples. For all the venom samples, the protein bands were observed in the 13 to 75 kDa molecular weight range (Figure 2.2).

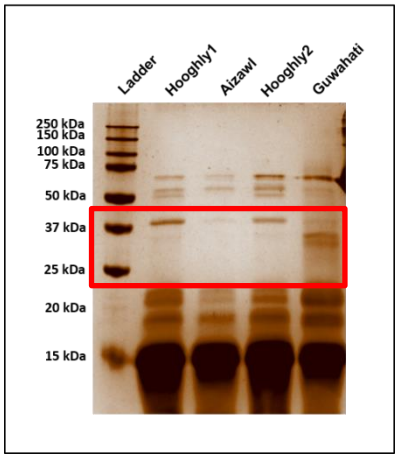

Figure 2.2: SDS-PAGE gel showing the venom profiles of *B. fasciatus*. The samples (5 µg) were reduced with beta-mercaptoethanol and 0.001% bromophenol blue and subjected to electrophoresis using 10% Tris-tricine gel and then visualized by silver staining. Pre-stained protein marker (PageRuler plus, Thermo Scientific, USA) was used as the protein ladder. The molecular weights of the proteins are marked (kDa). The difference in the banding pattern in the 25-37 kDa range is highlighted in a Red-colored box.

The protein bands in all the lanes were clustered in four molecular weight groups, lying in the 38-75, 25-37, 18-24 and 13-17 kDa range. However, comparing the samples from Hooghly1 and Hooghly2 with those from Guwahati and Aizawl revealed various disparities. For instance, two bands in the range of 25-37 kDa were viewed in the venom from Guwahati which was absent in all the other three samples. Similarly, the bands at 37 kDa exhibited a low intensity for the samples from Aizawl and Guwahati compared to that of Hooghly1 and Hooghly2 (Figure 2.2). On the other hand, dense patches of bands

were observed for all four samples at 13-17 kDa range, which represents a major fraction of the venom profile and may belong to the PLA_2 superfamily.

2.4.1.3 RP-HPLC profiling of crude venoms

For understanding the differences and similarities of the *B. fasciatus* venoms, these were subjected to RP-HPLC profiling. The congeneric *Bungarus caeruleus*, which is one of the "Big-Four" snakes, was also subjected to RP-HPLC profiling under the same chromatographic conditions for 120 min. The profiles of *B. fasciatus* and *B. caeruleus* were numbered consecutively. It was observed that for *B. fasciatus* a maximum of 9 peaks were obtained and for *B. caeruleus* a total of 22 peaks were obtained. The stacked profiles of all the venom samples were grouped into six zones (labelled A to F) for the convenience of description (Figure 2.3). In Zone A, no peak was observed for *B. caeruleus* venom and *B. fasciatus* venom sample from Aizawl, however, four peaks for *B. fasciatus* venom samples from Hooghly1 and Guwahati, and three peaks for Hooghly2 were observed.

