CHAPTER 4

Isolation and identification of poorly - immunodepleted proteins of *Bungarus fasciatus* venom from North-East India using third-generation antivenomics

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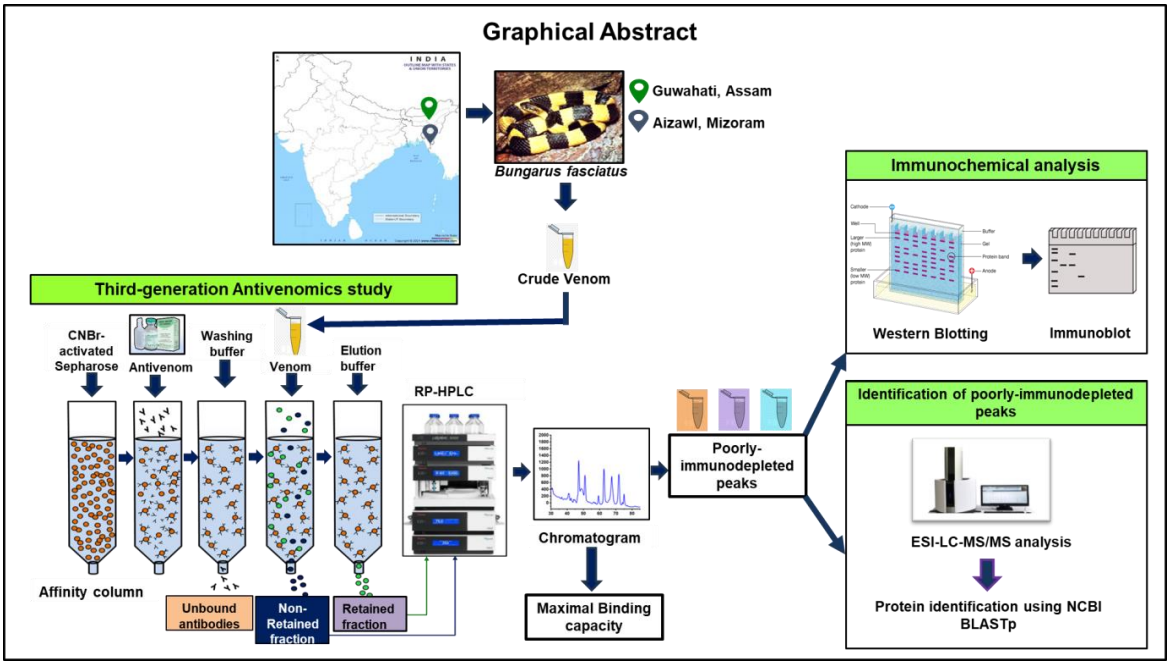

4.1 Introduction

In the Indian subcontinent, due to the unavailability of efficient venom detection kits, most of the countries depend on polyvalent antivenom over monovalent antivenom for the treatment of snakebite cases. Indian polyvalent antivenom fulfils much of this demand and various studies have reported the effectiveness of polyvalent antivenom manufactured in India to counter snake envenomation from India and neighbouring countries [186,204–206]. However, there are also reports which highlight the limitations of using polyvalent antivenom manufactured in India, such as inadequate cross-neutralization of venom toxins from various other snake species of the region, such as *Hypnale hypnale* [15], *H. nepa* [207], *Bungarus niger* [208], *B. sindanus*, *Naja naja*

[201], *N. kaouthia* [209] and *Daboia russelii* [210]. Variations in venom composition and differences in antigenicity of venom components are responsible for the inefficiency of polyvalent antivenom. Moreover, there is very limited information regarding the spectrum of efficacy for polyvalent antivenom manufactured in India against various medically important snake species from diverse geographical regions of India and surrounding countries.

The antivenomics approach is now being commonly used for quantitative molecular assessment of an antivenom's preclinical efficacy against different snake venom proteins. This approach, compared to other non-quantitative methods such as Western blotting and ELISA, is superior as the amount of non-immunodepleted proteins can be quantified. In the previous chapter (chapter 3), we utilized 2G antivenomics to determine the %NRi for the *B. fasciatus* venom samples from West Bengal, Assam and Mizoram against Bharat Serums polyvalent antivenom, in which the samples from Assam and Mizoram exhibited the maximum %NRi [177]. However, one of the major shortcoming of the 2G antivenomics approach is the fixed venom-antivenom ratio used (1:16 in our study), which highlights only a single aspect of the dynamic interaction process where various other intrinsic factors (such as antigen-antibody affinity) and extrinsic factors (such as density of antivenom bound to affinity column; venom load; proportion of some venom proteins in the overall venom) may also have a significantly important role [211].

The third-generation (3G) antivenomics technique was designed to mitigate the limoitations of the 2G antivenomics, which allows for accurate quantification of the immunorecognition profile of the antivenom against each snake venom protein as well as to determine its maximum snake venom binding capability [177,212]. As a result, appropriate and effective antivenom may be identified for further in vivo neutralization assays, which ultimately helps to limit the use of experimental animals [177]. In the 3G antivenomics approach, the antivenom's immunocapturing capability is estimated by running identical antivenom-bound affinity columns side-by-side with incremental load of venom till saturation point is achieved. This is followed by chromatographic estimation of bound and unbound venom fractions and the subsequent calculation of peak areas of the individual peaks. The amount of venom protein corresponding to the peak area can be determined. The calculated amount of venom protein can be utilized to

determine the antivenom's maximum binding capability of each venom peaks and the overall snake venom as well [212].

In India, Premium Serums is one of the most extensively distributed polyvalent antivenom and it is commonly utilized for treating snakebite cases in Eastern and North-East India as well. Previous studies involving medically important snake species from North-East India such as *Naja kaouthia* (Mizoram) against Premium Serums antivenom revealed poor immunodepletion [88]. Thakur et al., also reported poor immunorecognition by Premium Serums antivenom of low molecular weight venom proteins of *Trimerusurus erythrurus* from Aizawl (Mizoram) [213]. There are also reports of low binding efficacy of this antivenom against the venom of *B. fasciatus* from Eastern India (West Bengal) [78]. However, there is limited data available on the cross-reactivity of this antivenom for treating *B. fasciatus* envenomation from North-East India.

In this chapter, the immunodepletion capability of Indian polyvalent antivenom (Premium Serums) was determined against the venom of *B. fasciatus* samples from North-East India *i.e.,* Aizawl (Mizoram) and Guwahati (Assam) using 3G antivenomics approach and Maximum binding capability of the antivenom was calculated. This was followed by isolation and identification of the poorly-immunodepleted proteins from the venom sample from Guwahati using Liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach.

4.2 Materials

4.2.1 Snake venoms

B. fasciatus venoms from Guwahati and Aizawl were taken for this study (details mentioned in section 2.2.1).

4.2.2 Indian polyvalent antivenom

Polyvalent antivenom was obtained from Premium Serums & Vaccines Pvt. Ltd., Pune, Maharashtra for the study (details mentioned in chapter 3, section 3.2.2).

