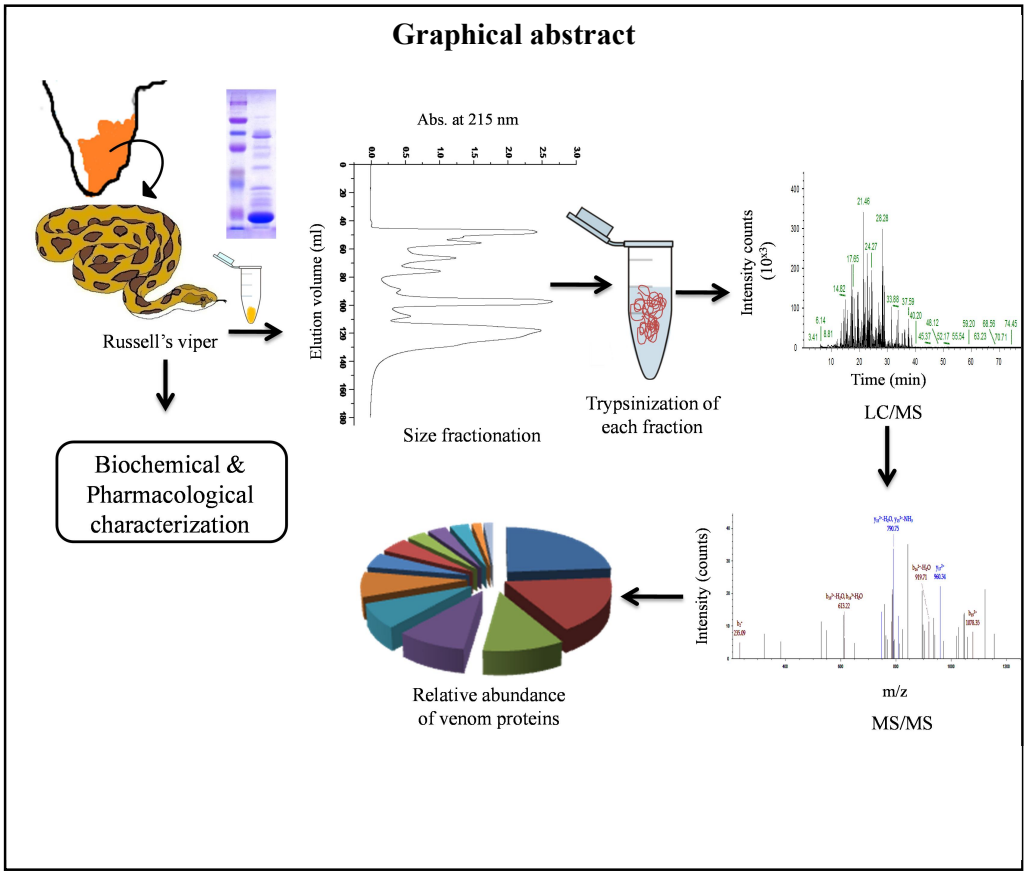
Chapter 3

Proteomics of *Daboia russelii* (Irula) venom and identification of a major protein



3.1 INTRODUCTION

Snake venom is a complex mixture of proteinaceous and non-proteinaceous components which are responsible for the manifestation of severe pathological effects in the envenomated victims. Snake envenomation leads to several incidences of morbidity and mortality every year especially in the tropical and sub-tropical countries of the world (90). Polyvalent antivenom is the only therapy accessible to the snakebite victims for treatment. However, it is often accompanied by various adverse side effects, questioning the specificity and efficacy of these antidotes (126,414).

Hence, it is very much essential to have comprehensive information about the complete venom proteome of venomous snakes to decipher their subtle pharmacological profile and design more effective antivenom to confront the snakebite challenges more apprehensively.

India, being an agricultural based tropical country, reports a massive number of snakebite cases every year especially in the rural areas (88,90,415). *Daboia russelii* is one of the most prevalent venomous snakes of the Indian subcontinent responsible for such casualties (9). Polyvalent antivenom used for the treatment of *Daboia russelii* envenomation is produced against the "Big Four" snake venoms of India viz., *Naja naja*, *Daboia russelii*, *Bungarus caeruleus*, *Echis carinatus* which is the only available treatment for snakebite patients. It is reported that nearly 90% of the venom requirement for antivenom production is supplied by Irula snake catcher's society, Tamil Nadu, which is an authorized venom supplier (92). Hence, it is essential to unveil the venom proteome of the snakes from this geographical region and analyse the effectiveness of the commercial polyvalent antivenom produced against the snakes of Irula snake catcher's society. Therefore, in this chapter, venom proteome of *Daboia russelii* venom from Irula and its correlation with the various biochemical and pharmacological activities and neutralization by commercial polyvalent antivenom has been undertaken.

3.2 MATERIALS

3.2.1 Venom

The lyophilized venom of *Daboia russelii* was purchased from Irula Snake Catchers Society, Tamil Nadu, India. Permission to purchase the venom for research purpose was obtained from Forest Range Officer, Wildlife Enforcement Range, Chennai-32 (enclosed in Appendix-V).

3.2.2 Chemicals and Reagents

Mass spectrometry: Dithiothreitol (DTT) was obtained from Gold biotechnology (Missouri, USA). Protease Max from Promega (Wisconsin, USA), trypsin, iodoacetamide (IAA) and ammonium bicarbonate were bought from Sigma (Missouri,

USA). Acetic acid was procured from VWR (Pennsylvania, USA). Polyvinylidene fluoride (PVDF) membrane was bought from GE Healthcare Life Sciences (Chicago, USA).

Assay kit: Secretory (s) PLA_2 assay kit was purchased from Cayman Chemical Company (Michigan, USA).

Coagulation reagents: Uniplastin for prothrombin time and liquecelin for activated partial thromboplastin time were procured from Tulip Diagnostics (P) Ltd. (Goa, India).

Cell culture: Human Embryonic Kidney (HEK)-293 and Michigan Cancer Foundation (MCF) -7 cell lines were purchased from National Centre for Cell Science (NCCS) (Pune, India). Dulbecco modified Eagle's media (DMEM) was obtained from Himedia (Mumbai, India), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue from Sigma Aldrich (Missouri, USA), fetal bovine serum (FBS), streptomycin-penicillin, trypsin/Ethylenediaminetetraacetic acid (EDTA) solution and dimethyl sulfoxide (DMSO) were procured from Thermo Fischer Scientific (Massachusetts, USA).

Others: Bovine plasma fibrinogen was obtained from Sigma (Missouri, USA) and Casein purified was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). PageRuler^{TM} prestained protein marker (catalog #SM0671) was purchased from Fermentas, Thermo Scientific (Massachusetts, USA).

All other chemicals and reagents used are described in material section of Chapter 2.

3.2.3 Column

Hiload^{TM} 16/600 Superdex 75 prep grade column (1x120 ml) was purchased from GE Healthcare life Sciences (Bucks, UK).

3.2.4 Animals

Both male and female Swiss albino mice weighing 40 ± 3 g were obtained from the Central animal facility, University of Mysore, Karnataka, India. The animals were kept under optimal housing conditions in well ventilated clean cages. The animals were supplied with proper food and water obtained from the centre. The temperature of the animal house was set at 25 ± 3˚C with alternative cycles of light/dark conditions after every 12 h. The *in-vivo* experiments were performed as per the

guidelines approved by the Animal Ethical Committee, University of Mysore, Karnataka, India (Animal Ethical committee approval no. UOM/IAEC/25/2011) (enclosed in Appendix-V).

3.3. METHODS

3.3.1 Protein estimation

Two milligrams of the lyophilized crude venom was dissolved in 1 ml of 20 mM of Tris-Cl buffer, pH 7.4 and protein content was estimated as described in section 2.3.1 of Chapter 2.

3.3.2 Gel filtration chromatography of crude venom of *Daboia russelii*

The lyophilized crude venom of *Daboia russelii* was subjected to gel filtration chromatography on Hiload^{TM} 16/600 Superdex 75 prep grade column (1x120 ml). Briefly, 100 mg of the crude venom was dissolved in 2.5 ml of 50 mM of Tris-Cl pH 7.4 and filtered through 0.2 µm nylon syringe filter. For single injection, 500 µl (40 mg/ml) of the filtrate was loaded onto the gel filtration column pre-equilibrated with 50 mM of Tris-Cl pH 7.4 using Äkta Purifier HPLC system, GE Healthcare (Uppsala, Sweden). Fractionation was carried out at a flow rate of 1 ml/min under isocratic conditions with the equilibration buffer. Elution of the fractions was monitored at 215 and 280 nm.

3.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of the crude *Daboia russelii* venom (15 µg) and its gel filtration peaks (7 µg each), was carried out as described in section 2.3.2 of Chapter 2.

3.3.4 Tandem mass spectrometry of the gel filtration peaks

The gel filtration peaks of the crude *Daboia russelii* venom were subjected to trypsinization in the presence of Protease Max (Promega) according to the instructions of the manufacturer. Briefly, 50 µg of each of the gel filtration peaks were reconstituted with 50 µl of Milli Q water to make up a concentration of 1 mg/ml. To each of them, 50 mM of ammonium bicarbonate (41.5 µl), 1% Protease Max (2 µl) and 0.5 M DTT (1 µl) were added and incubated at 56°C for 20 min. Following this, the reduced samples were alkylated with 0.55 M of IAA (2.7 µl) and incubated in

dark for 15 min. Next, 1% of Protease Max (1 µl) and 1.8 µl of Trypsin (1 µg/µl in 50 mM acetic acid) were added. After incubation at 37°C for 3 h, the reactions were quenched by addition of 100% TFA (0.5 µl) for 5 min at room temperature. Subsequently, the samples were centrifuged at 12,000 rpm for 10 min.