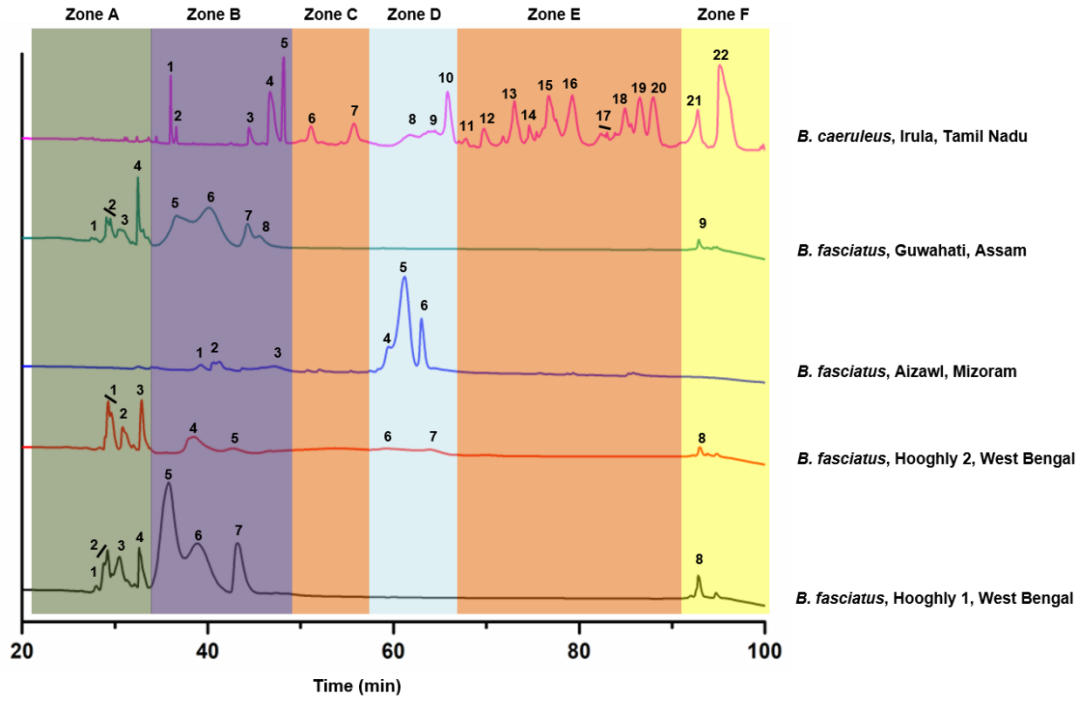

Figure 2.3: Chromatography profile of crude venoms of *B. fasciatus* and the congeneric "Big-Four" snake *B. caeruleus* (Tamil Nadu, India) after RP-HPLC analysis. The profiles were grouped into 6 zones (A to F) for the convenience of description.

Table 2.2: Number of peaks eluted in RP-HPLC profiles at different time-intervals (depicted as Zones).

RP-HPLC profile Zone	Elution Time	Number of peaks eluted in different zones				
		B. caeruleus (Irula, Tamil Nadu)	*B. fasciatus*			
			Guwahati	Aizawl	Hooghly2	Hooghly1
A	20-32 min	0	4	0	3	4
B	32-49 min	5	4	3	2	3
C	49-58 min	2	0	0	0	0
D	58-67 min	3	0	3	2	0
E	67-86 min	10	0	0	0	0
F	86-100 min	2	1	0	1	1

Zone B had 2-4 peaks of dissimilar pattern and peak height for the *B. fasciatus* venom samples, and five peaks were visible for the *B. caeruleus* sample. In Zone C, two peaks were visible for *B. caeruleus* venoms, and no peaks were observed from the *B. fasciatus* venoms. In Zone D, 3 peaks were observed each for *B. caeruleus* and *B. fasciatus* (Aizawl). Furthermore, two minor peaks were also observed for the venom of *B. fasciatus* from Hooghly2 but no peaks were observed for samples from Hooghly1 and Guwahati. Zone E exhibited the largest number of peaks (ten) for the *B. caeruleus* venom, on the contrary, no peaks for the venom from *B. fasciatus* were observed in this zone. The last zone (Zone F) represented two peaks for *B. caeruleus* and one small peak each for *B. fasciatus* from Hooghly1, Hooghly2 and Guwahati respectively. However, peaks were absent in Zone F for the *B. fasciatus* sample from Aizawl (Table 2.2).

2.4.2 Comparative biochemical analysis of crude venoms

2.4.2.1 Phospholipase A$_2$ activity of crude venoms

The *B. fasciatus* venoms from different locations were checked for PLA$_2$ activity in vitro using both turbidometric and colorimetric method.

Turbidometric method

The PLA$_2$ activity of crude *B. fasciatus* venoms were significantly higher than the positive control (22.53±0.26 µg/min/mg) and intra-specific variations in activity was observed. The highest activity was exhibited by the crude venom from Guwahati

(39.75±0.08 µg/min/mg), followed by that from Hooghly1 (38.25±0.25 µg/min/mg), Hooghly2 (34.95±1.12 µg/min/mg) and Aizawl (33.20±3.14 µg/min/mg) respectively (Figure 2.4).

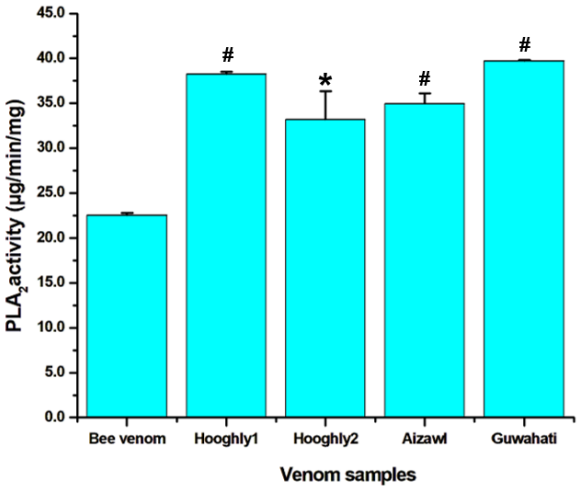

Figure 2.4: PLA_2 activity of *B. fasciatus* venoms estimated using turbidometric method. Venom doses of 1.0 µg was added to 60 µl assay buffer followed by 140 µl of egg yolk solution and then the absorbance was recorded at 740 nm for 10 min at 15 s interval. Bee venom was considered as the positive control. The level of significance ($^{\#}p<0.001$ and $^{*}p<0.05$) was examined with regard to the positive control. Each data-point depicts the mean ± S.E of three distinct experiments.