4.2.3 Chemicals and reagents

The chemical and reagents used for 3G antivenomics and RP-HPLC were the same as that used in chapter 3 (section 3.2.3).

4.2.4 Chromatographic Columns

For RP-HPLC analysis, a C18 column Aeris Widepore (150 x 2.1 mm, 3.6 µ, 200Å) (column volume ~4.15 ml) purchased from Phenomenex, USA was used.

4.3 Methods

4.3.1 3G antivenomics

The immunorecognition capability of polyvalent antivenom (Premium Serums) targeting the venom of *B. fasciatus* from Aizawl and Guwahati was determined and the maximum binding capability was calculated utilizing 3G antivenomics technique as per the methodology outlined by Pla et al. [212].

4.3.1.1 Affinity column preparation and collection of retained and non-retained fractions

Affinity columns were prepared by taking 350 mg of Premium Serums antivenom and then dissolving it in Purified water (Milli-Q) (10 ml). Once the antivenom dissolved, it (1 ml) was dialyzed in coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl; pH 8.3) for 3 changes of 2 h each at 4 ^oC. Antivenom bound immunoaffinity columns were prepared by first taking one gravity column in which CNBr-activated Sepharose 4B matrix was added and incubated with 10 CV of chilled 1 mM HCl and subsequently 2 CV of coupling buffer was added. Dialyzed antivenom (7.07 mg per column) was then incorporated in the matrix and was kept overnight with gentle shaking at 25 ^oC. The yield of antivenom adhered to each column (4.4 mg) was determined by estimating the amount of antivenom that did not bind and passed through the column, and then subtracting it from the initial antivenom load. Two control mock matrix were also prepared using the CNBr-activated Sepharose 4B matrix without the addition of the polyvalent antivenom, in order to determine if the snake venom binds to the matrix. Following 3 washes with coupling buffer, each column was treated with blocking buffer (0.1 M Tris-Cl; pH 8.5) for 4 h. Next, three alternate washes of acidic buffer (0.1 M acetate, 0.5 M NaCl; pH 4.0) and alkaline buffer (0.1 M Tris-Cl; pH 8.5) were given to the columns. This was followed by addition of 5 CV of equilibration buffer *i.e.*, 1X PBS (137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4; pH 7.4).

Subsequently, from each of the venom samples four varying quantities of venom (34, 68, 137 and 273 µg) were loaded to the columns to obtain a respective ratio of 1:128, 1:64, 1:32 and 1:16 for venom to antivenom. The venom was allowed to interact with the antivenom-bound to matrix overnight at 4 ^{o}C. The fractions which were non-retained were obtained after the matrix was washed 3 times with 2 CV of PBS. The retained fractions bound to the antivenom were obtained by washing the columns with 2 CV of elution buffer (100 mM glycine, pH 2.0) thrice. Neutralization of the retained fractions was done by the addition of 150 µl of 1 M Tris-Cl buffer (pH 9.0).

4.3.1.2 Chromatographic analysis of retained and non-retained venom portions

The retained and non-retained portions of venom proteins obtained after 3G antivenomics and their corresponding crude venom samples were analysed using RP-HPLC followed by the collection of individual peaks for subsequent studies. The samples were injected to the C18 column (Aeris Widepore 150 x 2.1 mm, 200Å, 3.6 µm) pre-equilibrated with 5 CV of 0.1% TFA. The RP-HPLC programme ran at a continuous flow rate of 100 µl/min containing buffer B (80% MeCN, 0.1% TFA) for 100 min. The MeCN gradient started from 0% to 20% (4 to 10 min), then 20% to 55% (10 to 70 min), 55% to 80% (70 to 84 min), and 80% to 100% (84 to 88 min). The gradient continued at 100% for 4 min (88 to 92 min) and then declined from 100% to 0% (92 to 96 min) and continued at 0% for 4 more min. Monitoring of protein elution was carried out at 215 nm, and the peaks were manually retrieved. Further, protein content in collected peaks was estimated using a NanoDrop 2000 spectrophotometer using type '1 Abs = 1 mg/ml' and 'A280' extinction coefficient.

4.3.1.3 Determination of maximum binding capability

The crude venom, retained and non-retained portions were profiled using RP-HPLC, and the method outlined by Pla et al. was utilized to ascertain polyvalent antivenom's (Premium Serums) maximum binding capability [212]. The peak area was calculated for the peaks present in the crude venom, as well as in the non-retained and retained portions utilizing the Chromeleon software (v6.0). From the %Relative Area of crude venom the calculation of protein content within each peak was done, since, the amount of injected venom was already known. Next, the amount of protein represented by the peak areas of the retained fraction was determined by comparing with the peak area of the crude

venom and corresponding protein amount. The total amount of venom proteins represented by the peak areas of crude venom and retained fractions are tabulated for all the venom loads (*i.e.*, 34, 68, 137 and 273 µg). The 'Sum of Retained fractions (Σ)' was determined by adding the venom protein content in each retained peak of the individual doses. From the sum, the maximum amount was chosen followed by further division by the antivenom quantity fixed in one column (4.4 mg). This helps in determining the maximum binding capability of antivenom for one unit of antivenom ($F(ab')_2$) fixed in the column. Moreover, from the maximum binding capability the venom protein content bound by one antivenom vial (~400 mg $F(ab')_2$) can also be calculated.

4.3.2 Gel electrophoresis and Western Blotting

Two gel electrophoresis (12.5% glycine gel) experiments were performed using crude *B. fasciatus* venom (Guwahati) and P5, P6 and P7 poorly-immunodepleted peaks (15 µg) as per the method of Laemmli [179]. Following gel electrophoresis, one gel was stained using Coomassie staining, and the other was processed to study the immune-crossreactivity of Premium Serums antivenom using western blotting technique described previously in chapter 3 (section 3.3.2).

4.3.3 Identification of proteins by ESI-LC-MS/MS

4.3.3.1 In-gel trypsin digestion

The bands visible in the Coomassie stained gel for peaks P5, P6 and P7 were excised using a scalpel, followed by reduction and alkylation steps, after which the bands were digested in-solution using trypsin following the methodology outlined by Babele et al. [214]. The excised bands were washed with 50% acetonitrile in 50 mM NH_4CO_3 repeatedly for 1 h at 25 ^oC for destaining the gel pieces properly. The gel pieces were then dried by incubation with acetonitrile and then treated with equilibration buffer A (2% SDS, 30% glycerol, 1% dithiothreitol and 6 M urea in 0.05 M Tris buffer; pH 8.8) and then adding equilibration buffer B (30% glycerol, 2% SDS, 4% indole-3-acetic acid and 6 M urea in 0.38 M Tris buffer; pH 8.8). Next, trypsin (Promega, USA) (0.1 µg) dissolved in 50 mM NH_4CO_3 was added to the excised bands and allowed to react overnight at 37 ^oC. The next day, the digested peptides were retrieved by means of a solution containing formic acid (0.1%) and acetonitrile (60%) followed by the desalting of the gel pieces.