For the tandem mass spectrometric (MS/MS) analysis of the tryptic digested samples, Accela LCQ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, Massachusetts, USA) was used (Figure 3.1). Approximately, 80 µl of each of the trypsinized supernatant of the gel filtration peaks was loaded onto a Hypersil Gold C_{18} column (50 x 2.1 mm, 1.9 µm) which was pre-equilibrated with 0.1% of formic acid. Fractionation was carried out at a flow rate of 200 µl/ min with a linear gradient of 100% MeCN in 0.1% formic acid from 0% to 40% (in 38 min) and then from 40% to 80% (in 18 min) of MeCN. Each eluent from liquid chromatography column was directed to the mass spectrometer. The spectra were recorded in MS/MS mode and scanned from 500 to 2000 m/z under positive ionization mode.

The MS/MS spectra obtained for each gel filtration peak were analysed using Proteome Discoverer 3.1 with Sequest program using the default parameters. *S*-carbamidomethylation of cysteine residues and oxidation of methionine residues were set as modifications (Figure 3.2). Based on m/z ratio and sequence similarity, each peptide fragment was assigned to a protein in the NCBI database. The validation of the identification and analysis of the proteins and peptides were carried out based on the presence of at least one unique peptides, Sequest protein score (~2-105) and coverage (~8-86) which were calculated by the Sequest program. Moreover, the peptides fragments with lower score and coverage were manually confirmed by NCBI blastp (blast.ncbi.nlm.nih.gov) search.

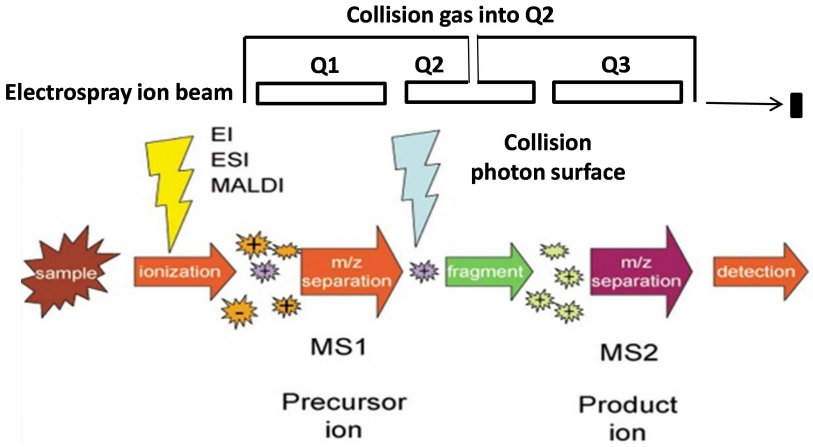


Figure 3.1: Pictorial representation of the various steps in tandem mass spectrometry (Adapted from www.slideshare.net/Nabiilah/mass-spectrometry-in-pharmacognosy). It consists of an ion source for producing gaseous ions from the sample under study. The ionization of the sample occurs by soft ionization techniques like ESI, MALDI or EI. The analyzer separates the ions based on their mass and charge by magnetic field deflection. These ions are further fragmented into smaller positively charged ions. Detector detects the ions formed and measures the relative abundance of the ions.

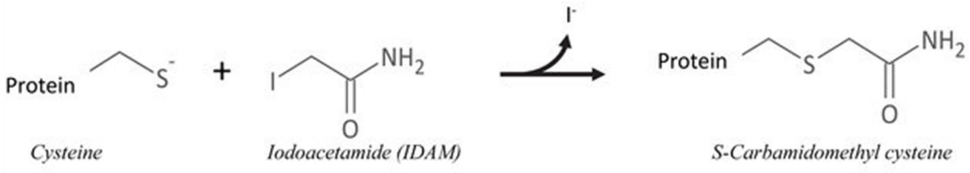


Figure 3.2: Mechanism of carbamidomethylation of cysteine residue in a protein by iodoacetamide (Adapted from Rombouts, I. et. al., Scientific reports, 2013)(416). IAA binds to the thiol group of a cysteine residue covalently to form S-carbamidomethyl cysteine.

3.3.5 *In-vitro* coagulation assays

3.3.5.1 Preparation of platelet poor plasma and recalcification assay

Preparation of platelet poor plasma and recalcification time assay were carried out as described in section 2.3.6.1 and 2.3.6.2 of Chapter 2 respectively.

3.3.5.2 Prothrombin time (PT)

The PT of the crude venom of *Daboia russelii* was performed using Uniplastin (PT reagent). Briefly, to different concentrations of the crude venom 50 µl of PP plasma was added and incubated for 2 min at 37°C. Consequently, 50 µl of PT reagent was added and clot formation was recorded using Coastat-1 Coagulation Analyser.

3.3.5.3 Activated partial thromboplastin time (APTT)

The APTT of crude *Daboia russelii* venom was tested using Liquecelin (APTT reagent). In brief, different concentrations of crude venom were pre-incubated with 50 µl of PP plasma and 50 µl of APTT reagent for 3 min at 37°C. Subsequently, 50 µl of 25 mM $CaCl_2$ was added to trigger clotting of plasma which was recorded using Coastat-1 Coagulation Analyser.

3.3.6 Fibrinogenolytic activity

The fibrinogenolytic activity of the crude venom of *Daboia russelii* was estimated using bovine plasma fibrinogen. Briefly, 2 mg/ml of fibrinogen was dissolved in 50 mM of Tris-Cl, pH 7.5 containing 0.15 M of NaCl (417). For the reaction, 300 µl of the dissolved fibrinogen was pre-incubated with 1 µg of the crude venom for 24 h at 37°C. Thrombin (10 units/ml) with fibrinogen was considered as positive control and fibrinogen with buffer was taken as the negative control. After 24 h of incubation, the reaction was stopped by addition of SDS-PAGE loading dye and subjected to 12.5% glycine SDS-PAGE as described in section 2.3.2 of Chapter 2.

3.3.7 Haemolytic assay

3.3.7.1 Preparation of the erythrocyte suspension

The pellet of RBC and WBC obtained from the centrifugation of the whole blood (discussed in section 3.3.5.1) was washed for 3 to 4 times with 0.9% NaCl at 3,000 rpm for 20 min at 16°C to get a pellet of RBC devoid of any other blood components (318). The washed pellets were suspended in 0.9% NaCl to make a suspension of 10% RBC which was used in the subsequent haemolytic assays to assess the effect of the crude venom.

3.3.7.2 Direct and indirect haemolytic activity

The direct haemolytic activity of the crude venom was estimated according to the method developed by Jeng and co-workers and as modified by Doley and Mukherjee (402,418). Briefly, 150 µl of 10% RBC was pre-incubated with different concentration of the crude *Daboia russelii* venom for 1 h in serological water bath, JSGW (Haryana, India) at 37^{o}C. For indirect haemolytic activity, 20 µl of egg yolk (re-suspended in 0.9% NaCl) was added to the mixture of 10% RBC and venom (described above) and incubated at 37^{o}C for 1 h. The RBC suspension with distilled water was taken as the positive control while RBC with 20 mM of Tris-Cl, pH 7.4 was taken as the negative control.

Following incubation, the reaction mixtures were centrifuged at 10,000 rpm for 10 min using Spinwin MC-02 table top centrifuge Tarsons (West Bengal, India). The optical density of the supernatant was measured at 540 nm using UV-Vis MultiSkan GO spectrophotometer (Thermo Scientific, Massachusetts, USA). The percentage of haemolysis was calculated considering haemolysis caused by water as 100%.

3.3.8 Phospholipase A_2 activity

The PLA_2 activity of the crude *Daboia russelii* venom and its gel filtration fractions were determined using $sPLA_2$ assay kit Cayman Chemical (Michigan, USA) according to the instructions of the manufacturer (Figure 3.3). Briefly, 0.01 µg of the crude venom and each of its gel filtration fractions were added to the reaction containing DTNB [5, 5'-dithio-bis-(2-nitrobenzoic acid)] and the reaction volume was adjusted to 20 µl with the assay buffer (25 mM Tris-Cl pH 7.5, 10 mM $CaCl_2$, 100 mM KCl, 0.3 mM Triton X-100). Following this, 1.66 mM of the substrate (200 µl of diheptanoyl thio-phosphatidylcholine) (diheptanoyl thio-PC) was added to initiate the hydrolytic reaction. As positive control, 0.001 µg/µl of bee venom PLA_2 enzyme was used. The rate of hydrolysis of the substrate was quantified at 414 nm for 10 min at room temperature using UV-Vis MultiSkan GO spectrophotometer, Thermo Scientific (Massachusetts, USA). Enzyme activity was expressed in micromoles of substrate hydrolysed per min (µm/min).