Colorimetric method

The colorimetric method also exhibited similar results for PLA_2 activity as that of the turbidometric method. The PLA_2 activity (in µmol/min/ml/µg) of venom samples were significantly higher than the positive control (3.97±0.49). The highest activity was exhibited by the crude *B. fasciatus* venom sample from Hooghly2 (11.55±0.25), followed by Guwahati (10.46±0.56), Hooghly1 (9.61±0.44) and Aizawl (8.22±0.86) respectively (Figure 2.5).

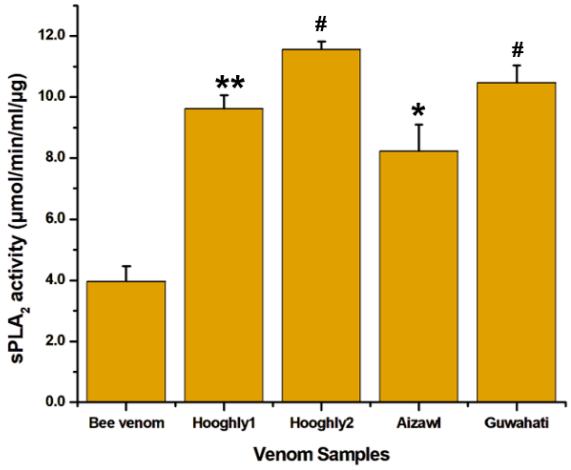

Figure 2.5: PLA_2 activity of *B. fasciatus* venoms estimated by $sPLA_2$ kit (colorimetric method). Venom doses of 1.0 µg was added to 7.5 µl assay buffer and 5 µl DTNB and then 100 µl of Diheptanoyl Thio-PC was added as substrate and absorbance was monitored at 414 nm for 10 min at 30 s interval. Bee venom served as the positive control. The p-value ($^{\#}p<0.001$, $^{**}p<0.01$ and $^{*}p<0.05$) examined with regard to the positive control. Each data-point depict the mean ± S.E of three distinct experiments.

2.4.2.2 Proteolytic activity of crude venoms

Fibrinogenolytic activity

The crude venoms of *B. fasciatus* did not exhibit any fibrinogenolytic activity after 1 h of incubation for any of the samples.

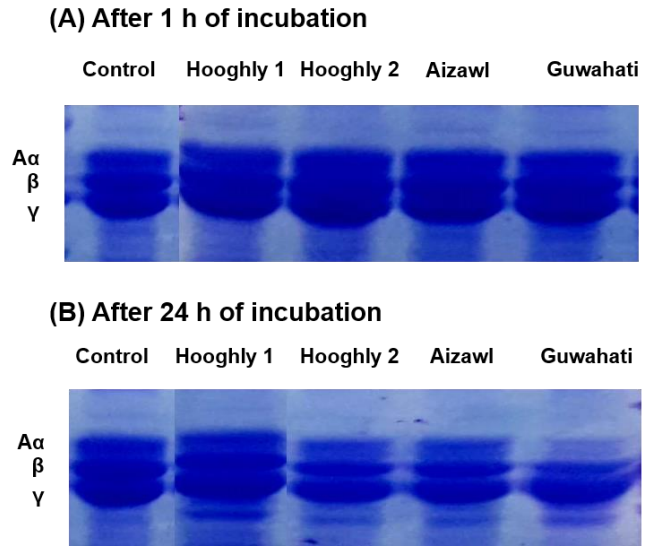

Figure 2.6: Fibrinogenolytic activity of crude venoms of *B. fasciatus* from Eastern and North-East India. Fibrinogen (2 mg/ml) was incubated with 50 mM Tris-Cl (pH 7.5) and 150 mM NaCl for 4 h at 37 ^{o}C. Next, 10 µg crude venom was added and incubated at 37 ^{o}C for different time intervals. **(**A). Incubation time of 1 h; (B). Incubation time of 24 h. The samples underwent electrophoresis in 10% SDS-PAGE and then Coomassie staining was performed for visualization. Fibrinogen incubated without any venom was taken as the control.