4.3.3.2 ESI-LC-MS/MS analysis

The peptides resulting from trypsin digestion of peaks (P5, P6 and P7) exhibiting inadequate immunodepletion were examined by ESI-LC-MS/MS following the methodology outlined by Babele et al. [215]. The Nano LC-Ultra 2D system (Eksigent, USA) was utilized for liquid chromatography, which was linked online to a Triple TOF 5600 mass spectrometer (MS) (Sciex, USA). The peptides were desalted using a C-18 column before prior to loading onto the Trap column (ChromXP C18-CL, 120 Å, 300 µm x 0.5 mm, 3 µm) and then onto another analytical micro column (3C18 CL, 120 Å, 300 µm x 15 mm, 3 µm). Next the samples were analysed using HPLC, 10 µg was injected with a flow rate set to 5.0 µl/min and the mobile phase consisted of sol. A and sol. B at a formic acid, acetonitrile and water ratio of 0.2:2:98 and 0.2:98:2 respectively. The gradient for the programme of sol. B is as follows: For first 2 min, 0% to 10%, then a linear gradient of 10% to 30% for the next 10 min, and for the next 18 min a linear gradient from 30% to 50%, next for elution of peptides the gradient was elevated to 90% for 1 min and continued for 3 min. The gradient was gradually brought back to 0% over the following min where it continued for additional 3 min.

For data-dependent acquisition, the MS (Triple TOF 5600) was employed with an electrospray ionization (ESI) source at 130 ^{o}C and 2.3 kV voltage containing nebular gas (20 psi), heater gas (10 psi) and curtain gas (25 psi). The resolution of MS scans was set at 30,000 FWHM and a 350 to 1250 mass-to-charge ratio (m/z). Simultaneously with the survey scans of 250 ms, ion scans for 35 products were conducted in a mode of high-sensitivity covering 100 to 1600 m/z. The acquisition limit was 120 scans per s, and charge states ranging from 2+ to 5+. The whole program duration consisted of 2.35 s. The Collision energy (CE) was automatically regulated for Collision-induced dissociation by including an IDA-CE program to optimize the disintegration process. Depending on the calculated CE a 5 eV collision energy spread was executed. A dynamic exclusion time was fixed to 3 s, after which the precursor was deleted from the exclusion list.

4.3.3.3 Peptide identification using database search

The obtained peptide sequences were identified using the NCBI database of *B. fasciatus* using the ProteinPilot 5.0 software (06-2022, 242 entries). The search parameters used

were **i.** sample categorization as identification; **ii.** alkylation of cysteine using iodoacetamide; **iii.** Trypsin digestion; **iv.** instrument of choice being the Triple TOF 5600; **v.** search conducted by ID. A reverse database search was utilized to ascertain the false discovery rate which was less than 1%. The peptides were subjected to further refinement and those possessing a minimum of 2 distinct peptides and a SEQUEST score of 2 or higher were chosen. Furthermore, the identified peptides were analysed for sequence alignment utilizing the NCBI-protein Basic Local Alignment Search Tool (BLASTp) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

4.4 Results

4.4.1 Maximum binding capability of antivenom targeting *B. fasciatus* venom from Aizawl (Mizoram)

The crude venom profile exhibited eight major peaks (Figure 4.1) and the %Maximum binding of different peaks were in the 0% to 91.65% range (Table 4.1). The peaks P3, P5 and P6 were non-immunodepleted, and peaks P2, P4 and P8 were poorly-immunodepleted (%Maximum binding <14%), and peak P7 was moderately immunodepleted (%Maximum binding <59%). However, for peak P1 the %Maximum binding was high (91.65%).

