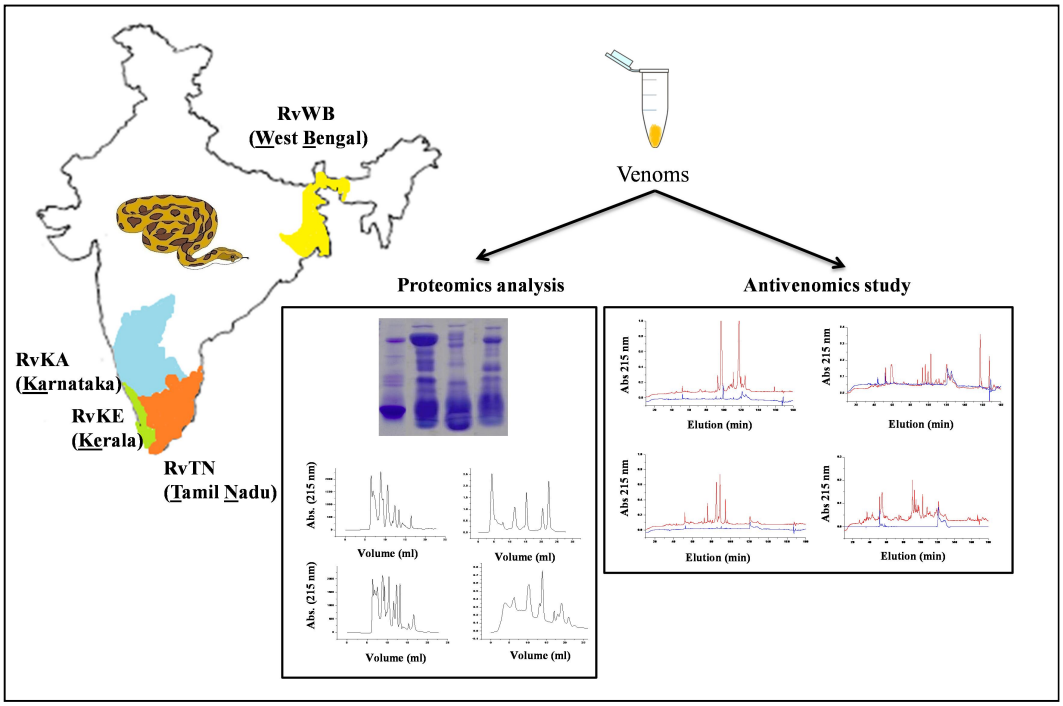
Chapter 2

Analysis of crude *Daboia russelii* venoms from different geographical locations of India



2.1 INTRODUCTION

Venom protein disparity due to inter and intra-species variation in venomous snake has made the study of snake venom very elusive (101-104). The diverse pharmacological profile observed post-envenomation by venomous snake is mainly due to this variation in venom composition. The in congruity in venom proteome within a species is mostly caused due to ontogenetic changes. sexual dimorphism and most prominently geographical location (111-114,397). As the habitat of a snake changes, it encounters different seasonal conditions along with a vast diversity of prey with altered morphology and physiology (105,107,115). This affects venom susceptibility towards different prey leading to natural selection and hence adaptive

evolution (115). The diverse pool of protein isoforms emerge due to evolutionary processes like gene duplication and accelerated point mutation in the protein coding regions, thus conferring various pharmacological functions to the venom proteins (290,398). Owing to this variation, the efficacy of the commercially available polyvalent antivenom raised against the snakes of a particular geographical location is highly compromised (100,113). This, in turn, might make snakebite management in victims very complicated and challenging (101,111). Therefore, comprehensive understanding of the complex snake venom proteome is important in understanding the pharmacological profile and for venom research.

In this chapter the variation in protein profile of *Daboia russelii* venom from four different geographical locations of India is analysed using proteomic tools. The efficacy of the commercially available polyvalent antivenom on the PLA_2 activity and coagulopathy of the four crude venoms were analysed by neutralization study. The immunoreactivity profile of the four crude venoms were also analysed by 2^{nd} generation antivenomics study which reveals the presence of N-toxins in them. The present study illustrates the qualitative and quantitative variation in the Indian *Daboia russelii* venoms which emphasizes the need to design regiospecific antivenom for better management of snakebite cases in India.

2.2 MATERIALS

2.2.1 Snake venoms

The crude venoms of *Daboia russelii* were obtained from four different geographical regions of India and designated with the name of the state from where it was obtained. The venom was milked from more than one individual snakes and pooled before lyophilisation. The venom obtained from Irula Snake catchers Society, Tamil Nadu was entitled as RvTN (Russell's viper Tamil Nadu); while from Kerala was milked from the snakes kept in Agadantantra snake park, Ayurveda Medical college, Thiruvananthapuram and labelled as RvKE (Russell's viper Kerala); crude venom from Karnataka was collected from Hassan, Western Ghats and designated as RvKA (Russell's viper Karnataka) while the venom from West Bengal was acquired from Kolkata Snake Park; named as RvWB (Russell's viper West Bengal). The crude

venom samples from West Bengal, Kerala and Karnataka (RvWB, RvKE and RvKA) were gifted by Dr. B.L. Dhananjaya and Dr. J.C. Menon.

2.2.2 Chemicals and reagents

Protein estimation: Albumin bovine fraction V powder was procured from Sisco Research Laboratories (Mumbai, India) and Folin Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany).

Antivenom: Commercial polyvalent antivenom (batch no. A5310028) manufactured by Bharat serums and vaccines limited was purchased from local medical supplier.

Electrophoresis: Ammonium persulphate (APS), acrylamide, bisacrylamide, N, N, N′, N′-tetramethylethylenediamine (TEMED), β-mercaptoethanol (βME) were obtained from Sigma (Missouri, USA). Sodium dodecyl sulphate (SDS) was purchased from Merck (Darmstadt, Germany). Coomassie brilliant blue R-250 (CBB R-250) was purchased from Merck (Darmstadt, Germany). The PageRuler^{TM} pre-stained protein marker (catalog # 26616) was purchased from Sisco Research Laboratories (Mumbai, India).

Others: Chemicals like Tris-base, $CaCl_2$, 12 N HCl, NaCl, glycine and HPLC grade solvents like acetonitrile (MeCN), methanol, acetic acid and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). Nitrocellulose membrane was bought from Whatman, GE healthcare life Sciences (Buckinghamshire, UK) while nylon syringe filter (0.2 µm) was purchased from Genetix, Biotech Asia Pvt. Ltd. (Delhi, India). Cyanogen bromide activated sepharose 4 fast flow was obtained from Sigma (Missouri, USA).