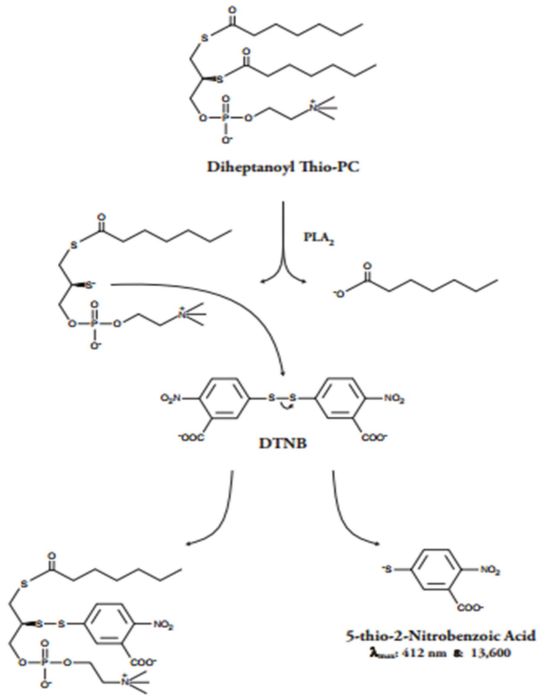


Figure 3.3: Schematic representation of the reaction mechanism of diheptanoyl thio-PC hydrolysis by PLA_2 enzymes (Adapted from Cayman Chemical $sPLA_2$ assay kit). PLA_2 enzymes catalyse the hydrolysis of the thio ester bond at the *sn-2* position releasing free thiols which are being detected in the presence of DTNB at 414 nm or 405 nm.

3.3.9 Proteolytic activity

The proteolytic activity of the crude venom was analysed according to the method developed by Ouyang and Teng with slight modification by Mukherjee and co-workers (417,419).

Preparation of 1% casein solution: Casein (1 g) was dissolved in 100 ml of 20 mM Tris-Cl pH 7.4. It was stirred for 30 min followed by warming at 37^{o}C for half an hour and stored overnight at 4^{o}C. Next day the solution was centrifuged at 10,000 rpm for 15 min at 4^{o}C using pre-conditioned Heraeus Multifuge X1R refrigerated centrifuge, Thermo Scientific (Massachusetts, USA).

Briefly, different concentration of crude venom was pre-incubated with 1% casein for 90 min at 37^{o}C. The reaction was stopped by the addition of 500 µl of 10% ice cold trichloroacetic acid (TCA) and incubated at 4^{o}C for 20 min. Following quenching, the

mixture was centrifuged at 5,000 rpm for 10 min using Spinwin MC-02 table top centrifuge Tarsons Product Pvt. Ltd. (West Bengal, India). For quantification of the amount of proteolysis, 1 ml of the supernatant was subjected to protein estimation as described in section 2.3.1 of Chapter 2.

The tyrosine standard curve was prepared using the Lowry's method. Briefly, a stock solution of 100 mg/ml of tyrosine was prepared in distilled water and heated gently until the tyrosine dissolved. Different amount of the dissolved tyrosine was subjected to the Lowry's method of protein estimation as described in section 2.3.1 of Chapter 2 to plot the standard graph. One unit of caseinolytic/proteolytic activity is defined as *n* mole equivalent of tyrosine formed per min per ml.

3.3.10 Edema inducing activity

The edema inducing effect of the crude venom of *Daboai russelii* was determined according to the modified method developed by Vishwanath and co-workers (420). Briefly, 45 µg of the crude venom was dissolved in 60 µl of phosphate buffer saline (PBS) to make up a concentration of 0.75 µg/µl. Dissolved venom (20 µl) was injected intra-mascularly (i.m.) into the right foot pad of male albino mice (n=3). As vehicle control, the left leg of the mice was injected with 20 µl of PBS. The animals were observed for 1 h following which they were euthanized by injecting 30 mg/kg of barbitone intra peritoneally. Following sacrifice, the feet of the mice were removed from the ankle joints and the increase in weight was evaluated using weighing balance (Wensar®, Tamil Nadu, India).

3.3.11 Haemorraghic activity

The haemorrhagic activity of the crude venom of *Daboia russelii* was determined according to the method developed by Kondo and co-workers (421). Briefly, 30 µl of the sample (0.5 mg/ml) was injected intradermally into the back of the male albino mice. As vehicle control, the mice were injected with equal volume of PBS. As positive control, 30 µl of Saw-scaled viper venom (0.1 µg/µl) dissolved in PBS was used. For each case, a set of three male albino Swiss mice were used. The treated animals were observed for 3 h following which they were sacrificed by injecting 30

mg/kg of barbitone (i.p). Subsequently, the dorsal skin of the sacrificed mice was removed and the inner surface was observed for any haemorrhagic damage.

3.3.12 Cytotoxicity assay

The cytotoxic effect of the crude *Daboia russelii* venom on the normal and cancerous cell lines were determined by the MTT based colorimetric method (422). Briefly, in two independent set of experiments, the cells lines of HEK-293 and MCF-7 were grown in DMEM media containing 10% FBS and 1% antibiotic (Strep-Pen). The cells were grown for 2-3 days under humified condition in CO_2 (5%) incubator, Eppendorf (Hamburg, Germany) at 37^{o}C until 70-80% confluency of the cells was reached. To evaluate the viability of the cells, they were stained with trypan blue dye. The viable cells were quantified on a haemocytometer and appropriate cell dilutions were prepared for the subsequent assay.

The re-suspended viable cells of HEK-293 and MCF-7 in fresh cell culture media were plated on 96 well plate at a concentration of $1x10^5$ cells/well under sterile condition using class-II biosafety cabinet, Daihan Labtech (Korea). The cells were incubated for 48 h at 37^{o}C in CO_2 (5%) incubator. Following incubation, the cells were treated with different concentration of crude venom for 24 h at 37^{o}C in CO_2 (5%) incubator. For vehicle control, cells were treated with 0.9% NaCl without addition of any venom sample. Next day, the morphological pattern of the treated cells were observed and documented using Inverted microscope (Axio Vert A1, Zeiss, Jena, Germany) at 10X magnification. Successively, 20 µl of MTT (5 mg/ml) was added to the cells and incubated at 37^{o}C for 2 h. Following incubation, 150 µl of MTT solution was added to each well to dissolve the formazan crystals formed by the viable cells. The amount of formazan formed by the viable cells was quantified at 590 nm using UV-Vis MultiSkan Go spectrophotometer (Thermo Scientific, MA, USA). The percentage of cell viability was calculated by considering the cells without the venom treatment as 100% viable. The results are mean ± SD of three independent experiments.

3.4 RESULTS

3.4.1 Protein estimation

The protein estimation of the pooled crude *Daboia russelii* venom from Irula revealed the presence of ~73% protein of the total dry weight.

3.4.2 Gel filtration chromatography

The fractionation of the crude venom of *Daboia russelii* on Superdex 75 pg column resolved it into 8 prominent protein peaks (P1-P8) (Figure 3.4). Gel filtration peak P1, P6 and P8 showed prominent protein peaks at both 215 and 280 nm. P6 displayed sharp protein peaks at both the wavelengths suggesting it to contain the major protein. Rest of eluted peaks did not show sharp peaks at 280 nm which might be due to the relatively lesser abundance of these proteins in this crude venom proteome. The total percentage recovery of proteins from gel filtration chromatography was 84.55% which showed a better efficiency of the technique employed.

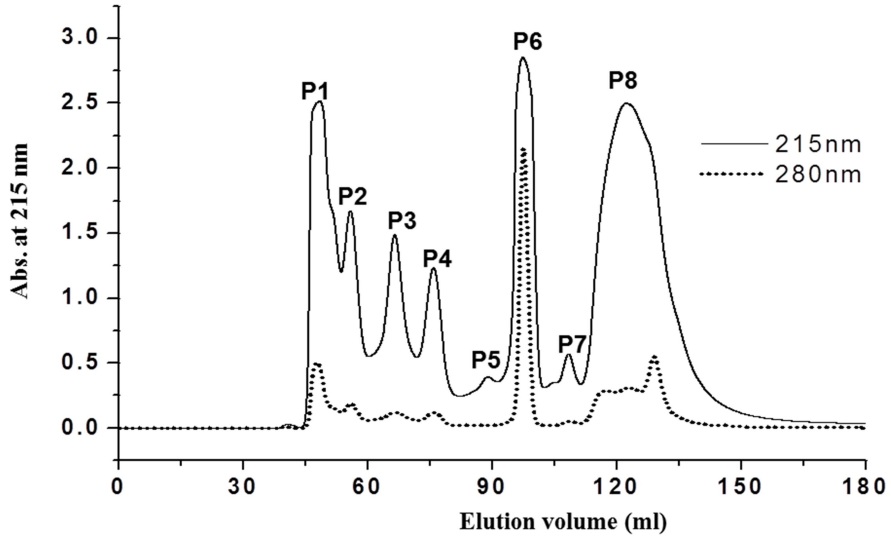


Figure 3.4: Size exclusion chromatography profile of crude *Daboia russelii* venom on Superdex 75 prep grade column. Fractionation was carried out using 50 mM Tris-Cl, pH 7.4 at a flow rate of 1 ml/min. The elution of crude venom protein was monitored at 215 and 280 nm.

Table 3.1 Summary of the protein content of the gel filtration peaks of crude *Daboia russelii* venom.

GF fractions	Total volume	Total protein (mg)	Protein % in crude venom
P1	50	10	11
P2	35	3.8	4.2
P3	50	4.5	5
P4	50	4	4.4
P5	25	2	2.2
P6	50	28	31
P7	25	2.25	2.5
P8	125	30	33

3.4.3 SDS-PAGE analysis

The electrophoretic profile of the crude *Daboia russelii* venom revealed the presence of both high and low molecular mass proteins ranging from 170-8 kDa (Figure 3.5 A). Bright protein bands at 70 and 15 kDa suggest the abundance of proteases and PLA_2 like enzymes in the crude venom.