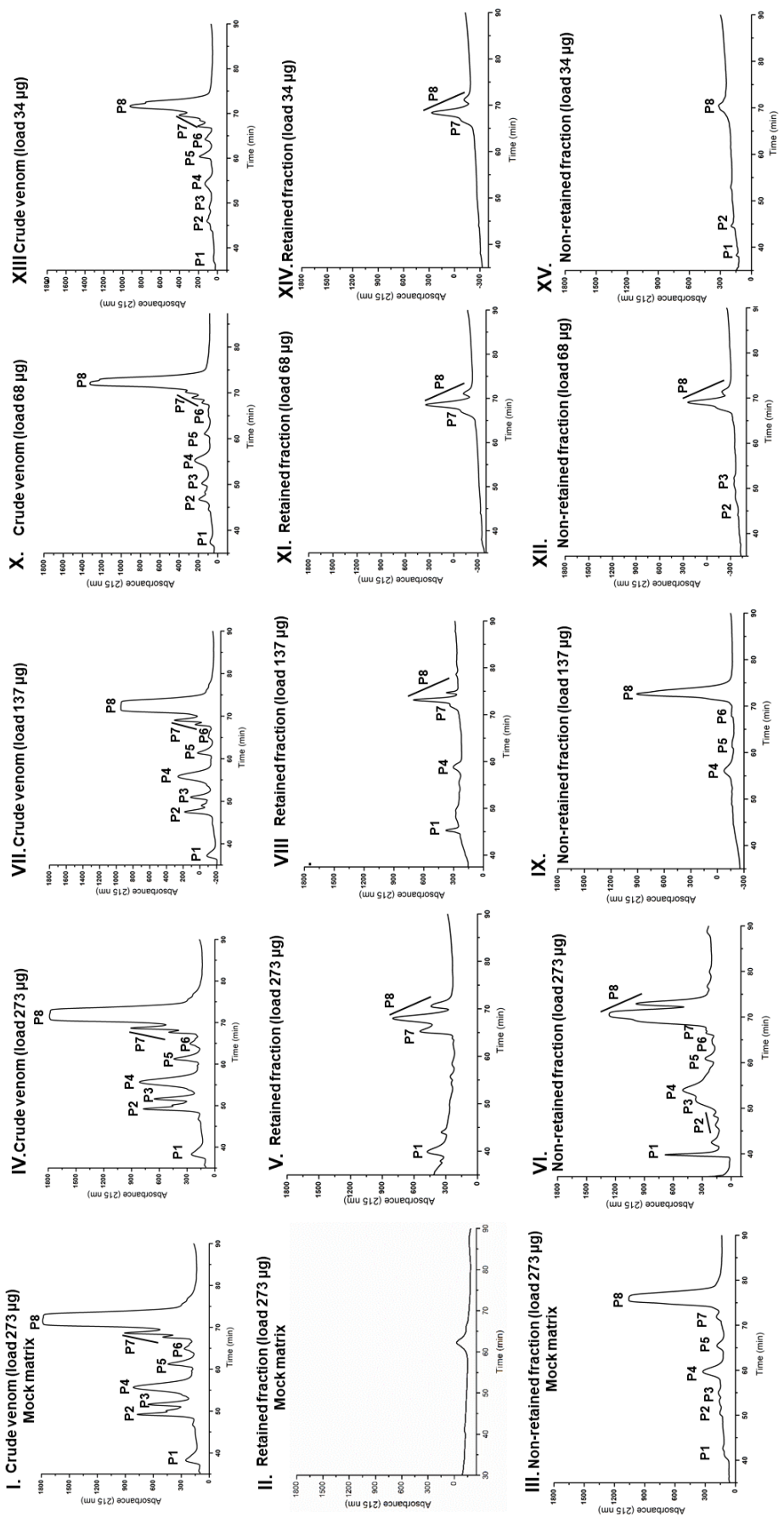

Figure 4.1: The immunodepletion profile of Premium Serums polyvalent antivenom against *B. fasciatus* venom from Aizawl, Mizoram. It illustrates the patterns of crude venom (I, IV, VII, and X), retained peaks (II, V, VIII, and XI), and non-retained peaks (III, VI, IX, and XII) following the addition of increasing venom sample loads. The crude venom load (XIII), retained fractions (XIV), and non-retained fractions (XV) were utilized as the control for the mock matrix.

Table 4.1: Amount of *B. fasciatus* venom (Aizawl, Mizoram) retained after immunodepletion using polyvalent antivenom (Premium Serums) and determination of %Maximum binding of individual peaks and venom.

Peak No.	RP-HPLC fraction	Amount of protein (µg)				%Maximum binding of individual peaks
		34 µg	68 µg	137 µg	273 µg	
P1	Total	0.45	1.03	4.66	8.74	
	Retained	0	0	2.35	**8.01**	91.65
P2	Total	1.49	3.08	9.15	18.32	
	Retained	0	0	0	**1.41**	7.70
P3	Total	0.35	1.31	4.55	9.56	
	Retained	0	0	0	0	0
P4	Total	2.75	6.90	20.88	42.29	
	Retained	0	0	**2.42**	0	11.59%
P5	Total	2.66	1.42	4.74	10.05	
	Retained	0	0	0	0	0
P6	Total	1.61	0.55	1.58	3.33	
	Retained	0	0	0	0	0
P7	Total	1.66	0.83	8.18	14.66	
	Retained	0.34	0.24	0.40	**8.57**	58.46
P8	Total	23.03	52.85	83.27	166.07	
	Retained	9.03	9.19	7.87	**22.78**	13.72
Sum of Retained fractions (Σ)=		8.01+1.41+8.57+22.78 = **40.77**				
Maximum binding capability = 40.77/4.4 = **9.3** mg venom proteins/g $F(ab')_2$						

Note: For individual peaks the maximum retained proteins are highlighted in Bold. The Maximum binding capability was calculated by division of the 'Sum of Retained fractions (Σ)' by the antibody content immobilized to one affinity column (4.4 mg).

From the 'Sum of Retained fraction (Σ)' (40.77 µg) bound in the immunoaffinity column the maximum binding capability was determined which equals to 9.3 mg venom proteins per g of antibody taken. Since, one vial of the antivenom typically contains ~400 mg $F(ab')_2$ fragments, hence, theoretically (9.3 x 400 = 3720 µg) ~3.7 mg of *B. fasciatus* venom proteins can be immunocaptured by each vial of the polyvalent antivenom.

4.4.2 Maximum binding capability of antivenom targeting *B. fasciatus* venom from Guwahati (Assam)

The maximum binding capability of polyvalent antivenom (Premium Serums) at four venom-antivenom ratio (1:16, 1:32, 1:64 and 1:128) was determined against the venom of *B. fasciatus* from Guwahati (Assam) utilizing 3G antivenomics approach. The crude venom profile exhibited seven major peaks (Figure 4.2) and the %Maximum binding of different peaks were in the 15.9% to 98.9% range (Table 4.2). For the P1, P2 , P5, P6 and P7 peaks, the %Maximum binding was <40% and that for peak P3 was 43.3%. When the venom load was the highest (273 µg), the binding sites of $F(ab')_2$ fragments must have been saturated except the peaks P2 and P5, which exhibited further increased binding in Table 4.2. From the 'Sum of Retained fraction (Σ)' (56.4 µg) bound per immunoaffinity column the maximal binding capability was determined which equals to 12.8 mg venom proteins per g of antibody taken. Since, one vial of the antivenom typically contains ~400 mg antibody fragments, hence, theoretically (12.8 x 400 = 5120 µg) ~5.1 mg of *B. fasciatus* venom proteins can be immunocaptured by each vial of the polyvalent antivenom.