All other chemicals used were of highest purity grade and obtained from either Merck Millipore (Massachusetts, USA) or Sigma (Missouri, USA).

2.2.3 Columns

For size exclusion chromatography, Shodex protein KW-803 column (8.0 mm x 300 mm) was purchased from Showa Denko (Tokyo, Japan). For reversed phase (Rp) HPLC, Jupiter C_{18} column (4.6 x 250 mm, 3 µm, 300 Å) was procured from Phenomenex (California, USA).

2.3 METHODS

2.3.1 Protein estimation

The protein estimation was carried out by the colorimetric method developed by Lowry (399). Samples were dissolved in 20 mM Tris-Cl buffer (pH 7.4) and the volume was adjusted to 25 µl in a 96 well plate. To this, 250 µl of freshly prepared alkaline $CuSO_4$ was added and incubated for 10 min. Subsequently, 25 µl of freshly prepared Folin Ciocalteu's phenol reagent (1:3) was added and incubated for 30 min. Following incubation, the amount of protein in each of the samples was quantified at 660 nm. For preparation of the protein standard curve, bovine serum albumin (BSA) was used.

To estimate the protein content of crude venoms (RvTN, RvKE, RvKA and RvWB), 2 mg was in dissolved in 1 ml of 20 mM Tris-Cl buffer (pH 7.4). Five microlitre of each of the dissolved venom were used for protein estimation as described above.

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

The SDS-PAGE was performed according to the method developed by Laemmli (400). Briefly, samples were treated with loading dye (0.5 M Tris-Cl, 6% Bromophenol blue, 30% glycerol) containing 0.45 µl of β-mercaptoethanol (βME) (as the reducing agent) for 3 min at 100^{o}C. Following reduction samples were loaded on 12.5% glycine SDS-PAGE along with PageRuler pre-stained standard protein marker. Electrophoresis was carried out with the reservoir buffer (191.82 mM glycine, 24.76 mM Tris-base, 10% SDS) at 15 mA for the stacking gel and at 25 mA for the resolving gel. After the electrophoretic run, the gel was stained with CBB R-250 dye (0.25% w/v) in methanol:water:acetic acid (40:50:10). The gel was destained using methanol:water:acetic acid (40:50:10) till the protein bands were visible and documented using ChemiDoc^{TM}XRS+, Biorad (California, United States). The electrophoretic separation of 15 µg of each of the crude venoms of *Daboia russelii* (RvTN, RvKE, RvKA and RvWB) were performed as described above.

2.3.3 Size exclusion chromatography

For the separation of the crude venom proteins based on molecular mass, 50 µg of each of the crude venoms (RvTN, RvKE, RvKA and RvWB) were subjected to gel

filtration chromatography on shodex column (one column volume ~15 ml) pre-equilibrated with 20 mM Tris-Cl buffer (pH 7.4). Fractionation was carried out with the same buffer at a flow rate of 0.5 ml/min using Waters HPLC system (Massachusetts, USA) for 1.5 column volume. Elution profile of the venom proteins were monitored at 215 nm.

2.3.4 Reversed phase-High Performance Liquid Chromatography (Rp-HPLC)

The crude venoms were also fractionated by Rp-HPLC using Waters HPLC system (Massachusetts, USA). Briefly, 200 µg of each of the crude venoms were loaded on the Jupiter C_{18} column (4.15 ml column volume) pre-equilibrated with 0.1% (v/v) TFA for 5 column volume. The proteins were eluted by a linear gradient of 80% (v/v) MeCN containing 0.1% (v/v) TFA at a flow rate of 0.8 ml/min for 180 min. The gradient of MeCN starts from 0% to 20% in 20 min and then from 20% to 70% in 120 min. Fractionation was monitored at 215 nm.

2.3.5 Phospholipase A_2 (PLA_2) activity

Turbidometric method: The PLA_2 activity of the crude venoms under study were quantified by the turbidometric method with minor modifications (401). Briefly, an egg yolk was suspended in 250 ml of freshly prepared 0.9% NaCl and its absorbance was adjusted to 1 optical density with 20 mM of Tris-Cl buffer (pH 7.4) at 740 nm. To 1 µg of crude venoms of *Daboia russelii*, 200 µl of re-suspended egg yolk (adjusted to 1 OD) was added. The final volume of the reaction mixture was adjusted to 250 µl with 20 mM Tris-Cl buffer (pH 7.4) to make a final concentration of each of the crude venoms as 4 µg/ml in the reaction mixture. The decrease in the optical density of the reaction was measured for 10 min at 740 nm using UV-Vis MultiSkan Go spectrophotometer (Thermo Scientific, Massachusetts, USA). One unit of PLA_2 activity is defined as the decrease in 0.01 absorbance in 10 min at 740 nm.

2.3.6 Coagulation assay

2.3.6.1 Preparation of blood plasma

Fresh goat blood was collected from a local butcher in a tube containing 3.2% tri-sodium citrate in the ratio of 9:1 (402). The platelet poor (PP) plasma was prepared from the whole blood by centrifuging it at 3,000 rpm for 20 min at 16°C. The light

yellowish supernatant, containing the plasma was aliquot into 2 ml eppendorf and stored at -20^{o}C for future assays.

2.3.6.2 Recalcification time

The effect of the crude *Daboia russelii* venoms on the PP plasma was determined according to the method developed by Langdell and colleagues with slight modifications (403). Briefly, different concentration of crude venoms of *Daboia russelii* (RvTN, RvKE, RvKA and RvWB) were incubated with 150 µl of PP plasma for 2 min at 37^{o}C. The plasma clot formation was initiated by addition of 100 µl of 50 mM $CaCl_2$ and monitored using Tulip Coastat-1 coaguloanalyser (Goa, India). For each set of experiment, clotting time of PP plasma with 20 mM Tris-Cl buffer (pH 7.4) was considered as the normal clotting time (NCT).

2.3.7 Neutralization study by polyvalent antivenom

To determine the effectiveness of the polyvalent antivenom, 1 µg of each of the crude venoms were pre-incubated with various amount of polyvalent antivenom at 37°C for 1 hr. Post incubations, inhibition of PLA_2 activity was assayed as described in section 2.3.5. The percentage inhibition was calculated considering the activity of the crude venom without antivenom as 100%.

For inhibition of recalcification time, 0.1 µg of each of the crude venoms were pre-incubated with different amount of polyvalent antivenom at 37°C for 1 h. The plasma clotting time was recorded as described in section 2.3.6.2.