The electrophoretic profile of the 8 gel filtration fractions of the crude venom provided an overview of the various proteins present in this venom (Figure 3.7 B). Gel filtration peak, P1 showed the presence of maximum number of proteins of the crude venom with prominent bands at 100, 70, 55, >25 and >15 kDa. Peak P2 revealed the presence of protein band at 70, 40, >25 and >15 kDa. The protein band at 40 kDa was relatively prominent compared to the rest of the three peaks in this peak. On the other hand, gel filtration peak P3 displayed faint protein bands at 70, 40 and >25 kDa with a bright band at >15 kDa. Peak P4 displayed faint bands at 70 ad 40 kDa with a prominent band at >25 kDa while P5 displayed very faint bands at 70 and 40 kDa. On the other hand, peak P6 displayed a very bright protein band below 15 kDa with faint bands at 70 and 40 kDa. The last two gel filtration peaks, P7 and P8 showed faint bands at 70 and >15 kDa. It is to be noted that, the SDS-PAGE profile of

P6 which displayed the most prominent protein band (>15 kDa) correlated well with its elution profile which showed the sharpest protein peak at both 215 and 280 nm. This suggests the presence of a major protein peak in this gel filtration fraction. The presence of a lesser number of proteins in P5 and P7 is evident from their elution profile as well as protein content which is only 2.2 and 2.5% of the total protein of the crude venom respectively (Figure 3.4 and Table 3.1).

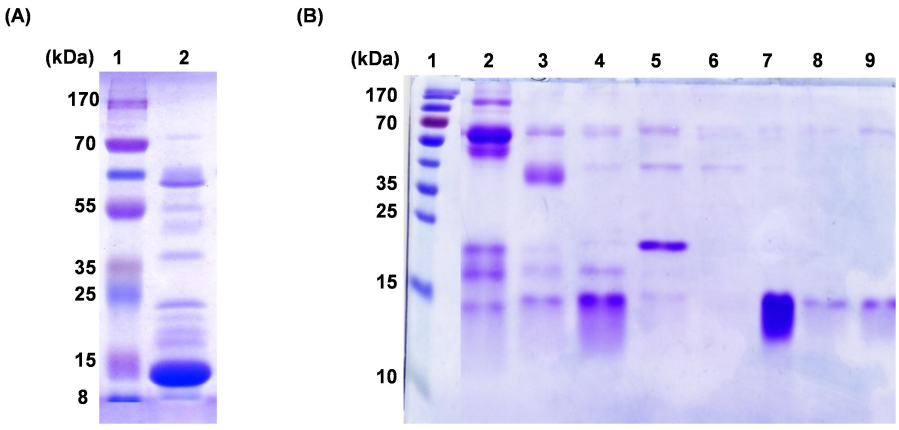


Figure 3.5: Electrophoretic profile of crude *Daboia russelii* venom and its gel filtration fractions (P1-P8) on 12.5% glycine SDS-PAGE under reduced conditions. (A): Crude venom profile, **Lane 1:** PageRuler^{TM} pre-stained protein marker (170-10 kDa). **Lane 2:** 15 µg of crude venom treated with βME. **(B):** 7 µg of each of the gel filtration fractions of the crude venom, **Lane 1:** PageRuler^{TM} pre-stained protein marker (170-10 kDa), **Lane 2:** P1, **Lane 3:** P2, **Lane 4:** P3, **Lane 5:** P4, **Lane 6:** P5, **Lane 7:** P6, **Lane 8:** P7, **Lane 9:** P8.

3.4.4 Tandem mass spectrometry

The MS/MS spectra for each of the gel filtration peaks of the crude venom of *Daboia russelii* revealed the presence of various isoforms of the proteins belonging to various snake venom proteins families. The peptide fragments obtained were analysed using homology search based on sequence similarity and m/z ratio with the existing viperidae NCBI database (http://www.ncbi.nlm.nih.gov/protein/?term=viperidae). Gel filtration peak P1 revealed the presence of peptide fragments having sequence similarity to 25 different snake venom proteins in the database. It consisted of 8 isoforms of SvMP, 4 isoforms of LAAO, 3 isoforms each of SvSP and Snaclec, 2 isoforms of each of PDE and 5'-NUC, and 1 isoform each of disintegrin and PLA_2

enzyme. Apart from these well characterized protein families, sequences homologous to a hypothetical protein were also identified in this peak. Gel filtration peak P2 on the other hand, showed homology to 22 proteins of the snake venom with 8 isoforms of snaclecs, 6 similar to SvSP, 4 homologous to LAAO, 3 similar to SvMP and 1 homologous to PLA_2. P3 consisted of 5 PLA_2 enzymes, 3 snaclec, 2 VEGF and 1 isoform each of SvMP, SvSP, LAAO and CRISP altogether forming 14 proteins. Gel filtration peak P4 revealed the presence of 21 proteins having similarity to 7 PLA_2 enzymes, 6 CRISP, 3 snaclec, 2 SvMP, 1 each of LAAO, VEGF and VNGF. P5 displayed 3 PLA_2 enzymes, 2 SvSP, 1 isoform each of SvMP and CRISP altogether constituting 7 proteins. Gel filtration peak P6 consisted of 7 proteins with 3 isoforms of PLA_2 enzymes, 2 KSPI and 1 each of SvSP and 5'-NUC. On the other hand, P7 consisted of only 1 PLA_2 isoform while P8 had 6 proteins with 4 PLA_2 enzymes, and 1 each of KSPI and disintegrins. A detailed summary of the various peptide fragments identified in each gel filtration peak which are categorized into the well-known snake venom protein families has been shown in Table 3.2. Sequence alignment of the various peptide fragments (obtained from tandem mass spectrometry) having homology with the snake venom protein families have been shown in Appendix-I. Table 3.3 summarizes the various isoforms of each protein family identified in this proteome while Figure 3.6 provides an overview of the various proteins families identified in each gel filtration peaks of the crude *Daboia russelii* venom.

Thorough analysis of the MS/MS spectra unveiled the presence of 63 different proteins belonging to 12 snake venom protein families viz., PLA_2 enzymes, SvSP and SvMP, LAAO, CRISP, snaclec, KSPI, disintegrin, 5'-NUC, PDE, VEGF and VNGF along with a hypothetical protein. The relative percentage of each protein family in the venom proteome was calculated considering the total number of proteins identified in the proteome as 100%. The summary of the relative abundance of each protein family in this venom proteome has been shown in Figure 3.7.

Table 3.2: List of peptide fragments belonging to various snake venom protein families obtained by MS/MS in each of the gel filtration peaks of crude *Daboia russelii* venom.

MH+ stands for mass/charge (m/z) of the protonated molecular ions (peptide), **Z** stands for the number of charges a peptide carries after ionization, **Score** implies the sum of all peptide cross correlation (Xcorr) values. **c*** stands for carbamidomethylation of cysteine residue; **m*** stands for oxidation of methionine residue.

Sl. No.	MS/MS derived Sequence	No. of peptides	Peptide ion m/z	MH+ [Da]	z	Coverage & Sequest Score	Protein/Acc. No./ Protein family	Homology with protein from
Gel filtration Peak 1								
1.	ARNEcDVPEHcTGQSAEcPR	2	792.19	2373.57	3	37.00 & 103.27	Factor X activator heavy chain (300079900) (SvMPs)	*Daboia russelii russellii*
	ASDLMTRKSHDNALLFTDMR	1	774.82	2321.47	3			
	cILYPPLR	1	1032.53	1031.53	1			
	KSHDNALLFTDMR	1	517.07	1548.22	3			
	LKPGAEcGNGLccYQcK	1	1008.63	2015.26	2			
	NEcDVPEHcTGQSAEcPR	1	1074.22	2146.45	2			
	NPCNmHYScmDQHKGMVDPGTKcEDGK	1	1057.98	3170.93	3			
	SVGIVQVQGNR	1	579.67	1157.35	2			
2.	ARDEcDVPEHcTGQSAEcPR	1	792.19	2373.57	3	49.41 & 97.67	Chain A, Crystal Structure of Russell's Viper Venom Metalloproteinase (162329887) (SvMPs)	*Daboia siamensis*
	DEcDVPEHcTGQSAEcPR	1	1074.58	2147.16	2			
	GYcYNGDcPIMR	1	1506.45	1505.45	1			
	KSHDNALLFTDMR	1	517.07	1548.22	3			
	LKPGAEcGNGLccYQcK	1	1008.63	2015.26	2			
	LVSTSAQFNK	1	548.58	1095.16	2			
	NPCNmHYScmDQHKGMVDPGTKcEDGK	1	1057.98	3170.93	3			
3.	KGSHLVSLHSR	1	611.46	1220.91	2	59.35 & 22.04	Coagulation factor X activating enzyme light chain, RVV-X-light chain (251205) (SvMPs)	*Daboia russelii*
	SMTcNFIAPVVcK	1	764.63	1527.27	2			
	VLDcPSGWLSYEQHcYK	1	1072.16	2142.32	2			