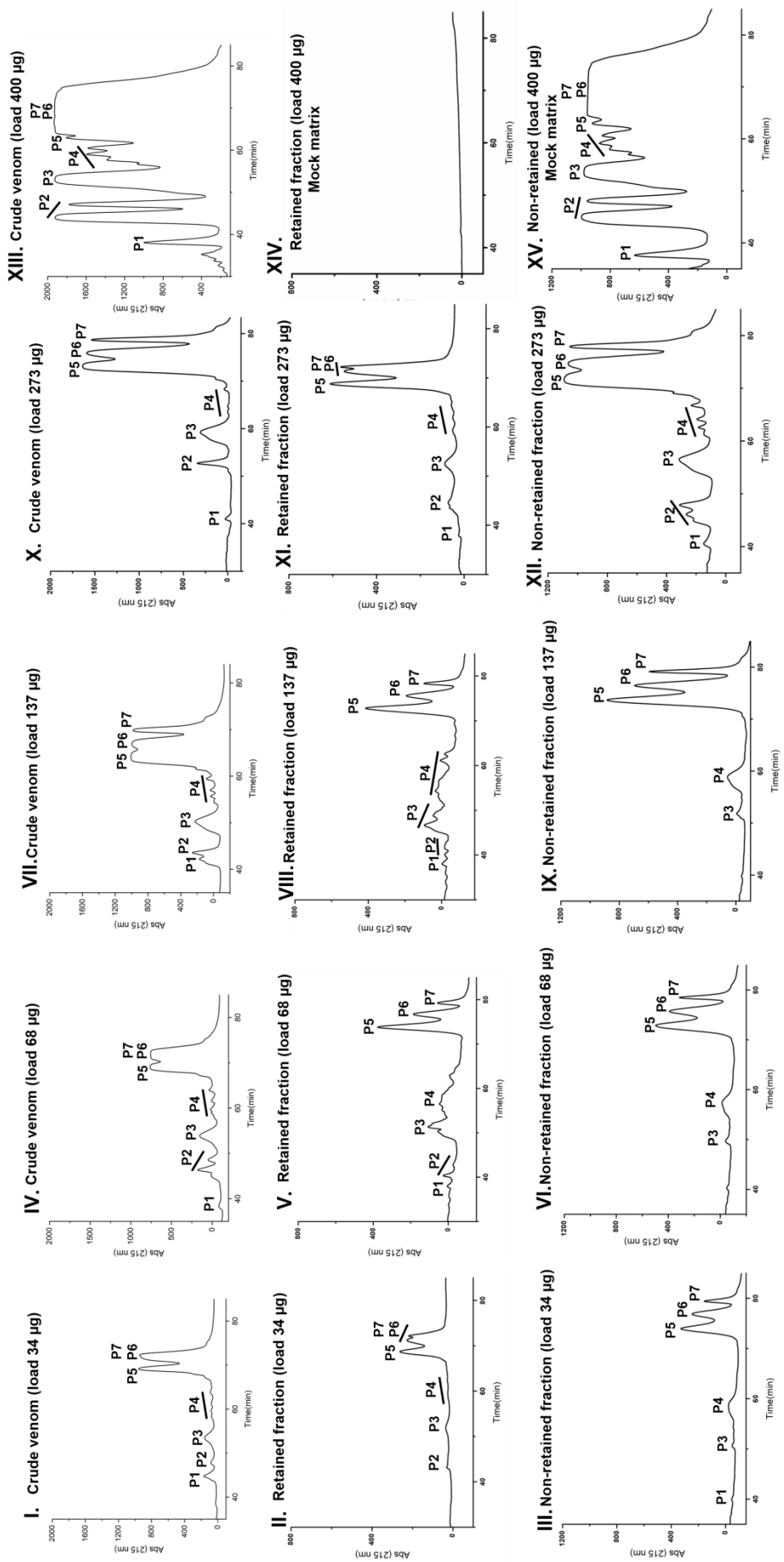

Figure 4.2: The immunodepletion profile of Premium Serums polyvalent antivenom against *B. fasciatus* venom originating from Guwahati, Assam. It demonstrates the patterns of crude venom (I, IV, VII, and X), retained peaks (II, V, VIII, and XI), and non-retained peaks (III, VI, IX, and XII) following the addition of escalating venom sample quantities. The crude venom load (XIII), retained fractions (XIV), and non-retained fractions (XV) were taken as the control for the mock matrix.

Table 4.2: Amount of venom from *B. fasciatus* venom (Guwahati) retained after immunodepletion utilizing the polyvalent antivenom (Premium Serums) and determination of %Maximum binding of each peaks and venom.

Peak No.	RP-HPLC fraction	Amount of protein (µg)				%Maximum binding of individual peaks
		34 µg	68 µg	137 µg	273 µg	
P1	Total	2.31	1.08	4.67	2.59	
	Retained	0	0.37	**0.8**	0.45	17.1
P2	Total	0.63	12.95	6.11	20.2	
	Retained	0.28	1.05	1.14	**5.3**	26.2
P3	Total	3.38	12.75	28.78	46.33	
	Retained	0.6	6.3	**12.47**	5.42	43.3
P4	Total	0.76	4.12	4.96	2.57	
	Retained	0.14	2.89	**4.91**	1.29	98.9
P5	Total	10.69	15.47	36.88	87.76	
	Retained	2.78	11.19	22.78	**24.73**	28.2
P6	Total	8.11	10.81	23.17	56.45	
	Retained	1.69	5.6	**9.16**	5.12	39.5
P7	Total	8.11	10.81	32.43	57.08	
	Retained	1.69	2.72	**5.14**	3.44	15.9
Sum of Retained fractions (Σ)=		0.8 + 1.14 + 12.47 + 4.91 + 22.78 + 9.16 + 5.14 = **56.4** µg				
Maximum binding capability = 56.4/4.4 = **12.8** mg venom proteins/g $F(ab')_2$						

Note: For individual peaks the maximum retained proteins are highlighted in Bold. The Maximum binding capability was calculated by division of the highest 'Sum of Retained fractions (Σ)' by the antibody content immobilized to one affinity column (4.4 mg).

4.4.3 Estimation of antivenom vials required to immunocapture all venom proteins in *B. fasciatus* envenomated victim and its associated cost

It has been previously reported that *B. fasciatus* may inject between 20-114 mg of venom in one bite [135]. For a victim bitten by *B. fasciatus* from Aizawl (Mizoram), theoretically the number of Premium Serums polyvalent antivenom vials (each vial containing around 400 mg of antibody fragments) required would be 6 to 30, and for venom of *B. fasciatus* from Guwahati (Assam), the number of vials required would be 4 to 23. The market price of Premium Serums antivenom in India in 2023-24 is around 750 INR per vial. Hence, in the first case (Aizawl) the estimated burden of antivenom cost

may range from 4,500 to 22,500 INR, and for the latter case (Guwahati) the cost may range from 3,000 to 17,250 INR (Table 4.3).

Table 4.3: Estimation of antivenom vials required and associated cost to immunocapture all venom proteins of *B. fasciatus* using Premium Serums polyvalent antivenom.

***Bungarus fasciatus* venom**	**Maximum binding capability per gram of antivenom (mg)**	**Amount of venom bound per vial (mg)**	**Estimation of vials required per bite**	**Estimated range of cost associated (in INR)**
Aizawl, Mizoram	9.3	3.7	6-30	4,500-22,500
Guwahati, Assam	12.8	5.1	4-23	3,000-17,250