2.3.8 Immunodepletion of venom proteins

The immunoreactivity of the antibodies of the polyvalent antivenom against the *Daboia russelii* venoms were determined by 2nd generation antivenomics study (132,404). In brief, 200 µl of 10 µg/µl of polyvalent antivenom was incubated with CNBr-activated matrix (0.1 g swollen overnight at room temperature in 12.5 ml of cold and filter sterile 1 mM HCl) for 16 h at 2-8^{o}C with continuous shaking. Next day, the resins were washed several times with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3-8.5) to remove any unbound antibodies. Succeeding this, the resins were

treated with blocking buffer (0.2 M glycine) for 2 h at room temperature to prevent any non-specific binding of the resin with venom proteins to be added subsequently. Following this, 200 µg of each of the crude venoms were added to the antivenom bound resin and incubated overnight at room temperature with gentle shaking. Thereafter, the flow-through containing the non-retained fraction was reloaded onto the column/resin several times to ensure sufficient venom-antivenom binding. Consequently, centrifugation was carried out at 3,000 rpm for 5 min and the supernatant was loaded onto Jupiter C_{18} Rp-HPLC column pre-equilibrated with 0.1% (v/v) TFA. The fractions were eluted by a linear gradient of 80% (v/v) MeCN and 0.1% (v/v) TFA at a flow rate of 0.8 ml/min. The gradient of MeCN starts from 0% to 20% in 20 min and then from 20% to 70% in 120 min and fractionation was monitored at 215 nm. A pictorial representation of the steps involved in this technique has been illustrated in figure 2.1.

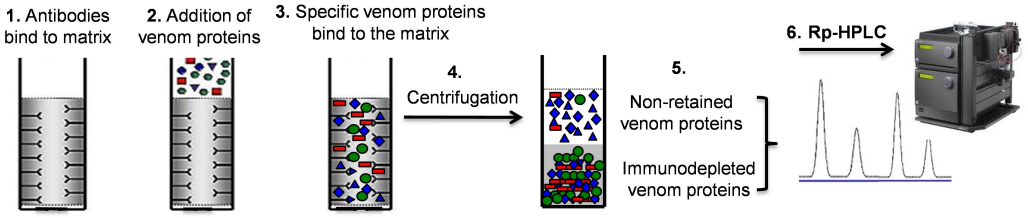


Figure 2.1: Schematic representation of the various steps involved in the 2nd generation antivenomics study. (1): Under alkaline conditions CNBr reacts with the hydroxyl group on the agarose beads to form the cyanate ester. These esters react with the primary amine group of the polyvalent antivenom to couple them covalently to the matrix. **(2):** Venom proteins are added to the polyvalent antivenom bound CNBr matrix. **(3): S**pecific venom proteins bind to the antivenom non-covalently **(4) & (5):** The bound and unbound venom-antivenom mixture is pellet down and separated. **(6):** The supernatant containing the unbound venom proteins is subjected to Rp-HPLC.

2.3.9 Statistical analysis

The results of each experiment are mean of three independent experiments and are represented in mean ± standard deviation (SD). Statistical analysis of the experimental data was carried out by student t-test using Microsoft Excel and p-value between ≤ 0.05 to ≤ 0.001 were considered significant for the analysis.

2.4 RESULTS

2.4.1 Quantitative and qualitative analysis of the four crude venom proteomes

2.4.1.1 Protein content

The protein estimation of the four venoms of *Daboia russelii* from different geographical locations of India (Figure 2.2) revealed considerable variation in protein content in each of them (Table 2.1). This advocates the quantitative difference in venom content though these four snake venoms under study belong to the same species.

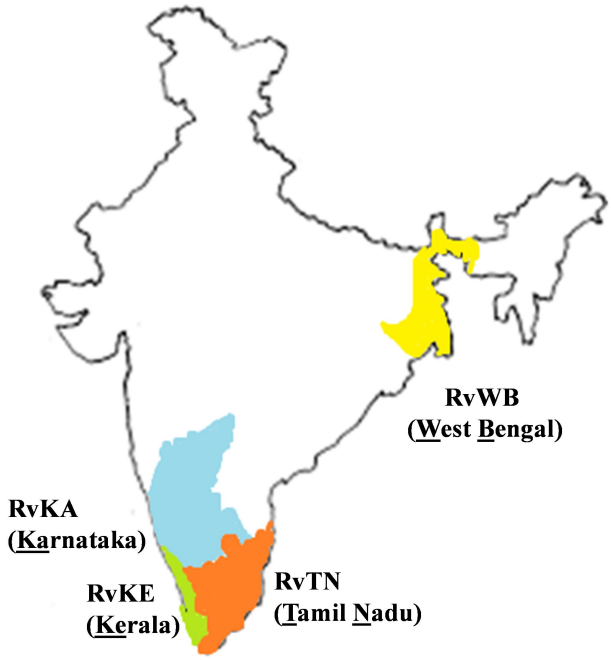


Figure 2.2: Map of India showing the geographical locations from which the crude *Daboia russelii* venoms were procured.

Table 2.1: Protein content of crude *Daboia russelii* venoms from different geographical locations. Amount of protein in the crude venoms were quantified using BSA standard curve.

Sl.No.	Crude venom of *Daboia russelii*	Percentage of protein in crude venom
1.	RvTN (Tamil Nadu)	73 ± 2%
2.	RvKE (Kerala)	95 ± 1.5%
3.	RvKA (Karnataka)	85 ± 2.3%
4.	RvWB (West Bengal)	75 ± 1.75%

2.4.1.2 SDS-PAGE analysis

Electrophoretic separation of the four crude venoms (RvTN, RvKE, RvKA and RvWB) have revealed the presence of different protein bands of varying intensity in the range of ~170 kDa to 10 kDa (Figure 2.3). The crude venom from Tamil Nadu, RvTN showed the presence of 9 protein bands with two most prominent bands at 70 and 15 kDa, relatively faint bands at ~55 kDa and four bands between ~25 to 15 kDa. Venom from Kerala, RvKE separated into 11 proteins with bright bands at 100 and ~15 kDa. It also displayed relatively low intensity but clear bands at 55, 35 and 25 kDa. The venom profile from Karnataka, RvKA displayed 13 proteins with a chunk of protein band between 15 and 10 kDa and faint protein bands at 70, 35 and ~25 kDa. In the venom profile from eastern India, RvWB, 11 protein bands were observed with thick bands at 70, 35, 25, 15 kDa and relatively faint bands at 130 and 55 kDa.