4.	DcQNPCCDAATCKLTPGAEcGNGLCCEKCK ENGRKIPcAPQDIK GSYYGYcR KNclcNDSScIMSAVLSSQPSK LFSNcSNHDYRR QcISLFGSRATVAEDSCFQENQK TAVIMAHELGHNLGMYHDR	2 1 1 1 1 1 1	1064.96 813.92 513.99 1244.35 1570.43 874.01 722.97	3191.89 1625.85 1025.98 2486.70 1569.43 2619.04 2165.90	3 2 2 2 1 3 3	25.98 & 15.15	Coagulation factor X-activating enzyme heavy chain (73621852) (SvMPs)	*Macrovipera lebetina*
5.	FcMEHPNNGHLVSVESmEEAEFVAK TWEAAER FcmEHPNNGHLVSVESmEEAEFVAK MWFNR YLFVcKVPPEc	1 1 1 1 1	969.87 863.39 975.75 754.41 706.68	2906.62 862.39 2924.27 753.41 1411.37	3 1 3 1 2	33.54 & 10.99	Factor X activator light chain 2 (300490458) (SvMPs)	*Daboia siamensis*
6.	IKDKEQEcSSEWSDGSSVSYDNLGK cEEPYPFVcK ESGYRMWFNHK mWFNHKCEEPYPFVcKVPPEC	1 1 1 1	950.67 665.26 728.11 886.62	2849.01 1328.53 1454.22 2656.86	3 2 2 3	41.77 & 9.12	Factor X activator light chain 2 (300079896) (SvMPs)	*Daboia russelii russelli*
7.	KcFGLEK GTGYRSWFNLNCEEPYPFVCKVPPNC YFcYR	1 1 1	882.36 1007.77 809.34	881.36 3020.32 808.34	1 3 1	24.05 & 5.64	Coagulation factor X-activating enzyme light chain 2 (73621141) (SvMPs)	*Macrovipera lebetina*
8.	DEcDVTEHcTGQSAEcPR IPcAPQDVK NQcISLFGSR VFSSCSYDDYRMYLAK	1 1 1 2	1076.02 1028.36 1182.43 975.07	2150.04 1027.36 1181.43 1948.14	2 1 1 2	22.83 & 9.68	Group III snake venom metalloproteinase (83523646) (SvMPs)	*Echis ocellatus*
9.	DLQTFcYPSIIQK EGWYANLGPMR HIVIVGAGMSGLSAAYVLAGAGHK IFFAGEYTANAHGWIDSTIK KDLQTFcYPSIIQK LNEFVQETENGWYFIK SAGQLYQESLGK VTVTYQTTQK	1 1 1 1 2 1 1 1	807.62 648.09 1140.60 1121.94 871.42 1009.67 1281.45 585.54	1613.24 1294.18 2279.20 2241.89 1740.85 2017.33 1280.45 1169.08	2 2 2 2 2 2 1 2	54.56 & 98.63	L-amino-acid oxidase (3954067960) (LAAO)	*Daboia russelii russelli*
10.	KFWEDDGIQGGK RFDEIVGGMDQLPTSMYR VYFAGEYTANAHGWIDSTIK	1 1 1	691.10 1058.70 748.54	1380.19 2115.41 2242.63	2 2 3	32.74 & 43.72	L-amino-acid oxidase (347602327) (LAAO)	*Echis ocellatus*

11.	EYLIK HDDIFAYEK SAGQLYEESLR	1 1 1	666.37 1138.40 627.03	665.37 1137.40 1252.06	1 1 2	29.31 & 28.94	L-amino acid oxidase (538259837) (LAAO)	*Protobothrops flavoviridis*
12.	HDDIFAYEK LNEFSQENDNAWYFIK SAGQLYEESLGK RFDEIVDGmDK	1 1 1 1	1138.40 673.78 641.86 671.09	1137.40 2018.35 1281.72 1340.19	1 3 2 2	22.49 & 27.10	Chain H, of L-amino acid oxidase (10120762) (LAAO)	*Calloselasma rhodostoma*
13.	cTERQAcCQDYEDTcVLPTQSWScSK DFYTFDSEGIVR FGPVSGEIIMALQMADR GKNEVTSFENIEVYNLMcDLLK NPFYNPSPAK	1 1 1 1 1	1072.02 725.31 918.72 873.30 568.78	3213.07 1448.62 1835.44 2616.90 1135.55	3 2 2 3 2	38.66 & 66.52	Phosphodiesterase (586829527) (PDE)	*Macrovipera lebetina*
14.	NLHNcVNLILLADHGMEEIScDR TFLPIFVNPVN VNLMVDQQWMAVR VRDVELLTGLNFYSGLK	2 1 1 1	908.92 1261.53 795.90 642.64	2723.77 1260.53 1589.80 1924.92	3 1 2 3	33.73 & 35.20	Phosphodiesterase (538259853) (PDE)	*Protobothrops flavoviridis*
15.	STHIAPLSLPSSPPSVGSVcR WDKDIMLIK DIMLIK	1 1 1	1126.31 1162.40 733.41	2250.62 1161.40 732.41	2 1 1	18.22 & 22.01	Serine protease VLSP-3 (380875417) (SvSPs)	*Macrovipera lebetina*
16.	FFcLSNK TSTYIAPLSLPSSPPR WDKDIMLIK	1 1 1	916.36 844.95 1162.40	915.36 1687.90 1161.40	1 2 1	20.70 & 26.99	Serine beta-fibrinogenase-like protein precursor (311223824) (SvSPs)	*Daboia siamensis*
17.	TLcAGILQGGIDTcK DIMLIK	1 1	1608.43 733.41	1607.43 732.41	1 1	10.12 & 22.05	Beta-fibrinogenase (380875421) (SvSPs)	*Macrovipera lebetina*
18.	AWNEGTNcFVFK GSHLLSLHNIAEADFVLK NHWSHMDcSSTHNFVcK QDcLSDWSFYEGYcYK	1 1 1 1	737.54 655.99 716.78 1061.43	1473.09 1964.99 2147.35 2120.85	2 3 3 2	57.43 & 22.60	Snaclec 5 (73620113) (Snaclec)	*Daboia siamensis*
19.	TWFNLScGDDYPFVcK NcFGLEK AFDEPKR NVIER SGDAEK	1 1 1 1 1	670.70 868.35 863.49 631.40 607.20	2009.11 867.35 862.49 630.40 606.20	3 1 1 1 1	26.28 & 12.33	Snaclec A14 (218526485) (Snaclec)	*Macrovipera lebetina*

20.	WSDGVNLDYK QDcLSDWSFYEGYcYK VFNEK	1 1 1	599.444 1061.43 637.35	1196.89 2120.85 636.35	2 2 1	20.67 & 11.74	Dabocetin beta subunit (300490464) (Snaclec)	*Daboia russelii russellii*
21.	LRQGAQCAEGLCCDQcRFmK MHMEAGEEcDCGSPGNPCCDAATCKLR MHMEAGEECDCGSPGNPcCDAATCK	1 1 1	778.84 962.66 872.90	2333.52 2884.98 2615.71	3 3 3	50.56 & 4.81	Adinbitor (50365991) (Disintegrin)	*Gloydius brevicaudus*
22.	cTGQDcYGGVAR YLGYLNVIFDDK AEVNK EVVKFMNSLR	1 1 1 1	673.05 730.89 561.21 612.72	1344.11 1459.77 560.21 1223.43	2 2 1 2	7.41 & 6.54	Ecto-5' nucleotidase (338855300) (5'-NUC)	*Crotalus adamanteus*
23.	FHEcNLGNLIcDAVIYNNLR DIPEDQVVK AQVNK	1 1 1	812.79 522.43 561.29	2435.38 1042.86 560.29	3 2 1	8.33 & 3.31	5'-nucleotidase, partial (586829529) (5'-NUC)	*Macrovipera lebetina*
24.	AcSGENGEQATSQNNSGDNER QLPCmHEFHFHCIDRWLSENSTcPICR RHPSITLDLQVRR	1 1 1	742.74 1126.14 796.09	2225.21 3375.43 1590.18	3 3 2	9.62 & 2.35	Hypothetical protein LOC100554767 (387016758)	*Crotalus adamanteus*
25.	YmLYSLLDcGEESEQc CCFVHDCCYGRVNGCDPK VAAIcFGENmNTYDK KTGIFGImSYIYYGcYCGWGGK	1 1 1 1	1007.56 674.08 1005.95 847.50	2013.12 2019.23 1748.94 2539.50	2 3 3 3	71.01 & 2.13	AmmodytinI1(C) variant (50874332) (PLA_2 enzyme)	*Vipera berus berus*
Gel Filtration Peak 2								
26.	DLQTFcYPSIIQK FDEIVGGMDQLPTSMYR HIVIVGAGMSGLSAAYVLAGAGHK IFFAGEYTANAHGWIDSTIK KFWEDDGIQGGK LNEFVQETENGWYFIK NPLEEcFREDDYEEFLEIAK SAGQLYQESLGK VTVLEASERPGGR VTVTYQTTQK YPVKPSEAGK	1 1 1 1 1 1 1 1 1 1 1	807.58 980.70 760.87 748.27 691.16 1009.81 849.86 641.69 686.47 585.06 539.15	1613.17 1959.39 2279.62 2241.82 1380.31 2017.61 2546.59 1281.38 1370.94 1168.12 1076.29	2 2 3 3 2 2 3 2 2 2 2	56.94 & 78.87	L-amino-acid oxidase (395406796) (LAAO)	*Daboia russelii russellii*
27.	FDEIVGGMDKLPTSMYR HDDIFAYEK SAGQLYEESLGK	2 1 1	654.31 570.14 642.22	1959.93 1138.27 1282.44	3 2 2	20.24 & 41.31	L-amino-acid oxidase (75570145) (LAAO)	*Gloydius blomhoffii*