4.4.4 SDS-PAGE and Western Blotting of poorly-immunodepleted peaks

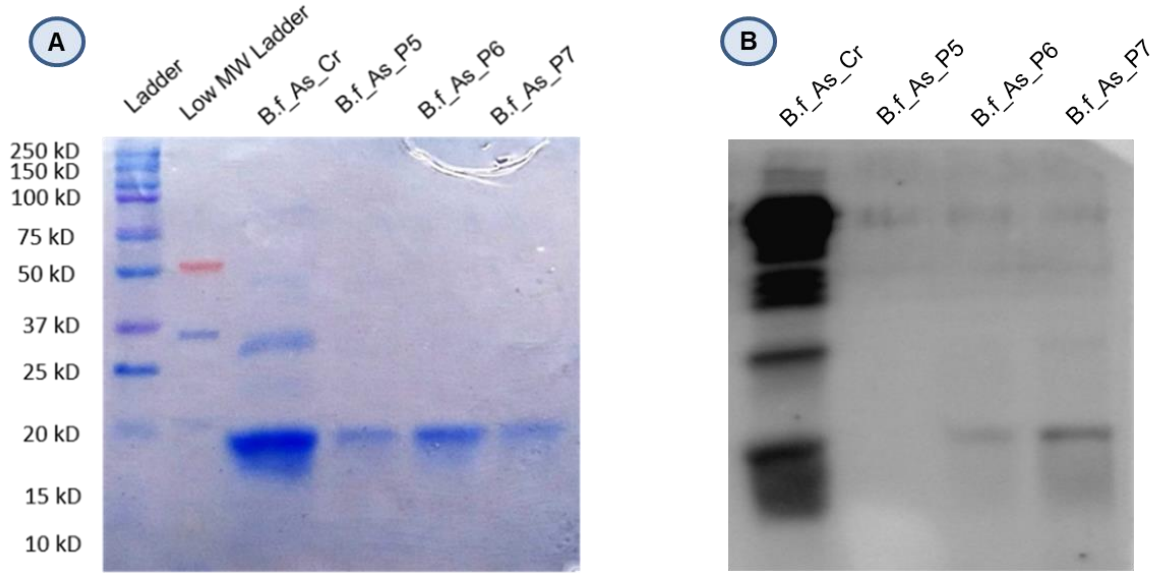

Figure 4.3: (A). 12.5% Glycine gel profile of crude venom and P5, P6 and P7 peaks from the venom of *B. fasciatus* (Guwahati, Assam). The wells were loaded with 15 µg of crude venom or poorly-immunodepleted proteins (P5, P6 and P7); **(B).** Immunoblot profile of crude venom and peaks (P5, P6 and P7) after western blotting utilizing Indian polyvalent antivenom (Premium Serums).

The peaks with inadequate immunodepletion (P5, P6 and P7), which exhibited saturation in 3G antivenomics analysis at a venom load of 273 µg, were run in 12.5% glycine

gel with reducing dye and were observed after Coomassie staining. From the gel low molecular weight bands ranging from 17-19 kDa were visible in all the peaks, which indicated the likelihood of these belonging to PLA_2 superfamily (Figure 4.3. A). It was followed by immunochemical analysis of the peaks using western blotting. The immunoblot analysis revealed that the crude venom bands were immunorecognized by the polyvalent antivenom, however, the peak P5 remained undetected, although it could faintly detect peaks P6 and P7 (Figure 4.3.B).

4.4.5 Identification of proteins by Liquid chromatography-tandem mass spectrometry

According to the findings of the 3G antivenomics analysis, the peaks P5, P6 and P7 exhibited inadequate immunodepletion despite a venom-antivenom ratio of 1:128 (venom load 34 µg). Hence, ESI-LC-MS/MS analysis was utilized for identification of these peaks (Table 4.4). From the three peaks, the partial sequences were identified using NCBI-BLASTp as seven PLA_2 proteins which were earlier documented from the venom of *B. fasciatus* from India and other East Asian nations like China and Taiwan [27,122]. The identified proteins from peak P5 include Basic phospholipase A_2 II (Uniprot ID Q90WA8), Acidic phospholipase A_2 KBf-grIB (Uniprot ID P0C551) and Basic phospholipase A_2 X (Uniprot ID P14411), Peak P6 contained two PLA_2 proteins, namely Phospholipase A_2 precursor BF-16 (Uniprot ID ABU63161) and Basic phospholipase A_2 I (Uniprot ID Q90WA7). Similarly, peak P7 also contained two PLA_2 enzymes, namely Phospholipase A_2 precursor BF-40 (ABU63164) and Basic phospholipase A_2 (A6MEY4). This was followed by pairwise sequence alignment of the identified proteins using NCBI-BLASTp (Table 4.5).

Table 4.4: Summary of proteins/peptides identified from poorly-immunodepleted peaks from the venom of *B. fasciatus* (Guwahati) after examination by ESI-LC-MS/MS.

HPLC fraction	Protein name	MS/MS derived sequence	m/z	Charge (z)	Score	Best NCBI match	Protein family
P5	Basic phospholipase A_2 II	NLLQFKNMIECAGTR	604.30	3	13	*B. fasciatus* (Q90WA8)	PLA_2
		CCQTHDQCYDNAK	841.80	2	17		
		CCQTHDQCYDNAKK	567.21	3	17		
		KKFGNCIPYFK	709.34	2	8		
		KFGNCIPYFK	425.22	3	9		
		FGNCIPYFK	651.32	2	11		

		KTYVYTCNKPDITCTGAK	707.33	3	7		
		TYVYTCNKPDITCTGAK	707.33	3	8		
		TYVYTCNKPDITCTGAKG	683.65	3	17		
		TYVYTCNKPDITCTGAKGSCGR	628.04	4	12		
		NKPDITCTGAK	402.21	3	9		
		APYNLANFGINK	661.35	2	9		
		NLANFGINK	495.77	2	9		
P5	Basic phospholipase A_2 X	GGSGTPVDQLDR	601.29	2	7	*B. fasciatus* (P14411)	PLA_2
		GSGTPVDQLDR	601.29	2	8		
		RFGKCNPYFK	694.34	2	8		
		FGKCNPYFK	581.27	2	4		
		CNPYFK	406.18	2	6		
		TYEYTCNKPNLTCR	607.28	3	14		
P5	Acidic phospholipase A_2 KBf-grIB	GTPVDALDR	472.25	2	9	*B. fasciatus* (P0C551)	PLA_2
		CSSLLNVPYVK	638.31	2	8		
		TAALCFAEVPYKR	685.34	2	10		
P6	Basic phospholipase A_2 I	NIPPQPLNLLQFK	761.42	2	7	*B. fasciatus* (Q90WA7)	PLA_2
		NMIQCAGSR	518.73	2	10		
P6	Phospholipase A_2 precursor BF-16	YACYCGPGGTGTPLDELDR	739.32	3	7	*B. fasciatus* (ABU63161)	PLA_2
		ACYCGPGGTGTPLDELDR	661.33	3	9		
		PGGTGTPLDELDR	692.83	2	11		
		GTPLDELDR	508.25	2	7		
		TPLDELDR	508.25	2	7		
		FGSCIPYFK	572.79	2	7		
		KFGSCIPYFK	623.78	2	5		
P7	Basic phospholipase A_2	YGCYCGPGGTGTPLDQLDR	664.95	3	9	*B. fasciatus* (A6MEY4)	PLA_2
		GPGGTGTPLDQLDR	692.35	2	9		
		PGGTGTPLDQLDR	692.35	2	8		
		TGTPLDQLDR	586.80	2	7		
		GTPLDQLDR	507.76	2	6		
		CCQTHDHCYDNAK	564.54	3	14		

		CCQTHDHCYDNAKK	564.54	3	14		
		TYEYTCNKPDLTCTDAK	731.66	3	9		
		TYEYTCNKPDLTCTDAKG	712.98	3	10		
		LTCTDAK	404.70	2	7		
		NVCDCDR	441.17	2	5		
		AAAICFAAAPY	563.27	2	8		
		AAAICFAAAPYNLAN	769.38	2	9		
P7	Phospholipase A_2 precursor BF-40	YGCYCGYGGSGTPVDELDR	709.28	3	6	*B. fasciatus* (ABU63164)	PLA_2
		TYSYTCNKPDLTCTDAK	689.30	3	18		
		FVCDCDR	486.19	2	7		

Table 4.5: Pairwise sequence alignment of partial peptide fragments from inadequately immunodepleted peaks (P5, P6 and P7) with their homologous protein (using NCBI BLASTp).