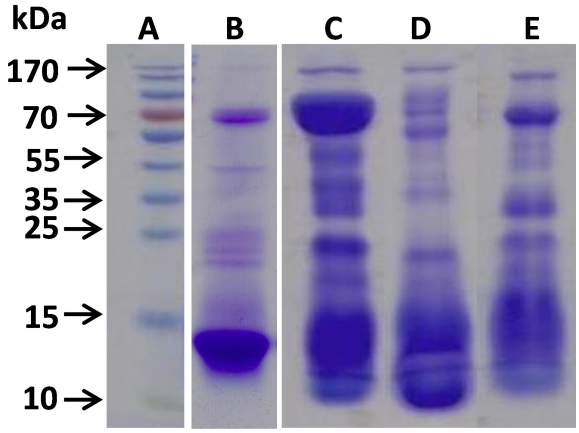


Figure 2.3: 12.5% Glycine SDS-PAGE profile of crude *Daboia russelii* venoms (15 µg) from different geographical locations of India under reduced condition. **Lane A:** Standard pre-stained protein marker, **Lane B:** RvTN, **Lane C:** RvKE, **Lane D:** RvKA and **LaneE:** RvWB.

2.4.1.3 Chromatographic analysis

The gel filtration profile of the four geographically isolated crude venoms of Indian *Daboia russelii* showed disparity in elution pattern on Shodex column (Figure 2.4). RvTN separated into 10 protein peaks with peak 1, 3 and 5 most prominent. On the other hand, elution profile of RvKE revealed only 6 protein peaks of which peak 1, 4

and 6 were major while RvKA resolved into 13 peaks with peak 1, 4, 7, 9 and 10 were noteworthy. RvWB, the eastern Indian venom separated into 10 protein peaks of which peak 3 and 5 were most notable compared to the other peaks.

The compositional variation in the four crude venoms was further analysed by Rp-HPLC which also displayed distinct elution pattern, further validating the existence of substantial variation in crude venoms under study (Figure 2.5). The venom procured from Tamil Nadu and Karnataka revealed the presence of unique protein peaks (starred mark in figure 2.5) which were not observed in the other crude venoms.

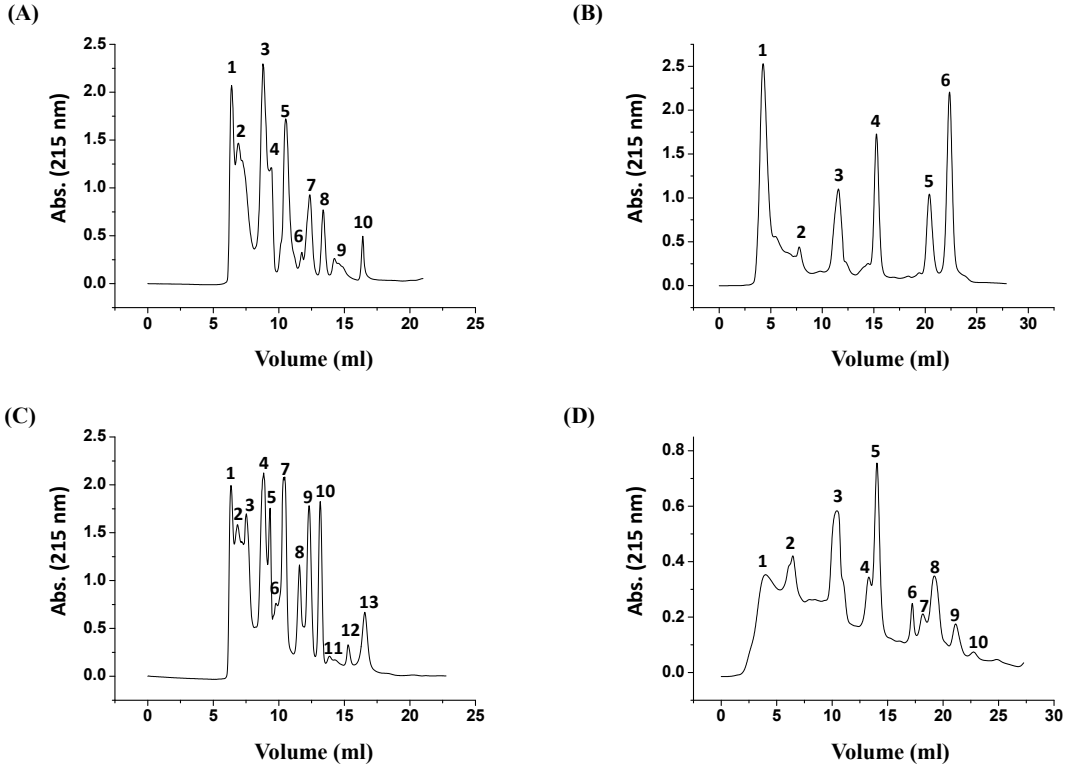


Figure 2.4: Gel filtration chromatography profile of crude venoms of *Daboia russelii* on Shodex column. Elution profile of **(A)** RvTN; **(B)** RvKE; **(C)** RvKA and **(D)** RvWB. Elution of the venom proteins was carried out using the equilibration buffer and monitored at 215 nm.

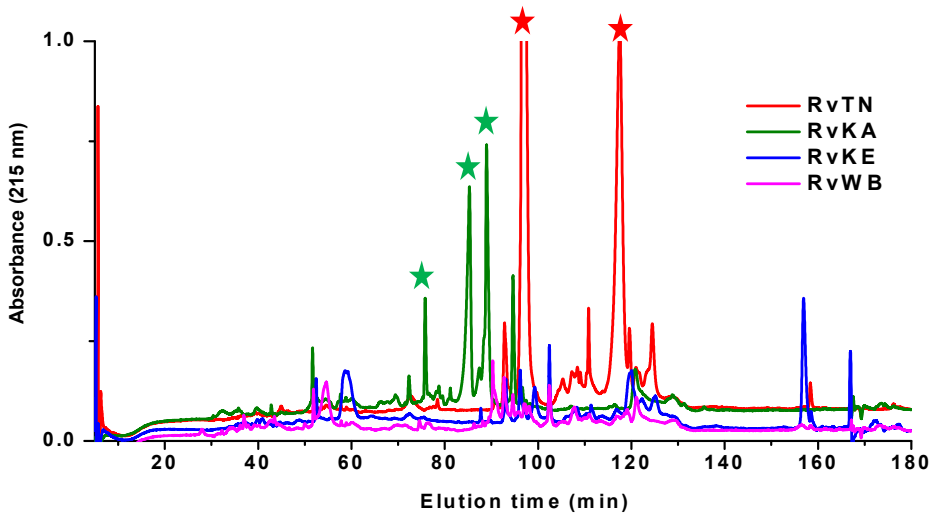


Figure 2.5: Comparative Rp-HPLC elution profile of the four crude venoms from different geographical locations of India on a Jupiter C_{18} column monitored at 215 nm. Starred indicates unique peak.