28.	EGWYANLGPMR FDEIVGGMDQLPTSMYR VTVLEASER	1 2 1	647.94 980.70 503.10	1293.87 1959.39 1004.20	2 2 2	19.21 & 34.56	L-amino-acid oxidase (347602330) (LAAO)	*Vipera ammodytes ammodytes*
29.	EGWYANLGPMR HDDIFAYEK SAGQLYEESLR	1 1 1	647.94 570.14 627.73	1293.87 1138.27 1253.47	2 2 2	21.39 & 30.57	L-amino acid oxidase (538259837) (LAAO)	*Protobothrops flavoviridis*
30.	FDFFWIGLR QDcLSDWSFYEGYcYK SSEEMDFVIR TTDNQWLR TWEDAEK WSDGVNLDYK	1 1 1 1 1 1	601.58 1061.73 607.18 518.03 880.36 599.40	1201.16 2121.47 1212.37 1034.07 879.36 1196.81	2 2 2 2 1 2	54.00 & 49.10	Dabocetin beta subunit (300490464) (Snaclec)	*Daboia russelii russellii*
31.	GFDcPFGWSSYEGYcYK AWSGKSYcLVSK mNWEDAESFcR TTNNEWLSMDCSR	1 1 1 1	708.79 693.46 731.62 779.25	2123.38 1384.91 1461.24 1556.50	3 2 2 2	59.20 & 2.48	Alboaggregin-A subunit beta (3023231) (Snaclec)	*Trimeresurus albolabris*
32.	NPFIcK TWEDAEK YHAWIGLR	1 1 1	779.33 880.36 508.94	778.33 879.36 1015.88	1 1 2	27.92 & 24.74	Dabocetin subunit alpha (123899657) (Snaclec)	*Daboia siamensis*
33.	FGSVWIGLNDPWHNcNWEWSDNAR GSHLASIHSSEEEAFVSK IFWFNRGcEKFVSFVcK RPYcTVMVLKPDR	1 1 1 1	987.90 958.72 742.46 817.95	2960.70 1915.44 2224.39 1633.91	3 2 3 2	48.00 & 19.28	P31 beta subunit (300490484) (Snaclec)	*Daboia siamensis*
34.	AWNEGTNcFVFK GSHLLSLHNIAEADFVLK QDcLSDWSFYEGYcYK TWEDAEK	1 1 1 1	737.53 655.93 1061.73 880.36	1473.07 1964.79 2121.47 879.36	2 3 2 1	56.08 & 16.42	Snaclec 5 (73620113) (Snaclec)	*Daboia siamensis*
35.	DGIYVWIGLR FcTEQANSGHLVSIK WDYVNcAEHYR	1 1 1	596.97 846.62 757.51	1191.94 1691.25 1513.03	2 2 2	34.18 & 15.14	P31 alpha subunit (300490478) (Snaclec)	*Daboia russelii limitis*
36.	TWFNLScGDDYPFVcK AFDEPK TWFNLScGDDYPFVcKFPPRc FPPR FIKNCFGLEKESDYR	1 1 1 1 2	1005.84 707.36 890.02 516.45 889.45	2009.68 706.36 2667.05 515.45 2665.34	2 1 3 1 3	17.09 & 8.76	P68 alpha subunit (300490470) (Snaclec)	*Daboia siamensis*

37.	KGSHLVSLHSR VFTEEmNWADAEKFCTEQKK MEWSDR	1 1 1	611.72 817.79 824.36	1221.45 2450.36 823.36	2 3 1	24.66 & 8.59	Snaclec 4 (73620112) (Snaclec)	*Daboia siamensis*
38.	DIMLIK FFcLSNK IMGWGAITSPNETFPGVTHcANINILPYSVcR TSTYIAPLSLPSSPPR	1 1 1 1	733.42 916.41 1193.23 563.60	732.42 915.41 3576.69 1687.80	1 1 3 3	32.03 15.47	Serine beta-fibrinogenase-like protein precursor (311223824) (SvSPs)	*Daboia siamensis*
39.	DIMLIR NMEIYLGVHSK NMEIYLGVHSKK	1 1 1	761.49 646.35 710.12	760.49 1290.71 1418.24	1 2 2	23.61 & 11.51	Thrombin-like enzyme elegaxobin-1 (90116798) (SvSPs)	*Protobothrops elegans*
40.	TSTHIAPLSLPSSPPSVGSVcR DIMLIK SSELVIGGDECNINEHR	1 1 1	751.25 733.42 937.24	2250.75 732.42 1872.48	3 1 2	17.44 & 8.67	Serine protease VLSP-3 (380875417) (SvSPs)	*Macrovipera lebetina*
41.	SIIAGNTAATcPP FGAHSQKVLNEDEQIRNPK NNEVLDKDImLIK	1 1 1	637.65 737.10 521.01	1273.29 2210.10 1560.04	2 3 3	17.44 & 7.51	Snake venom serine protease 1 (13959617) (SvSPs)	*Trimeresurus gramineus*
42.	FHcSGTLLNEEWVLTAAHCDmENmQIYLGVHDK DIMLIR KAYGGLPEK TLCAGVLQGGIDTCLADSGGPLIcNGQFQGIVAWGR	1 1 1 1	1298.61 761.49 963.46 1216.93	3892.83 760.49 962.46 3647.79	3 1 1 3	32.31 & 7.35	Serine protease VLSP-1 (381141431) (SvSPs)	*Macrovipera lebetina*
43.	KDDENDKDIMLIR SSELVIGGDECNINEHR	1 1	536.08 937.24	1605.23 1872.48	3 2	11.63 & 4.69	Hypothetical like protein (406609998) (SvSPs)	*Gloydius blomhoffii*
44.	VLDcPSGWLSYEQHcYK KGSHLVSLHSR MEWSDR	1 1 1	1072.35 611.72 824.36	2142.70 1221.45 823.36	2 2 1	27.64 & 9.44	RVV-X-light chain (251205) (SvMPs)	*Daboia russelii*
45.	DQLQQNGQPcQNNR DScFQENLK NEcDVPEHcTGQSAEcPR NQcISLFGSR	1 1 1 1	851.18 571.74 1074.16 592.07	1700.36 1141.47 2146.32 1182.14	2 2 2 2	24.39 & 45.24	Factor X activator heavy chain (300079900) (SvMPs)	*Daboia russelii russellii*
46.	cEEPYPFVcK EEFRKcFVLQK FcMEHPNNGHLVSIESMEEAEFVAK VPPEc	1 1 1 1	665.33 742.59 969.75 602.28	1328.65 1483.18 2906.26 601.28	2 2 3 1	32.28 & 13.27	Factor X activator light chain 2 (300079896) (SvMPs)	*Daboia russelii russellii*

47.	EAVHSYAIYGcYcGWGGQGR AVCECDRAAAICLGENVNTYDK CCFAQDCcYGR	1 1 1	764.95 787.11 664.10	2291.86 2358.34 1326.20	3 3 2	43.44 & 7.36	Phospholipase A_2 acidic subunit (1408314) (PLA_2 enzyme)	*Vipera aspis*
Gel Filtration Peak 3								
48.	AAAIcLGENVNTYDK CcFAQDCcYGR EAVHSYAIYGcYcGWGGQGR NLFQFGDmILQK NYEYYSISHcTEESEQc	1 2 1 1 1	820.68 691.917 764.781 735.999 1100.68	1639.36 1381.83 2291.34 1469.10 2199.37	2 2 3 2 2	80.33 & 31.58	Phospholipase A_2 acidic subunit (1408314) (PLA_2 enzyme)	*Vipera aspis*
49.	AAAIcLGQNVNTYDK EVVHSYAIYGcYcGWGGQGR NYEYYSISHcTEESEQc	1 1 1	547.12 774.15 1100.68	1638.36 2319.46 2199.37	3 3 2	69.57 & 22.98	Acidic phospholipase A_2RV-7 (400714) (PLA_2 enzyme)	*Daboia siamensis*
50.	ccFVHDccYGKLWSCSPK GTWCEKQIcEcDKAAAIcFR NGAIVcARGTWCEKQIcECDK	1 1 2	770.266 816.331 1221.11	2307.80 2445.10 2440.23	3 3 2	33.33 & 11.15	Basic phospholipase A_2 Tpu-G6D49 (123907686) (PLA_2 enzyme)	*Trimeresurus puniceus*
51.	ccFVHDCCYGNLPDcNPK LAIPSYSSYGCYcGWGGKGTPK VNGAIVcEKGTScENR YmLYPDFLCKGELRC	1 1 1 1	734.72 784.90 898.40 934.10	2201.15 2351.71 1794.81 1866.20	3 3 2 2	68.61 & 5.97	Basic phospholipase A_2DsM-S1 (408407675) (PLA_2 enzyme)	*Daboia siamensis*
52.	CCLVHDCcYTRVGDCSPK EAAICLGENVNTYDK VGDCSPKmTLYSYRFENGDIICDNK	1 1 1	687.16 547.70 962.35	2058.47 1640.11 2884.05	3 3 3	46.67 & 4.99	Chain A, Acidic Phospholipase A_2 (40889259) (PLA_2 enzyme)	*Echis carinatus*
53.	FDFFWIGLR QDcLSDWSFYEGYcYK SSEEMDFVIR TTDNQWLR WSDGVNLDYK	1 1 1 1 1	601.52 1061.63 607.56 517.85 599.75	1201.04 2121.26 1213.12 1033.70 1197.50	2 2 2 2 2	51.33 & 26.92	Dabocetin beta subunit (300490464) (Snaclec)	*Daboia russelii russellii*
54.	NPFIcK YHAWIGLR YHEWITLPcGDKNPFIcK	1 1 1	779.36 508.96 760.33	778.36 1015.91 2277.10	1 2 3	38.31 & 18.80	Dabocetin subunit alpha (123899657) (Snaclec)	*Daboia siamensis*
55.	FDYK FGSVWIGLNDPWHNcNWEWSDNAR GSHLASIHSSEEEAFVSK	1 1 1	573.402 987.923 958.684	572.40247 2960.76999 1915.36894	1 3 2	37.33 & 15.35	P31 beta subunit (300490484) (Snaclec)	*Daboia siamensis*