Peak P5

1. Basic phospholipase A_2 II (Accession No. Q90WA8)

Sequence:
NLLQFKNMIECAGTRTWMAYVKYGCYCGPGGTGTPLDELDRCCQTHDQCYDNAKKFGNCIPYFKTYVYTCN
KPDITCTGAKGSCGRTVCDCDRAAALCFAAAPYNLANFGINKETHCQ

This study:
NLLQFKNMIECAGTRTWMAYVKYGCYCGPGGTGTPLDELDRCCQTHDQCYDNAKKFGNCIPYFKTYVYTCN
KPDITCTGAKGSCGRxxxxxxxxxxxxxxxx**APYNLANFGINK**xxxxx

2. Basic phospholipase A_2 X (Accession No. P14411)

Sequence:
NLYQFKNMIQCAGTQLWVAYVNYGCYCGKGGSGTPVDQLDRCCQTHDHCYHNAKRFGKCNPYFKTYEYTCN
KPNLTCRGAKGSCGRNVCDCDRAAAICFAAAPYNLSNFGINKKTHCK

This study:
xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**GGSGTPVDQLDR**xxxxxxxxxxxxxxx**RFGKCNPYFKTYEYTCN**
KPNL TCRxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

3. Acidic phospholipase A_2 KBf-grIB (Accession No. P0C551)

Sequence:
DLLQFNEMIECTIPGSFPLLDYMDYGCYCGTGGRGTPVDALDRCCKEHDDCYAQIKENPKCSSLLNVPYVK
QYSYTCSEGNLTCSADNDECAAFICNCDRTAALCFAEVPYKRRNFRIDYKSRCQ

This study:
xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**GTPVDALDR**xxxxxxxxxxxxxxxxxx**CSSLLNVPYVK**
xxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**TAALCFAEVPYKR**xxxxxxxxxxxxx

Peak: P6

1. Basic phospholipase A_2 I (Accession No. Q90WA7)

Sequence:
NLLQFKNMIQCAGSRLWVAYVKYGCYCGPGGTGTPLDQLDRCCQTHDHCYDNAKKFGNCIPYFKTYEYTCN
KPDLTCTDAKGSCARNVCDCDRAAAICFAAAPYNLANFGINKETHCQ

This study:
NLLQFKNMIQCAGSRLWVAYVKxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Peak: P6

2. Phospholipase A_2 precursor BFx16 (Accession No. ABU63161)

Sequence:
NLYQFKNMIECAGTRTWLAYVKYACYCGPGGTGTPLDELDRCCQTHDHCYDNAKKFGSCIPYFKTYEYTCN
KPDITCTDAKGSCGRTVCDCDRAAAICFAAAPYNLANFGIDKEKHCQ

This study:
xxxxxxxxxxxxxxxxxxxxxxYACYCGPGGTGTPLDELDRxxxxxxxxxxxxxxFGSCIPYFKxxxxxxx
xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx

Peak: P7

1. Basic phospholipase A_2 (Accession No. A6MEY4)

Sequence:
NLYQFKNMIQCAGTQLCVAYVKYGCYCGPGGTGTPLDQLDRCCQTHDHCYDNAKKFGNCIPYFKTYEYTCN
KPDLTCTDAKGSCARNVCDCDRAAAICFAAAPYNLANFGINKETHCQ

This study:
xxxxxxxxxxxxxxxxxxxxxxYGCYCGPGGTGTPLDQLDRCCQTHDCYDNAKKxxxxxxxxxxTYEYTCN
KPDL TCTDAKGxxxxNVCDCDRAAAICFAAAPYNLANxxxxxxxxxx

2. Phospholipase A_2 precursor BFx40 (Accession No. ABU63164)

Sequence:
NLFQFKNMIECAGTRSWTHYVSYGCYCGYGGSGTPVDELDRCCYVHDHCYGEAEKIFKCNPKTKTYSYTCN
KPDLTCTDAKGTCERFVCDCDRAAAICFAAAPYNNNNFMMKPKTHC

This study:
xxxxxxxxxxxxxxxxxxxxxxYGCYCGYGGSGTPVDELDRxxxxxxxxxxxxxxxxxxxxxxxTYSYTCN
KPDL TCTDAKxxxxxFVCDCDRxxxxxxxxxxxxxxxxxxxxxxxx

Note: The identified PLA_2 enzymes with Lys (K) or Pro (P) residue at position 31 is highlighted in yellow and green background respectively. The residues which are missing in the identified proteins are highlighted in blue color and marked as 'x'.

4.5 Discussion

Antivenom are produced in healthy animals injected with various snake venoms which act as antigens and generate an immune response resulting in the production of toxin-specific antibodies [216]. However, these antigens may also cross-react with other immunogens than the ones used. Antivenom may exhibit paraspecificity in which they cross-react with snake venom toxins not used in antivenom development due to presence of epitopes of similar conformation [217].

WHO suggests preclinical studies utilizing in vivo experimental animal models as well as antivenomics assessment in vitro is to estimate the effectiveness of an antivenom [188]. The 3G antivenomics approach was first designed to assess the EchiTAb-Plus-ICP antivenom's immunodepletion capability against the *Bitis arietans* venom from Ghana by Pla et al [212]. The parameters of antivenomics study, such as immunorecognition profile of an antivenom, maximum binding capability as well as presence of the toxin-specific fraction of antibodies in the antivenom helps in developing a suitable and effective antivenom [177]. The *B. fasciatus* venom is not a part of the venom pool which is used for polyvalent antivenom production in India and hence, their immunocapturing capability against this venom from Guwahati, Assam is assessed utilizing a 3G antivenomics approach. The study represents the first effort to estimate the maximum binding capability of polyvalent antivenom manufactured in India against the venom of *B. fasciatus* from North-East India.

There are previous reports of envenomation in humans by *B. fasciatus* both from Mizoram and Assam [16,45]. Kakati et al. have reported in their retrospective study two cases of *B. fasciatus* envenomation from Demow Model Hospital (Sivasagar, Assam) from April to August 2022. The pathophysiology in both the victims involved ptosis, dysarthria, dysphagia, lethargy, blurring of vision and dizziness. However, other neuroparalytic symptoms such as difficulty in breathing, loss of consciousness, dysgeusia, aphonia and hypertension were not observed in the victims. The patients were treated with VINS polyvalent antivenom along with intravenous injection of 10% calcium gluconate. Further local wound was treated with sterile dressing and antibiotics, and tetanus toxoid was also administered for immune support and subsequently both the patients recovered and were discharged on the 6^{th} day [16]. Biakzuala et al. attributed three cases of snakebite associated death known through verbal autopsy to possible

envenomation by *B. fasciatus* in Mizoram, however, the pathophysiological features of the envenomation was not described [45]. An observational study from Kerala, India by Kumar et al. reported two cases of *B. fasciatus* envenomation who were admitted to the Government Medical College, Kozhikode, Kerala between January to October 2016. Both the patients exhibited symptoms such as ptosis and ophthalmoplegia, and one of them exhibited blurred vision, paraesthesia, respiratory distress (single breathe count of <20) and respiratory distress requiring ventilator. However, no death was reported for the envenomation [218].