2.4.2 Biochemical assays

2.4.2.1 PLA_2 activity

Differential level of PLA_2 activity was observed in the four crude venoms when tested using egg yolk as the substrate (Figure 2.6). The venoms from southern India exhibited significantly (p-value < 0.001) lesser hydrolytic activity compared to the venom from eastern India. 4 µg/ml of RvKA exhibited 18.7 ± 2 units of PLA_2 activity followed by RvKE with 7.12 ± 1.34 units and RvTN with least activity of only 5.0 ± 1 units. On the other hand, the same amount of RvWB showed the highest activity of 58.18 ± 2.5 units.

2.4.2.2 Recalcification time

RvKA and RvWB displayed procoagulant activity on goat plasma in a dose dependent manner. On the other hand, RvKE reduced the clotting time to 16.23 ± 0.98 s at 0.033 µg/ml but further there was no or negligible change in clotting time upto 33.33 µg/ml (Figure 2.7). Nevertheless, RvTN exhibited procoagulant activity, but with the increase in venom amount a gradual delay in clotting time was observed from 0.33 µg/ml to 33.33 µg/ml. The procoagulant activity of RvKE was significant with respect

to RvTN (p-value < 0.001) and RvKA (p-value ≤ 0.01). However, the procoagulant activity of RvKE was found to be significant (p-value < 0.01) up to 0.33 µg/ml with respect to RvWB but beyond this there was no significant change in the activity.

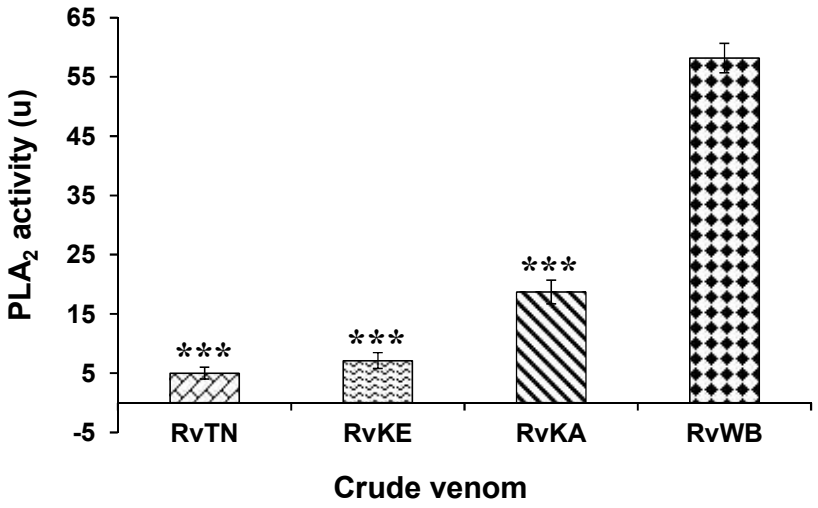


Figure 2.6: Phospholipase A_2 activity of *Daboia russelii* crude venoms (4 µg/ml) from different geographical locations of India using egg yolk as the substrate. One unit (u) of PLA_2 activity is defined as the decrease in 0.01 absorbance in 10 min at 740 nm. The data is mean ± SD of three independent experiments. *** denotes p-value < 0.001 with respect to the PLA_2 activity of RvWB.

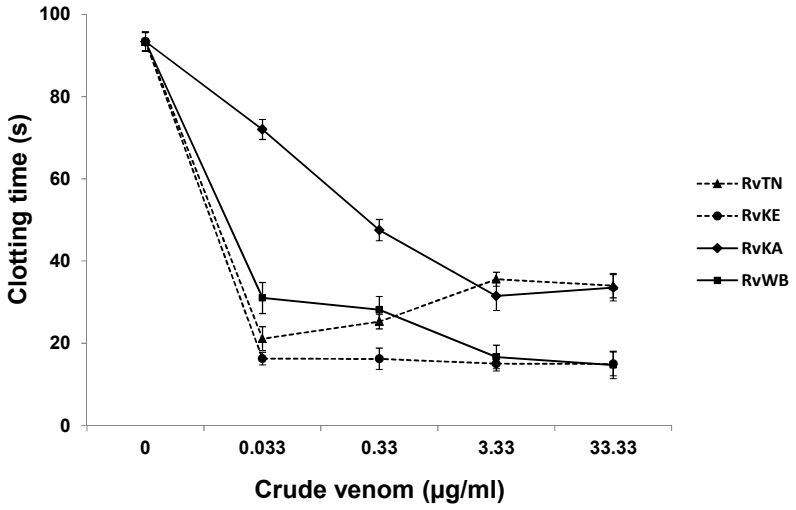


Figure 2.7: Recalcification time of *Daboia russelii* crude venoms from different geographical locations of India on PP plasma. The clotting time was initiated by $CaCl_2$ and recorded on Coastat-1 coagulation analyser. Results are mean ± SD of three independent experiments.

2.4.3 Neutralization study

The commercial polyvalent antivenom could differentially neutralize the PLA_2 activity of the four crude venoms under study (Figure 2.8). At a ratio of 1:1 (venom to antivenom), the tested polyvalent antivenom could not neutralize the PLA_2 activity of any of the crude venoms significantly. However, at 1:100 ratio, it could significantly (p-value < 0.001) lower the percentage residual PLA_2 activity of the four crude venoms (RvKA 77.34 ± 1.05%, RvWB 44.8 ± 2.1%, RvKE 33.09 ± 1.23% and RvTN 5.64 ± 1.8%). Overall it was observed that the polyvalent antivenom was effective in neutralizing the PLA_2 activity of RvTN followed by RvKE and RvWB while least effective on RvKA.