However, after 24 h of incubation, dissolution of the Aα-chain was exhibited in the Coomassie stained 10% SDS-PAGE gel for the sample from Guwahati (Assam). The remaining three samples from Hooghly1, Hooghly2 and Aizawl did not exhibit any cleavage of any of the three chains Aα, Bβ or γ (Figure 2.6).

Caseinolytic activity

The caseinolytic activity of crude venoms from *B. fasciatus* demonstrated significantly low activity compared to the venom of *Daboia russelii* (positive control) (9.005 U/mg). The *B. fasciatus* venom sample demonstrated a low caseinolytic activity for the venoms from Hooghly1 (0.884 U/mg) and Guwahati (0.304 U/mg). However, the venoms from Hooghly2 and Aizawl did not exhibit any caseinolytic activity.

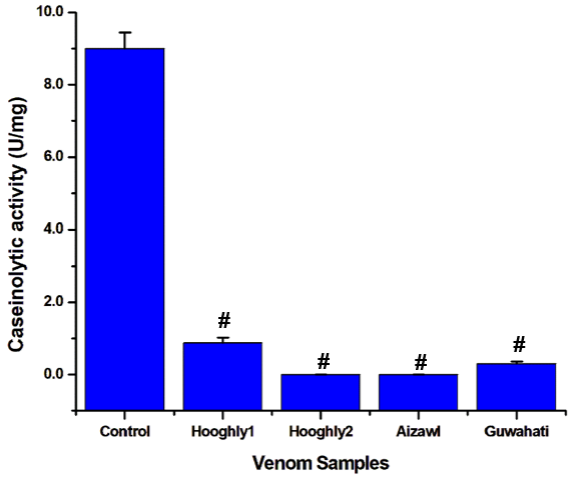

Figure 2.7: Caseinolytic activity of crude *B. fasciatus* venoms. Incubation of 20 µg venom samples along with casein solution (1%) at 37 ^{o}C for 1.5 h was followed by the addition of TCA solution (10%) to halt the reaction. The mixture was subjected to centrifugation at 5000 rpm for 30 min at 4 ^{o}C and tyrosine content in the supernatant was estimated by Lowry's methodology of protein estimation. The venom of *Daboia russelii* was taken as the positive control. The p-value ($^{\#}p<0.001$) was considered with respect to the positive control. The data-points depict the mean ± S.E of three distinct experiments.

2.4.2.3 Hemolytic activity of crude venoms

The crude *B. fasciatus* venom from different locations was incubated in a dose-dependent manner with RBCs. After incubation, negligible direct hemolytic activity (<1%) was exhibited for all the venom samples compared to the positive control (distilled water) which was taken as 100% (Figure 2.8).

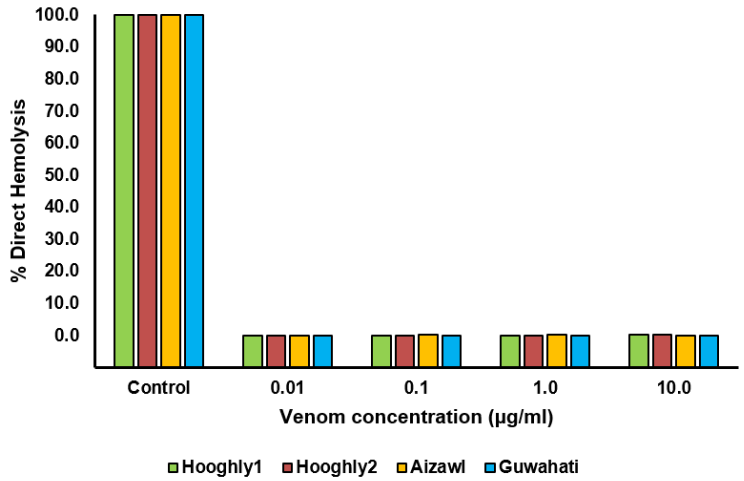

Figure 2.8: Direct hemolytic activity of crude *B. fasciatus* venoms. Crude venom of different concentrations was incubated with 10% RBC suspension (150 µl) and volume adjusted up to 2 ml. The mixture was incubated for 1 h at 37 ^{o}C and then subjected to centrifugation at 10,000 rpm for 10 min. The supernatant was obtained and absorbance was monitored (540 nm). RBCs mixed with distilled water was taken as the positive control.