The venoms of *B. fasciatus* from Aizawl and Guwahati exhibited differential concentration-dependent crossreactivity of venom proteins to immunoaffinity columns with 4.4 mg of bound antibody fragments of polyvalent antivenom (Premium Serums) (Figure 4.1 and 4.2). After RP-HPLC profiling of non-retained fractions, it was observed that the peaks P2, P4 and P8 for Mizoram sample, and P5, P6 and P7 for Guwahati sample, remain poorly immunodepleted even at the lowest venom-antivenom ratio of 1:16 (*i.e.,* venom load of 273 µg) which reflect the presence of inadequate $F(ab')_2$ fragments against these venom proteins (Table 4.1 and 4.2). It was interesting to note that multiple peaks from Mizoram sample were non-immunodepleted (P3, P5 and P6) unlike the samples from Guwahati, where all the samples were immunorecognized, although to a lower degree for P1, P2, P5, P6 and P7 peaks. The venom binding sites present in the antivenom were saturated at a high venom load (273 µg) for the peaks P1, P3, P4, P6 and P7 (Guwahati sample). The maximum binding capability of the polyvalent antivenom reflects a higher degree of overall immunorecognition for the samples from Guwahati (12.8 mg/g antibody fragments) than the one from Aizawl (9.3 mg/g antibody fragments). Hence, the theoretical estimation of the number of polyvalent antivenom vials that would be required for neutralize *B. fasciatus* bite is larger for the Aizawl sample (6-30 vials) than the Guwahati sample (4-23 vials). The poor binding of venom proteins by the antivenom suggest a possible poor performance in its ability to neutralize *B. fasciatus* envenomation associated pathophysiology. The findings of the antivenomics study was supported by the results of the western blot analysis, where the P5, P6 and P7 peaks were inadequately detected by the antivenom resulting in faint bands in the immunoblot, which implies poor cross-reactivity.

Snakebite is considered an occupational hazard where most of the victims belong to weaker sections such as agricultural workers, labourers, fishermen and forest dwellers from tropical areas of Asia, Africa and Latin America [219–221]. Also, known as a 'disease of poverty' [222] incidents of snakebites trigger a vicious cycle of poverty for the victims and their family, which in one hand would negatively affect their working ability, and on the other hand would put a financial burden due to the costly treatment [223]. Requirement of multiple antivenom vials would significantly raise the overall treatment cost for the *B. fasciatus* envenomated victim in North-East India and would worsen the financial situation of such victims already facing a precarious income source. The use of a larger dose of polyvalent antivenom leads to the occurrence of adverse effects like anaphylactic reactions and pyrogenic fever in victims [59,187].

The poor recognition of venom toxins by the antivenom in the western immunoblot supports the findings of the 3G antivenomics study. However, it should also be considered that cross-reactivity does not always translate to in vivo neutralization due to the presence of many non-neutralizing antibodies, which may competitively bind to the targets of the antibodies neutralizing venom proteins [75]. Identification of the three poorly-immunodepleted peaks using LC-MS/MS analysis revealed partial sequences of seven PLA_2 enzymes. The PLA_2 enzymes break glycerophospholipids at sn-2 bond into lysophospholipids and fatty acids, however, the pharmacological effects of PLA_2s does not always depend on its enzymatic activity. PLA_2 enzymes represent among the most lethal constituents in snake venom, playing a crucial part in the capture or immobilization of prey, and they can induce their toxicity either on their own or by forming complexes. Further, they also induce a variety of pathophysiological effects in their envenomated prey/victims [224]. The same effect can be mediated by different PLA_2 enzymes by following different mechanisms. For instance, some PLA_2 enzyme may exhibit neurotoxicity based on enzymatic activity while some other PLA_2 may exhibit similar neurotoxicity following a non-enzymatic mechanism [225]. Activities such as lysis of membrane proteins or membrane-associated target proteins, with the subsequent release of lysophospholipids and fatty acids depend on the enzymatic activity. It changes the fluidity of cell membrane and disrupts the phospholipid bilayer making it selectively permeable through which ions and drugs can enter. The lysophospholipids could undergo metabolism to form platelet-activating factors, which mediate inflammation and may also cause cell lysis [226,227]. Fatty acids such as

arachidonic acid may form secondary messengers like prostaglandins and thromboxanes, which may interact with various receptors, ligands and enzymes of the cell, and modulate cell behaviour [225]. On the other hand, the PLA_2 enzymes may bind to target proteins and act as an agonist or antagonist and affect the ligand-binding capacity of the protein or alter the secondary messenger release from target cells independent of its enzymatic activity [228]. However, it is important to note that various pharmacological manifestations occur due to a combination of enzymatic as well as non-enzymatic interactions [228].

The abundance of PLA_2 enzymes in the snake venom composition contributes significantly to its toxic nature and also the pathophysiology in snakebite patients, such as neurotoxicity, myocardial toxicity, blood coagulation, clot formation, edema, hemolytic activity, and tissue or organ injury [229–232]. These effects are however not observed in the case of all PLA_2s as their activity is target-specific. For instance, β-bungarotoxin, which is isolated from *Bungarus multicinctus* exhibits presynaptic neurotoxic effects, but lacks post-synaptic or anticoagulant effects, whereas *Ophiophagus hannah* venom acidic PLA_2 (OHVA-PLA_2) exhibits cardiotoxic, myotoxic and anti-platelet effects [233–236]. Identification of these target proteins may significantly contribute in understanding the mechanism by which the PLA_2 enzymes induce their toxicity and also shed light on the physiological importance of the target proteins.

4.6 Conclusion

Incomplete immunodepletion of venom from *B. fasciatus* from North-East India (Mizoram and Assam) using polyvalent antivenom manufactured in India is a cause of concern which requires immediate attention. The 3G antivenomics approach helped in determination of the maximum binding capability of these polyvalent antivenom. Subsequent identification of the three poorly-immunodepleted venom proteins utilizing liquid chromatography-tandem mass spectrometry resulted in identification of seven PLA_2 proteins which might be enzymatically active. These venom proteins may be characterized and assessed to know if they have any direct or indirect role in pathophysiological manifestations in envenomated victims. Inclusion of these PLA_2 enzymes in adequate amount in the venom pool used during antivenom preparation may result in the development of more effective polyvalent antivenom.