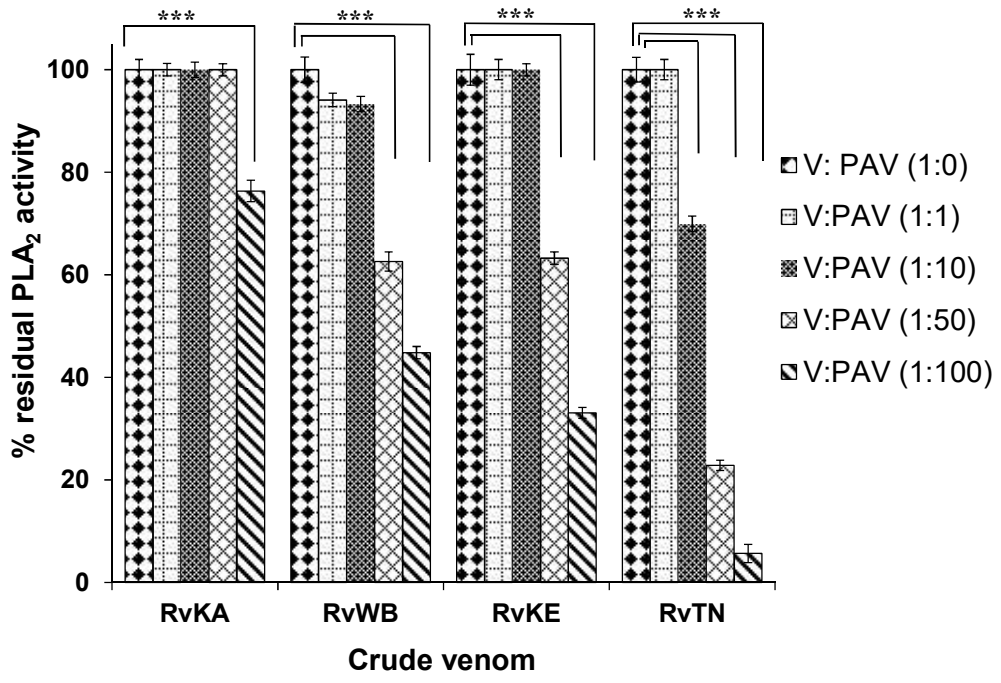


Figure 2.8: Percentage residual phospholipase A_2 activity of crude *Daboia russelii* venoms (1 µg) pre-incubated with different amounts (µg) of polyvalent antivenom. The percentage residual activity was calculated considering the activity of the respective crude venoms without antivenom as 100%. Results are mean ± SD of three independent experiments. *** indicates p-value < 0.001.

Similarly, the neutralizing ability of the polyvalent antivenom on the procoagulant activity of the crude *Daboia russelii* venoms on goat plasma was also noticeably variable (Figure 2.9). At 1:1 ratio, the polyvalent antivenom could neutralize the procoagulant effect of RvTN significantly (p-value < 0.001) while was insignificant for RvKA, RvWB and RvKE. At a ratio of 1:100 ratio, the procoagulant effect of RvTN and RvKA were significantly (p-value < 0.001) prolonged up to 76.4 ± 2.68 s and 66.6 ± 1.3 s respectively. However, the same ratio of polyvalent antivenom could not neutralize the procoagulant effect of RvWB (14.6 ± 1.6 s) and RvKE (19.7 ± 1.3 s).

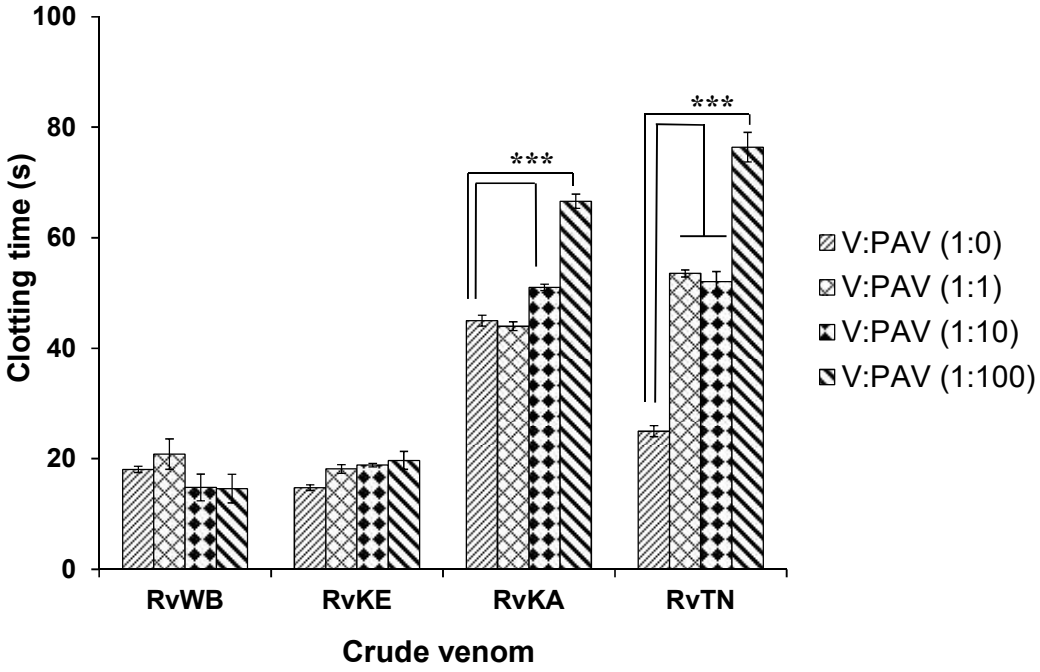


Figure 2.9: Neutralization of recalcification time of crude *Daboia russelii* venoms (0.1 µg) pre-incubated with the different amount (µg) of polyvalent antivenom. The normal clotting time was 93.36 ±1.02 s and clotting time in presence of only polyvalent antivenom was 92 ±2.5 s. Results are mean ± SD of three independent experiments. *** indicates p-value < 0.001.

2.4.4 Immunodepletion study

The antivenomics study reveals the presence of immunogenic and non-immunogenic proteins in each of the crude venom under study. Based on the immunoreactivity of

the venom proteins towards the antibodies of the polyvalent antivenom, venom proteins are categorized into completely immunodepleted toxins (C-toxins), partly immunodepleted toxins (P-toxins) and non-immunodepleted toxins (N-toxins). The Rp-HPLC elution profile of the non-retained fraction of each of the crude venom was compared with the respective crude venom profile to identify the presence of these C, P and N- toxins (Figure 2.10). The flow-through of RvTN revealed the presence of 5 N-toxins and one P-toxin. The elution profile of the flow-through of RvKE and RvWB revealed four N-toxins while RvKA revealed the presence of three N-toxins and one P-toxin.