	IFWFNRGcEK	1	679.656	1357.31120	2			
56.	EDDYEEFLEIAKNGLK EGWYANLGPmRVPEK LNEFVQETENGWYFIK SAGQLYQESLGK	1 1 1 1	638.52 588.35 1009.83 641.43	1912.56 1762.05 2017.67 1280.87	3 3 2 2	26.19 24.15	L-amino-acid oxidase (395406796) (LAAO)	*Daboia russelii russellii*
57.	DEcDVPEHcTGQSAEcPR DQLQQNGKPcQNNR NPcNMHYScMDQHK YKPKCIFNPPLR	2 1 1 1	716.74 850.92 608.54 738.67	2147.22 1699.84 1822.63 1475.35	3 2 3 2	20.84 & 6.97	Chain A,Russell's Viper Venom Metalloproteinase (162329887) (SvMPs)	*Daboia siamensis*
58.	TLcAGILEGGKDSCR TLCAGILEGGK	1 1	791.37 531.40	1580.73 1060.80	2 2	6.44 & 4.06	Beta-fibrinogenase brevinase (82117246) (SvSPs)	*Gloydius blomhoffii*
59.	SLLQQDSCQDAGmQSKcSASCFCQNK SVDFDSESPR VLEGIKcGENIYMSPNPMK YTNcKSLLQQDSCQDAGMQSKcSASCFCQNK	1 1 1 1	961.837 570.742 1090.99 1178.63	2882.51 1139.48 2179.97 3532.88	3 2 2 3	41.82 & 5.05	Da-CRPa (190195337) (CRISP)	*Deinagkistrodon acutus*
60.	cSGccTDESMK FMEHTAcEcRPR HTADIQIMR MEVMKFmEHTACEcRPR WKQGEPEGPK	1 2 1 1 1	668.472 798.092 543.665 724.447 579.19	1334.94 1594.18 1085.33 2170.34 1156.38	2 2 2 3 2	56.94 & 32.54	Snake venom vascular endothelial growth factor toxin (327478537) (VEGF)	*Daboia russelii russellii*
61.	QQGEVISFLTVYER QENHcEPCSERR CSCKFTDSRcK	1 1 1	835.685 773.384 1335.28	1669.37 1544.77 1334.28	2 2 1	19.27 & 6.28	Vascular endothelial growth factor A (327488518) VEGF	*Vipera ammodytes ammodytes*
Gel Filtration Peak 4								
62.	cGENIYMSPYPMK cILNHSPYNSR RPEIQNEIVDLHNSLR RSVTPTASNMLK WTAIIHEWHK	2 1 1 1 1	796.09 1361.59 967.78 653.69 661.46	1590.18 1360.59 1933.56 1305.38 1320.92	2 1 2 2 2	53.56 & 69.14	Dr-CRPK (190195321) (CRISP)	*Daboia russelii*
63.	KPEIQNEIVDLHNSLR SLVQQAGcEDKQIQSDcSAICFCQNKII SVDFDSESPRKPEIQNEIVDLHNSLR	1 1 1	953.80 1062.84 1009.40	1905.59 3185.53 3025.20	2 3 3	61.09 & 28.64	Ch-CRPKa (190195307) (CRISP)	*Crotalus horridus*
64.	KPEIQNKIVDLHNFLR mEWYPEAAANAER SGPPCGDCPSACDNGLCTNPCTK	1 2 1	655.69 777.78 747.93	1964.08 1553.55 2240.80	3 2 3	54.30 & 24.21	Cv-CRP (190195319) (CRISP)	*Crotalus viridis*

65.	DFKYGVGAVPSNAATGHYTQIVWYK mEWYPEAAANAER SYRGGcAAAYCPSSK GGcAAAYcPSSK	1 2 1 1	925.08 777.78 790.05 615.52	2772.24 1553.55 1578.10 1229.03	3 2 2 2	28.96 & 12.38	Prepro-cysteine-rich venom protein (1778013) (CRISP)	*Protobothrops mucrosquamatus*
66.	Cildhspynsr mEWYPEAAANAER SVDFDSESPR	1 2 1	1363.45 777.78 570.71	1362.45 1553.55 1139.43	1 2 2	46.15 & 21.81	Dr-CRPB (190195323) (CRISP)	*Daboia russelii*
67.	IVDLHNSLR mEWYPEAAANAER YTNcNSLVQKSGCQDTWmQSNcPAICFCQNK	1 2 1	534.61 777.78 1216.06	1067.23 1553.55 3645.19	2 2 3	22.08 & 11.43	Pg-CRP (190195329) (CRISP)	*Cerrophidiong odmani*
68.	AAAIcLGENVNTYDK EAVHSYAIYGcYcGWGGQGR NLFQFGDmILQK NYEYYSISHcTEESEQc	1 1 1 1	820.60 764.78 735.66 1100.56	1639.21 2291.33 1469.31 2199.13	2 3 2 2	86.07 & 23.82	Phospholipase A_2 acidic subunit (1408314) PLA_2 enzyme	*Vipera aspis*
69.	AAAIcLGQNVNTYDK NLFQFGEMILQK NYEYYSISHcTEESEQc	1 1 1	820.14 735.35 1100.56	1638.29 1468.71 2199.13	2 2 2	69.67 & 18.33	Chain G, of Rv4RV7 complex (37927199) (PLA_2 enzyme)	*Daboia siamensis*
70.	VNGAIVcEQGTScENR ccFVHDCCYGNLPDcNPK LAVPFYSSYGcYcGWGGK RVNGAIVcEQGTScENR	1 1 1 1	898.08 734.95 691.81 651.18	1794.16 2201.86 2072.43 1950.54	2 3 3 3	58.68 & 14.85	Basic phospholipase A_2 (298351762) (PLA_2 enzyme)	*Daboia russelii russellii*
71.	NLFQFAEMIVK GKPQDATDRccFVHDccYEKVK	1 1	670.94 925.11	1339.88 2772.33	2 3	27.27 & 6.27	Basic phospholipase A_2 RVV-VD (3914259) PLA_2 enzyme	*Daboia russelii russellii*
72.	SPIFSYGDYGcYcGWGGK VAAIcFGENVNTYDKK CcFVHDcCYGR	1 1 1	692.49 915.12 710.63	2074.47 1828.24 1419.27	3 2 2	37.50 & 2.39	Acidic phospholipase A_2 (129506) (PLA_2 enzyme)	*Cerastes cerastes*
73.	ccFVHDccYGKATGCDPKK	1	769.94	2306.81	3	13.67 & 2.39	Acidic phospholipase A_2 Tgc-E6 (403399517) (PLA_2 enzyme)	*Trimeresurus gracilis*
74.	ccFVHDccYGKLAK DATDRCcFVHDCCYGK GTWCEEQICEcDRVAAECLR	1 1 1	910.15 631.73 791.42	1818.30 1892.18 2371.26	2 3 3	57.38 & 2.01	Basic PLA_2 (223635543) (PLA_2 enzyme)	*Crotalus durissus ruruima*

75.	YHEWITLPcGDKNPFIcK cFGLNK TWEDAEK	1 1 1	760.66 739.34 879.34	2278.98 738.34 878.34	3 1 1	37.01 & 17.16	Dabocetin subunit alpha (123899657) (Snaclec)	*Daboia siamensis*
76.	TWEDAEWFCTK cFGLK	1 1	708.56 625.37	1415.13 624.37	2 1	10.13 & 6.14	Crotocetin-1 (82129809) (Snaclec)	*Crotalus durissus terrificus*
77.	TTDNQWLR KTWEDAEK VFNEK	1 1 1	517.88 504.39 637.31	1033.76 1006.79 636.31	2 2 1	48.00 & 11.53	Dabocetin beta subunit (300490464) (Snaclec)	*Daboia russelii russellii*
78.	GNVVTVMVDVNLNNNVYK INTAcVcVISR HWNSYcTTTDTFVR THEALKTSRNTDQHYPAPNK	1 1 1 1	997.28 647.37 597.15 770.36	1992.56 1292.74 1788.45 2308.09	2 2 3 3	41.98 & 10.75	Venom nerve growth factor 2 (335892642) (VNGF)	*Daboia russelii*
79.	SAGQLYQESLGK LNEFVQETENGWYFIK HIVIVGAGMSGLSAAYVLAGAGHK EGWYANLGPMR VTVLEASERPGGR	1 1 1 1 1	641.50 1009.73 760.66 648.10 686.64	1280.10 2017.47 2278.98 1294.21 1371.29	2 2 3 2 2	23.41 & 9.39	L-amino-acid oxidase (395406796) (LAAO)	*Daboia russelii russellii*
80.	cSGccTDESMK cTPVGKHTADIQIMRMNPR HTADIQIMR QGEPEGPKEPR	2 2 1 1	668.50 1113.64 543.66 613.25	1335.00 2225.29 1085.32 1224.51	2 2 2 2	43.75 & 8.07	Snake venom vascular endothelial growth factor toxin (327478537) (VEGF)	*Daboia russelii russellii*
81.	ATVAEDAcFQFNRLGSDYGYcRK DECDmADLCNGQSDEcPK DENKGmVEPGTKcENGK NGHPcQNNNGYcYNGK	2 2 1 1	910.39 682.86 955.70 949.62	2728.17 2045.58 1909.40 1897.25	3 3 2 2	36.27 & 7.34	Metalloproteinase (297593790) (SvMPs)	*Echis carinatus sochureki*
82.	ARNECDVPEHCTGQSAECPR cEDGKVcNNK DQLQQNGQPcQNNR GMVDPGTK LFSNcSIHDYQRYLTR	1 1 1 2 1	735.19 612.52 851.12 805.37 691.81	2202.56 1223.05 1700.23 804.37 2072.43	3 2 2 1 3	22.94 & 5.53	Factor X activator heavy chain (300079900) (SvMPs)	*Daboia russelii russelii*