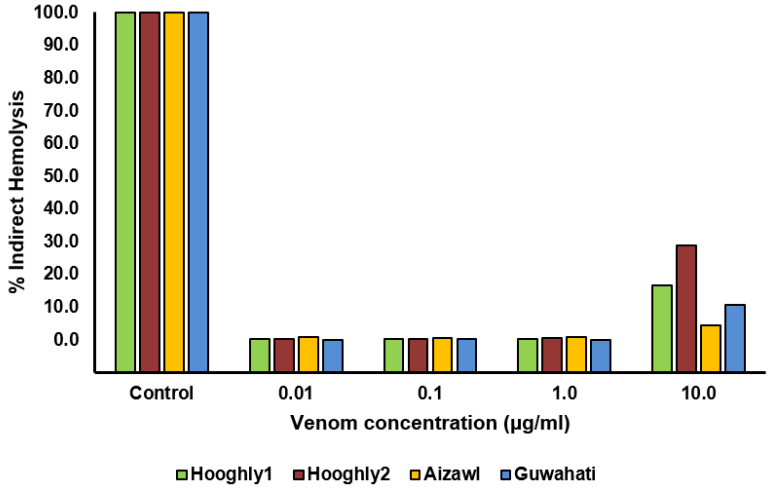

Figure 2.9: Indirect hemolytic activity of crude *B. fasciatus* venoms. Crude venom of different concentrations was incubated with 150 µl of 10% RBC suspension and 20 µl of egg yolk solution and volume adjusted to 2 ml. The mixture was kept for 1 h at 37 ^{o}C and then subjected to centrifugation at 10,000 rpm for 10 min. The supernatant was obtained and absorbance was checked (540 nm). RBCs mixed with egg yolk solution and distilled water was considered as the positive control.

The *B. fasciatus* venom samples exhibited very poor indirect hemolytic activity (<1%) at low doses (0.01, 0.1 and 1.0 µg/ml), however, at a higher dose of 10.0 µg/ml, the samples exhibited a relatively higher indirect hemolytic activity. The highest activity was exhibited by the venom sample from Hooghly2 (28.68%), followed by the samples from Hooghly1 (16.52%), Guwahati (10.72%) and Aizawl (4.35%) (Figure 2.9).

2.4.2.4 Coagulation activity of crude venoms

From the results of RT, a concentration dependent (0.01, 0.1 and 10.0 µg/ml) increase in time required for clotting was obtained for all the *B. fasciatus* venoms. At 10.0 µg/ml concentration, the highest delay in clotting time from NCT (145.0±5.0 s) was observed for the venom from Guwahati (256.7±1.7 s), followed by the samples from Aizawl (245.0±13.2 s), Hooghly2 (238.3±8.8 s) and Hooghly1 (205.0±10.0 s) respectively (Figure 2.10). At the dose of 10.0 µg/ml, the *p*-value for Hooghly 1 and Hooghly2 was 0.01 and that of Aizawl and Guwahati was 0.001 with respect to NCT.

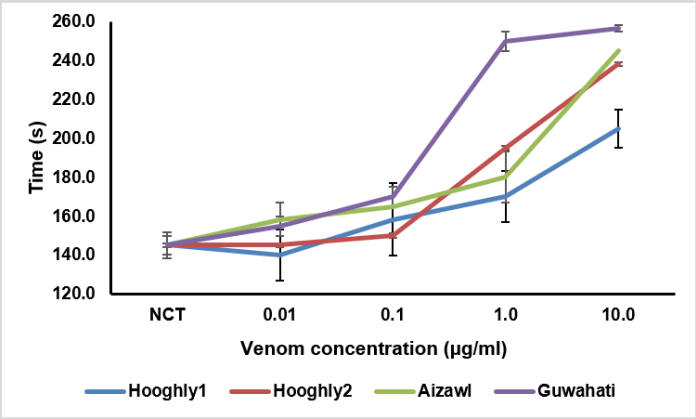

Figure 2.10: Recalcification Time of crude *B. fasciatus* venoms. Crude venom of different concentrations was incubated with 20 mM Tris-Cl (pH 7.4) and 50 µl PPP at 37 ^{o}C for 120 s. Initiation of clot development was started by adding 50 mM $CaCl_2$ and the absorbance was recorded at 405 nm for 15 min. The NCT of PPP represents the time taken it takes to clot in the presence of buffer. The data-points depict the mean ± S.E of three distinct experiments.

The crude venoms of *B. fasciatus* exhibited a dose-dependent increase in PT from the NCT of 28.0±1.15 s. At the dose of 10.0 µg/ml, the highest increase in PT was observed in the sample from Aizawl (37.0±1.76 s), Hooghly2 (34.7±0.66 s), followed by the samples from Hooghly1 (31.3±2.90 s), and Guwahati (28.0±1.15 s) respectively (Figure 2.11). However, no significant increase ($p<0.05$) was observed for the samples from Hooghly1 and Guwahati, although the increase for Hooghly2 ($p<0.01$) and Aizawl ($p<0.01$) were significant with respect to the NCT.