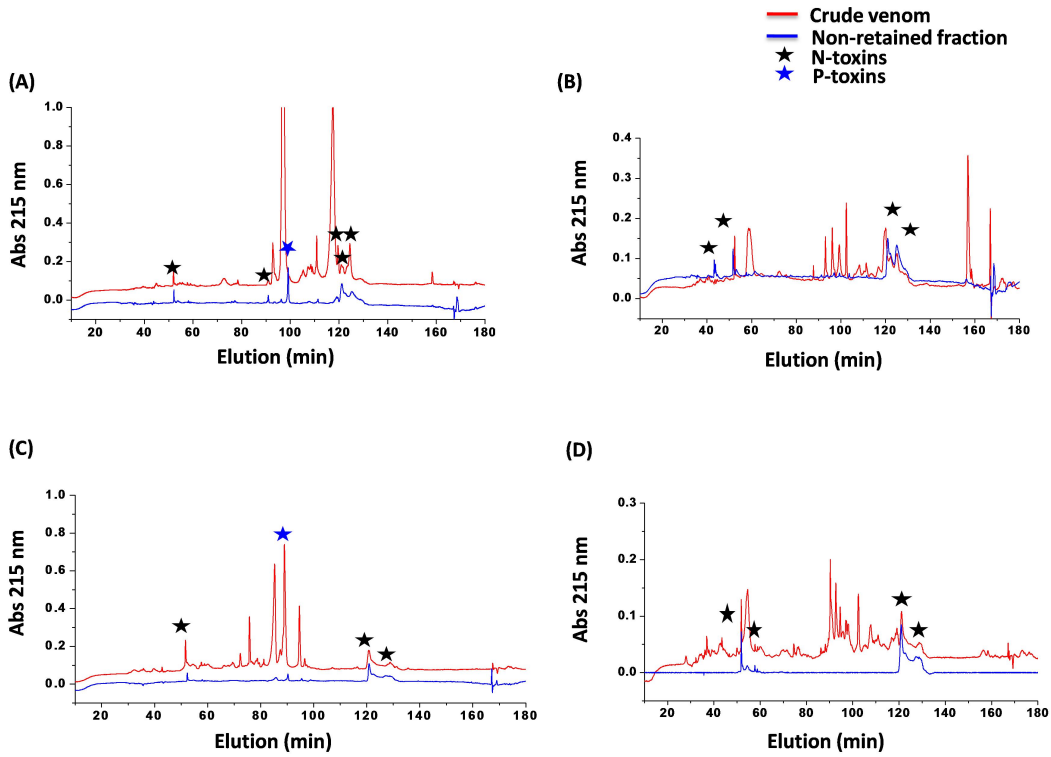


Figure 2.10: Comparison of Rp-HPLC profile of crude venom with respective flow through fractions. Rp-HPLC profile of **(A)** RvTN, **(B)** RvKE, **(C)** RvKA and **(D)** RvWB. Fractionation was carried out on Jupiter C_{18} column using waters HPLC system and monitored at 215 nm.

2.5 DISCUSSION

The variation in venom composition of venomous snakes has been an intriguing concern in venom research and snakebite management. Like other venomous snake,

the venom proteome of *Daboia russelii*, one of the most important venomous snakes of the Indian sub-continent (398,405) also shows profound variation with geographical locations (128,371,372). This, in turn, leads to demonstration of diverse clinical pathologies post-envenomation which might make the management scenario very critical (398,406). The variation in clinical manifestations of Russell's viper envenomation based on geographical locations has been well documented in the literature. For example, the Burmese Russell's viper envenomation is reported to manifest conjunctival edema, acute pituitary infarction (398) while the Indian viper presented acute pituitary infarction along with rhabdomyolysis and neurotoxicity (398). On the other hand, Sri Lankan viper envenomation is reported to cause rhabdomyolysis, neurotoxicity (363,398) pulmonary edema (407), delayed anuria, agrochemical nephropathy (365) and ischemic stroke followed by bilateral blindness (370). Interestingly, the Taiwan viper presented systemic thrombosis apart from other common pathological signs but was devoid of any neurotoxic effects (408). As such it is very much crucial to have an inclusive understanding of the venom composition of venomous snakes belonging to different geographical regions for better management and design of effective snake antidotes.

In the present study, the lyophilized crude venoms of *Daboia russelii* were procured from four different regions/states of India which varied in climatic conditions and geographical locations. The three geographically nearby states of southern India, viz., Tamil Nadu a land of hills and coastal area with rich vegetation, Karnataka a land of hills, plains and coastal regions with tropical climate while Kerala experiencing tropical equatorial climate with heavy rainfalls and have high and mid lands with coastal areas. On the other hand, West Bengal, an eastern Indian state, more than 1,300 km away from South India experiences tropical and sub-tropical climatic conditions and is surrounded by the mountains in the north, and delta in the south and plateaus in the west. Such variation in geographical location and climatic condition might have an impact on the type and availability of prey which in turn, might affect the venom composition. Analyses of the proteome profile of these crude venoms using various proteomic tools have revealed the presence of considerable variation in the venom composition both qualitatively and quantitatively. SDS-PAGE analysis shows the existence of various high and low molecular weight proteins in all the

venoms under reduced condition with different band intensities. This underscores the probable variation in the level of protein expression in the respective venom glands. Based on the comparative analysis of the electrophoretic profile with standard protein marker, it was observed that RvTN is rich in low molecular weight proteins like PLA_2 enzymes and KSPI followed by proteases and LAAO in its proteome. A very intense band at 100 and 15 kDa in the SDS-PAGE profile of RvKE suggests its richness in high molecular mass proteins like PDE, 5'-NUC and low molecular weight proteins like PLA_2 enzymes. The protein profile of RvKE displayed high band intensities suggesting the relative abundance of proteins in its proteome compared to the rest of the crude venoms under study. This co-relates well with its protein content which is 95% of the total protein of the crude venom. On the other hand, the venom from Karnataka, RvKA was found to be rich in proteins having molecular masses between 15 and 10 kDa compared to the high molecular mass proteins proposing the predominance of PLA_2 enzymes and other low molecular mass proteins like disintegrins and KSPI. Last but not the least, the venom proteome from West Bengal, RvWB displayed prominent bands at 70, 25 kDa with a thick protein band at 15 kDa indicating the abundance of LAAO, proteases, PLA_2 enzymes and other low molecular weight proteins. Noteworthy that, all the four crude venoms of *Daboia russelii* showed the relative abundance of PLA_2 enzymes in its proteome accentuating the prominent role of this protein family in offensive, defensive and digestive functions of this venom irrespective of their geographical origin (46).