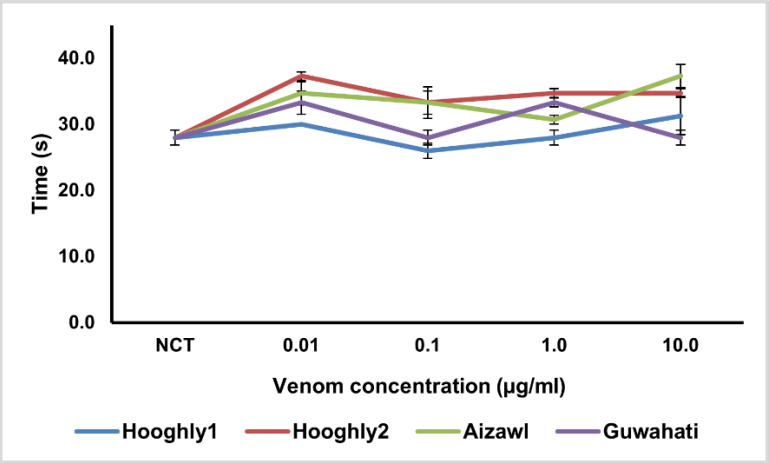

Figure 2.11: Prothrombin Time of crude *B. fasciatus* venoms. Crude venom of different concentrations was incubated with 50 µl PPP at 37 ^{o}C for 120 s. Clot formation was started by adding 50 µl Uniplastin and the absorbance was monitored at 405 nm for 2 min. The NCT represents the time taken by PPP for clot formation in the presence of Uniplastin. The data-points depict the mean ± S.E of three distinct experiments.

For APTT, the crude venoms of *B. fasciatus* did not exhibit any significant increase (p<0.05) of NCT (25.3±0.66 s) for the venom samples from Hooghly1 (28.0±0.1.15 s), Hooghly2 (24.7±0.66 s) and Aizawl (26.7±1.76 s), however, for the sample from Guwahati (31.3±0.66 s), a significant (p<0.01) increase in APTT was observed at a concentration of 10.0 µg/ml (Figure 2.12).

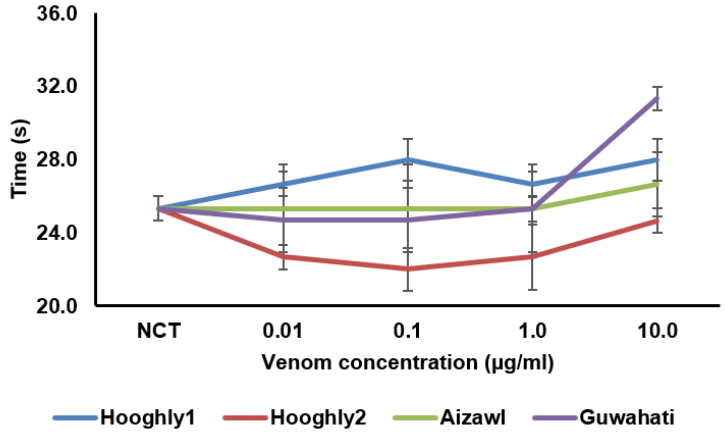

Figure 2.12: APTT of crude *B. fasciatus* venoms. Crude venom of different concentrations was incubated with PPP and Liquicelin-E (1:1 ratio v/v) at 37 ^{o}C for 3 min. Clot formation was started by adding 25 mM $CaCl_2$ and the absorbance was recorded at 405 nm for 5 min. The NCT represents the time taken by PPP for clot formation with Liquicelin-E and 25 mM $CaCl_2$. The data-points depict the mean ± S.E of three distinct experiments.

2.5 Discussion

Intra-species venom compositional variation has been reported for different snake species based on geography, prey, habitat, age, sex and season [131,132]. This intra-

species and inter-species venom variation may be responsible for a lower efficacy of polyvalent antivenom, which is used as a primary treatment modality in case of snakebites [151,152]. There are prior reports detailing the contents of crude venom of *B. fasciatus* sourced from Eastern India (West Bengal) [27,78]. However, there are no reports on the venom composition of *B. fasciatus* and its associated biochemical activities from any of the North-East Indian states which raise an important question of whether the venom profile of *B. fasciatus* and their associated biochemical activities from North-Eastern states is similar to that reported from Eastern India.

In the 10% SDS-PAGE Tris-tricine gel, the distribution of protein bands of a wide molecular weight range (13-75 kDa) suggests the possible presence of different snake venom protein superfamilies previously reported from *B. fasciatus* venoms such as PLA_2, 3FTx, SVMP, LAAO, SVSP, KSPI, CRiSP, Nucleotidases and Phosphodiesterases [58,78,129]. The bands observed in Hooghly1 and Hooghly2 are mostly similar which may be due to the closeness of the two locations (less than 8 km). Variations in the SDS-PAGE profile of *B. fasciatus* are also observed when compared to previous reports. For instance, in this study, some protein bands were observed between 20-50 kDa in all the *B. fasciatus* samples. Similar results were also observed by Senji Laxme et al. when a faint band between 20-50 kDa was also observed in a 12.5% electrophoresis gel from a *B. fasciatus* venom sample from West Bengal [78]. On the contrary, a 12% electrophoresis gel profile of venom from the same species from Malaysia, and a 15% SDS-PAGE profiles from Malaysia, Thailand, Indonesia, Myanmar and China did not exhibit any protein bands in this range [18,58]. Hence, qualitative differences in venom composition assessed through SDS-PAGE profiling are observed among *B. fasciatus* venoms from within India and also with other countries where *B. fasciatus* is distributed.

In the RP-HPLC profiles, venom samples of *B. fasciatus* sourced from different locations differ in the number of peaks and also their corresponding elution time, which reflect the differences in protein expression levels. Previously, Tsai et al. outlined variations in PLA_2 and 3FTx expression in venom within the same species from two individual snakes of *B. fasciatus* from Kolkata (West Bengal) after purification by gel filtration chromatography using an FPLC system followed by spectrometric identification of the protein fractions [27]. Moreover, the venom variation observed in this study between *B.*

fasciatus and *B. caeruleus* also highlights the existence of inter-species variations and these variations among medically important snakes are a matter of concern as the Indian polyvalent antivenom are only partially effective in neutralizing the effects of snakes other than the "Big-Four" [153]. Thus, the proteomics analysis for medically relevant snake venoms from different regions may prove to be beneficial for understanding the pharmacological manifestations of the venom in snakebite victims.

The PLA_2 proteins comprise majority (>44.0%) of the *B. fasciatus* crude venom as per various reports using samples from countries like China, Myanmar, Thailand, Vietnam and Malaysia [58,128,129]. However, the same is not the case for the crude venom reported from Indonesia where the composition of PLA_2 proteins was less than 30.0% [58]. In this study, a relatively high PLA_2 activity was estimated by turbidometric and colorimetric methods which also corresponds with the high abundance of protein bands at 15 kDa molecular weight observed in 10% SDS-PAGE (Figure 2.2). The presence of PLA_2 proteins is mainly responsible for neurotoxicity in envenomated patients, however, the inconsequential content of β-Bungarotoxin (typically <5%) reported in the venom of *B. fasciatus* may be the reason for which their venom is not as potently neurotoxic compared to congeneric kraits, like *B. sindanus*, *B. multicinctus* and *B. caeruleus* [58,154,155]. The less toxicity compared to other kraits may also be due to the absence of conventional 3FTx which are replaced by non-conventional or orphan 3FTx in *B. fasciatus* venom resulting in a low median lethal dose (<0.2 μg/g) [156,157]. Similarly, for other elapid species, such as the Asiatic cobras (*Naja* sp.), subgenera *Boulengerina* (eg. *N. annulata*) and subgenera *Afronaja* (eg. *N. nigricolis*), moderate to high (12-33%) abundance of PLA_2 proteins have been noted previously, however, for the subgenera *Uraeus* the abundance in extremely low (4%) [158].

Snake venom superfamilies such as SVSPs and SVMPs are mostly responsible for proteolytic activity in envenomated victims [159,160]. *B. fasciatus* venom from Taiwan has been previously reported to have no proteolytic activity (5 μg/ml) when both casein and hemoglobin were used as substrates [161]. The low proteolytic (both fibrinogenolytic and caseinolytic) activity exhibited in this study could be attributed to the occurrence of small quantities of these protein families. Previous reports of low abundance of SVSPs and SVMPs from *B. fasciatus* venom have been reported from West Bengal (India), and also from countries such as Vietnam, Malaysia, Thailand,

Indonesia, Myanmar and China [58,78,128,129]. Similarly, the venom of *B. candidus* from Eastern, North-Eastern and Southern regions of Thailand also exhibited a low proteolytic activity [162]. However, the congeneric *B. caeruleus* venom (Tamil Nadu, India) has been reported to have high fibrinogenolytic and caseinolytic activity [163].