The elution profiles of the gel filtration and Rp-HPLC of the crude venoms under study further validated the existence of variation in the venom composition with respect to protein families as well as the level of protein expression. Gel filtration profiles of the venoms from Tamil Nadu and Karnataka had comparatively similar elution pattern whereas the elution profile from Kerala and West Bengal were considerably different. The Rp-HPLC profile of each of the crude venoms eluted with different retention time for venom proteins further supporting the observed variation in the protein pool. Interestingly, unique protein peaks were observed in the Rp-HPLC profile of RvTN and RvKA which were not observed in the other venoms emphasizing the venom variation due to geographical location.

Similar to the proteomic results, the functional aspects of the four crude venoms also exhibited the differential level of activities. As the venom proteomes were observed to be rich in PLA_2 enzymes and proteases irrespective of the geographical locations, so the two most prominent biochemical activities of *Daboia russelii* venom were taken into consideration, viz: PLA_2 activity and recalcification time for further analysis. All the four venoms under study exhibited differential level of PLA_2 activity. The hydrolytic activity was highest in the venom from West Bengal followed by Karnataka, Kerala and Tamil Nadu (RvWB > RvKA >RvKE > RvTN). This can be correlated with the SDS-PAGE profile of the venoms discussed above. RvTN displayed a low intensity protein band at 15 kDa compared to the rest of the crude venoms suggesting lesser availability of PLA_2 isoforms in its proteome. However, all the crude venoms exhibited procoagulant activity on goat plasma although with different mode of action. RvKA and RvWB displayed procoagulant activity in a dose dependent-manner. Nonetheless, under the same experimental conditions, the procoagulant activity of RvKE venom saturated at a lower concentration, signifying the relative abundance of procoagulant proteins in its proteome which is also evident from its SDS-PAGE profile showing a number of prominent protein bands in the molecular mass between 55 to 25 kDa. Conversely, RvTN exhibited procoagulant activity at lower concentrations, but the clotting time was gradually prolonged with increasing concentration suggesting the predominance of anticoagulant components like PLA_2 enzymes, serine protease inhibitors, LAAO (184) which might have surpassed the activity of the procoagulant components.

From the present proteomic and functional study we have observed extensive variation in the venom composition and activities of the four crude venoms of *Daboia russelii* under investigation. This might lead to noticeable differences in pathophysiological effects in victims upon envenomation, posing a great challenge to the efficacy of the antivenom produced against the venom of a particular geographical location. For the clinical management of snakebite victims, polyvalent antivenom produced against the “Big four” snake venoms (*Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus*) is the only available treatment for snakebite victims. Paradoxically, polyvalent antivenom is not always efficient to neutralize or prevent the pathophysiological effects of envenomation (126,363,405,409). Although

there is a discrepancy regarding the dosage of polyvalent antivenom administration, 10-20 vials of polyvalent antivenom are generally injected intravenously to the victims for the treatment (410). However, this is often accompanied by several anaphylactic reactions like nausea, vomiting, chills, hypotension, respiratory distress and late serum sickness (121,122,126). This might be due to the presence of large repertoire of non-specific antibodies (from the other three snakes) in the polyvalent antivenom or absence of certain proteins in the crude venom which is used to raise the antivenom (121). Prasad and colleagues reported considerable variation in diffusion and cross-reactivity pattern in polyclonal antibodies produced against *Daboia russelii* venom from south India when compared venoms from other geographically isolated snakes (372). On the other hand, Madhu Kumar and co-workers reported that the antibodies prepared against the venom of eastern India did not cross-react with all the gel filtration fractions of the western and southern Indian viper venom (128). Further, Shashidharamurthy and co-workers also documented the ineffectiveness of the polyvalent antivenom against *Naja naja* venom from other geographical locations (129).

Based on this investigation, the neutralization of PLA_2 and procoagulant activity of the crude venoms was tested using the available polyvalent antivenom. In the neutralization study it was observed that the commercial polyvalent antivenom differentially reversed the PLA_2 and procoagulant activity of the four crude venoms of *Daboia russelii.* At a higher ratio of 1:100, the polyvalent antivenom could effectively neutralize the PLA_2 activity of RvWB followed by RvKE, RvTN and RvKA. Similarly, at 1:100 ratio the polyvalent antivenom inhibited the procoagulant activity of RvKA and RvTN venom but the same amount of polyvalent antivenom had least effect on the procoagulant activity of RvKA and RvWB venom. Calvete and colleagues proposed a new generation proteomic approach termed “Antivenomics” in which an antivenom recognizes the venom proteins containing the specific epitopes (132,411). The principle underlying this technique is that upon incubation of crude venom with antivenom, only the epitope bearing antigenic proteins immunodeplete forming the antigen-antibody complex but not the other proteins. The partial or non-immunodepleted venom proteins are the ones against which either low affinity or no antibodies are developed respectively during hyperimmunization. The comparison of

the Rp-HPLC profile of the supernatant and the pellet fractions after antivenomics with the crude venom in turn helps in unveiling the immunoreactivity profile qualitatively. Comparative analysis of Rp-HPLC profile of respective crude venoms with the non-retained supernatant obtained from immunodepletion study has revealed the presence of partially immunodepleted and non-immunodepleted peaks. Noteworthy observation is that, highly hydrophobic proteins in all the venoms were least immunodepleted by the polyvalent antivenom. This highlights the inefficacy of the polyvalent antivenom to recognize and immunodeplete the venom components completely. This could be due to the variation in venom composition of the geographically isolated snakes and non-immunogenicity of some of the venom proteins as venom from a particular geographical location is generally used for the production of polyvalent antivenom (412). The differential expression of venom proteins and its isoforms within the same species and the presence of unique toxins might be the cause for the presence of a large number of non-antigenic proteins in the venoms (109,114,372,413). Based on these analyses it could be concluded that the inability to neutralize or immunodeplete the crude venom proteins suggests the absence of specific antibodies for the epitopes of the venom components in the polyvalent antivenom. The absence of specific antibody repertoire might, in turn, be one of the major limitations of the available polyvalent antivenom to confront the pathophysiological challenges manifested upon envenomation.

Hence, the present study states that venom variation and non-immunogenicity based on geographical location should be considered as important criteria for antivenom production. Proper efforts and emphasis have to be made to design regiospecific antivenom for better management of pathological challenges in snakebite victims.