Low molecular weight venom proteins like cytotoxins or cardiotoxins binds to the anionic lipids of cell membranes and causes membrane damage [164,165]. The Indirect hemolytic activity observed in this study using egg yolk solution as a substrate was due to the formation of lytic products such as free fatty acids (eg. arachidonic acid) and lysophospholipids, which are products of the hydrolysis of phospholipids due to the presence of PLA_2 enzymes in the venom [145,166]. Similar results have also been reported for the congeneric *B. caeruleus* where direct hemolytic activity was not observed but indirect hemolytic activity in the presence of horse serum was prominently observed [167]. However, for elapid snakes such as *Naja naja* and *N. kaouthia* apart from indirect hemolytic activity their venom also exhibits direct hemolytic activity as they contain direct lytic factors (DLFs) which works synergistically with PLA_2 proteins inducing a direct hemolysis [145,168,169].

Snake venom commonly contains anticoagulant proteins which may belong to the enzymatic (PLA_2, Metalloproteinase, Serine proteinase and LAAO) or non-enzymatic superfamilies (Snaclecs and 3FTx) [170]. Anticoagulant activity of crude venom of elapid snakes *Naja naja* and *N. kaouthia* have been previously reported from our lab [138]. From the results of this study (RT, PT and APTT), observations indicate that the venom of *B. fasciatus* from Aizawl (Mizoram), Hooghly (West Bengal) and Guwahati (Assam) displays prominent anticoagulant properties. The delay in coagulation time was prominent in RT and APTT compared to the minor delay in PT which indicates that the venom mainly affects the intrinsic coagulation pathway. Similar reports have been previously reported from crude venom of *B. fasciatus* and *B. multicinctus* which exhibited anticoagulant activity by increasing the coagulation time of APTT but not PT. Two anticoagulant proteins BF-IV-8 (13.12 kDa mol. wt.) of 118 amino acids and BF-V-4 (6.95 kDa mol. wt.) of 65 amino acids were isolated from the *B. fasciatus* venom and identified using MALDI-TOF mass spectroscopy as a PLA_2 protein and a KSPI respectively [171]. On the contrary, previously Chen et al. have isolated two anticoagulant proteins (BF-AC1 and BF-AC2) targeting the external tissue factor

pathway, which were isoforms of β-Bungarotoxin having a large PLA_2 component and a small KSPI component. The proteins significantly prolonged PT at a concentration of 10 μg/ml, whereas, it had a minor effect on APTT [172]. The nature of *B. fasciatus* venom may be either procoagulant or anticoagulant depending on the geographic origin of the venom. For instance, Zhang et al. have reported that the *B. fasciatus* venoms from Guanxi, China and Cambodia had an anticoagulant effect, whereas, the ones from India and Yunnan, China had a procoagulant effect on the coagulation cascade [121].

A major target of snake venom toxins is the activation or inhibition of factor X to factor Xa. A procoagulant serine protease inhibitor of ~70 kDa was isolated from the venom of *B. fasciatus* which exhibited factor X activation and shortened the RT in a dose-dependent manner [121]. Another anti-coagulant protein (Fasxiator) which inhibits the factor XIa of blood coagulation cascade was isolated by Chen et al [126]. The procoagulant and anticoagulant nature of snake venom toxins, which are hemostatically active can influence the blood coagulation pathway by inhibition or promotion of clot formation. This may lead to excessive blood loss or the formation of undesired clots in envenomated victims [173].

2.6 Conclusion

In this chapter, the crude venom profile of *B. fasciatus* from different localities of Eastern and North-East India was quantitatively and qualitatively analyzed using the techniques of protein estimation, gel electrophoresis and RP-HPLC. This was followed by the comparative biochemical analysis of crude *B. fasciatus* venom samples where the PLA_2 activity, proteolytic activity (fibrinogenolytic and caseinolytic), hemolytic activity (direct and indirect), and coagulation activity (RT, PT and APTT) was determined and compared. The *B. fasciatus* samples exhibited intra-population (between Hooghly1 and Hooghly2), intra-specific (geographical variation between the three states) and inter-specific (between *B. fasciatus* and *B. caeruleus* venom) venom variation. Although the geographically distinct locations share a similar climatic condition, however, the availability of diverse prey in different terrains and localities may play a role for this variation. The regional differences in venom composition had resulted in considerable disparities in the in vitro biochemical activities which may be responsible for varying degrees of pathological manifestations in envenomated victims and the response to standard treatment methods available.