Gel Filtration Peak 5								
83.	LAIPSYSSYGcYcGWGGK MILEETGK VNGAIVcEKGTScENR YMLYPDFLcK	1 1 1 2	676.48 921.38 898.045 675.90	2026.43 920.38 1794.09 1349.80	3 1 2 2	76.86 & 45.27	Basic phospholipase A_2VRV-PL-VIIIa (24638087) (PLA_2 enzyme)	*Daboia russelii russellii*
84.	LAVPFYSSYGcYcGWGGK RVNGAIVcEQGTScENR SLLEFGMMILEETGK	1 1 1	1037.08 976.28 850.14	2072.16 1950.57 1698.29	2 2 2	66.94 & 36.61	PLA_2 enzyme Basic phospholipase $A_2$3 (298351762)	*Daboia russelii russellii*
85.	AAAIcLGQNVNTYDK TATYSYSFENGDIVcGDNDLcLR EVVHSYAIYGcYcGWGGQGRAQDATDR	1 1 1	820.14 1336.56 1026.42	1638.27 2671.12 3076.25	2 2 3	67.39 & 5.24	(Viperotoxin non-toxic acidic component) RV-7 (400714) (PLA_2 enzyme)	*Daboia siamensis*
86.	IPcAPEDVK IQNDADSSASISACNGLKGHFK LYcFDNLPEHK MPQcILIKPSR	1 1 1 1	514.81 755.25 718.95 672.77	1027.63 2262.76 1435.91 1343.55	2 3 2 2	16.29 & 10.04	Zinc- metalloproteinase-disintegrin-like Eoc1 (123896981) (SvMP)	*Echis ocellatus*
87.	EKFFcLSSK FFcLSSK FYcAGTLINQEWVLTAAR NVPNEDQQIRVPK TLCAGILQGGIDSCK	1 1 1 1 1	1146.44 889.316 1057.87 769.85 739.94	1145.44 888.31 2113.75 1537.70 1477.87	1 1 2 2 2	21.15 & 7.21	Venom serine proteinase-like protein 2 (13959655) (SvSP)	*Macrovipera lebetina*
88.	WDKDIMLIK TLcAGVLEGGIDScK VPAEKVFCVSSKTYTR	1 1 1	582.21 790.62 606.29	1162.43 1579.24 1815.87	2 2 3	16.88 & 6.22	Serine protease (297593764) (SvSP)	*Echis carinatus sochureki*
89.	RPEIQNEIVDLHNSLR cPAScFcHNEII	1 2	967.74 754.89	1933.48 1507.77	2 2	11.72 & 4.86	Dr-CRPK (190195321) (CRISP)	*Daboia russelii*
Gel Filtration Peak 6								
90.	KYmLYPDFLcK LAIPSYSSYGcYcGWGGK QNLNTYSK VNGAIVcEK SLLEFGKMILEETGK VNGAIVcEKGTScENR	3 3 1 1 1 2	748.16 676.18 968.43 991.39 848.59 898.15	1494.32 2025.53 967.43 990.39 1695.18 1794.30	2 3 1 1 2 2	82.64 & 72.67	Basic phospholipase A_2 VRV-PL-VIIIa (24638087) (PLA_2 enzyme)	*Daboia russelii russellii*

91.	IcECDKAAAICFRR IYmLYPDFLcK LAVPFYSSYGcYcGWGGK SLLEFGmMILEETGK VNGAIVcEQGTScENR	1 3 1 1 2	829.30 740.44 1037.08 858.14 898.15	1656.61 1478.89 2072.16 1714.28 1794.30	2 2 2 2 2	81.82 & 45.98	Basic phospholipase A_2 3 (298351762) (PLA_2 enzyme)	*Daboia russelii russellii*
92.	CcFVHDccYGKVNGCNPK EVCECDKAAAIcFRDNK NVAGR	1 1 1	721.11 987.01 516.37	2160.33 1972.02 515.37	3 2 1	32.79 & 6.21	Acidic phospholipase A_2 (3914268) (PLA_2 enzyme)	*Trimeresurus gramineus*
93.	FPNGLDKDIMLIR RPVTYSTHIAPVSLPSR WcEPLYPWVPADSR	1 1 1	767.17 941.69 889.10	1532.34 1881.39 1776.19	2 2 2	47.03 & 26.89	Factor V activator RVV-V alpha (134129) (SvSP)	*Daboia siamensis*
94.	cKEFIYGGcHGNANKFPSR cRQTcGASAKGRPT EFIYGGcHGNANK FcYLPADPGEcLAHMR	1 1 1 1	748.64 775.39 734.58 646.89	2242.91 1548.79 1467.16 1937.69	3 2 2 3	65.56 & 15.16	Trypsin inhibitor-5 precursor (159883540) (KSPI)	*Daboia siamensis*
95.	FcHLPVDSGIcR DQcRHTcGGK	1 1	731.39 609.77	1460.78 1217.54	2 2	26.19 & 2.25	Kunitz protease inhibitor 4 (123913154) (KSPI)	*Daboia russelliirussellii*
96.	HANFPILSANIRPK ASGNPILLNK ETPVLSNPGPYLEFRDEVEELQNHANK	1 1 1	527.25 514.50 1042.91	1578.80 1026.99 3125.74	3 2 3	9.86 & 8.65	5' nucleotidase, partial (538259847) (5'-NUC)	*Protobothrops flavoviridis*
Gel Filtration Peak 7								
97.	IYmLYPDFLcK LAVPFYSSYGcYcGWGGK RVNGAIVcEQGTScENR SLLEFGmMILEETGK	2 1 2 2	740.38 1037.13 651.08 857.89	1478.76 2072.26 1950.26 1713.78	2 2 3 2	76.86 & 50.08	PLA_2 enzyme Basic phospholipase A_2 (298351762) (PLA_2 enzyme)	*Daboia russelii russellii*
Gel Filtration Peak 8								
98.	SLLEFGmmILEETGK LAVPFYSSYGcYcGWGGK IYmLYPDFLcK RVNGAIVcEQGTScENR	3 2 2 2	866.11 1037.36 740.28 976.225	1730.22 2072.72 1478.56 1950.45	2 2 2 2	86.78 & 104.89	Basic phospholipase A_2 (298351762) (PLA_2 enzyme)	*Daboia russelii russellii*
99.	VNGAIVcEKGTScENR LAIPSYSSYGcYcGWGGK KYMLYPDFLcK	2 1 3	1795.25 1014.34 740.28	1794.25 2026.68 1478.56	1 2 2	82.64 & 59.37	Basic phospholipase A_2 VRV-PL-VIIIa (24638087) (PLA_2 enzyme)	*Daboia russelii russellii*

100.	FAIIAYSNYGcYcGWGGK ccFVHDccYGR	1 1	696.876 7 767.965	2087.63 1533.93	3 2	21.01 & 3.70	Phospholipase A_2 (13936543) (PLA_2 enzyme)	*Echis coloratus*
101.	SPIFSYGDYGcYcGWGGK ccFVHDccYGR mIFKMTGK	2 1 1	692.283 3 767.965 973.32	2073.85 1533.93 972.32	3 2 1	30.83 & 2.33	Acidic phospholipase A_2 (129506) (PLA_2 enzyme)	*Cerastes cerastes*
102.	**TS**VSSHYcTGR CTTGPCCRQcK LKPAGTTcW**R**	1 1 1	628.6 629.085 596.145	1255.20 1256.17 1190.29	2 2 2	74.42 & 8.67	Disintegrin CV short precursor (123916448) (Disintegrin)	*Cerastes vipera*
103.	FcYLPADPGEcMAYIR SFYYDSESK cRQTcRAPR	1 1 1	982.81 563.925 603.185	1963.62 1125.85 1204.37	2 2 2	37.78 & 4.57	Trypsin inhibitor-4 precursor (159883524) (KSPI)	*Daboia siamensis*

Table 3.3: Summary of snake venom protein families with the number of isoforms identified in the proteome of crude *Daboia russelii* venom.

Family	PLA_2	RVVX	SvSP	Snaclec	LAAO	Crisp	5'NUC	KSPI	Dis	PDE	VNGF	VEGF
No. of isoforms	15	6	11	6	5	7	3	3	2	2	1	2

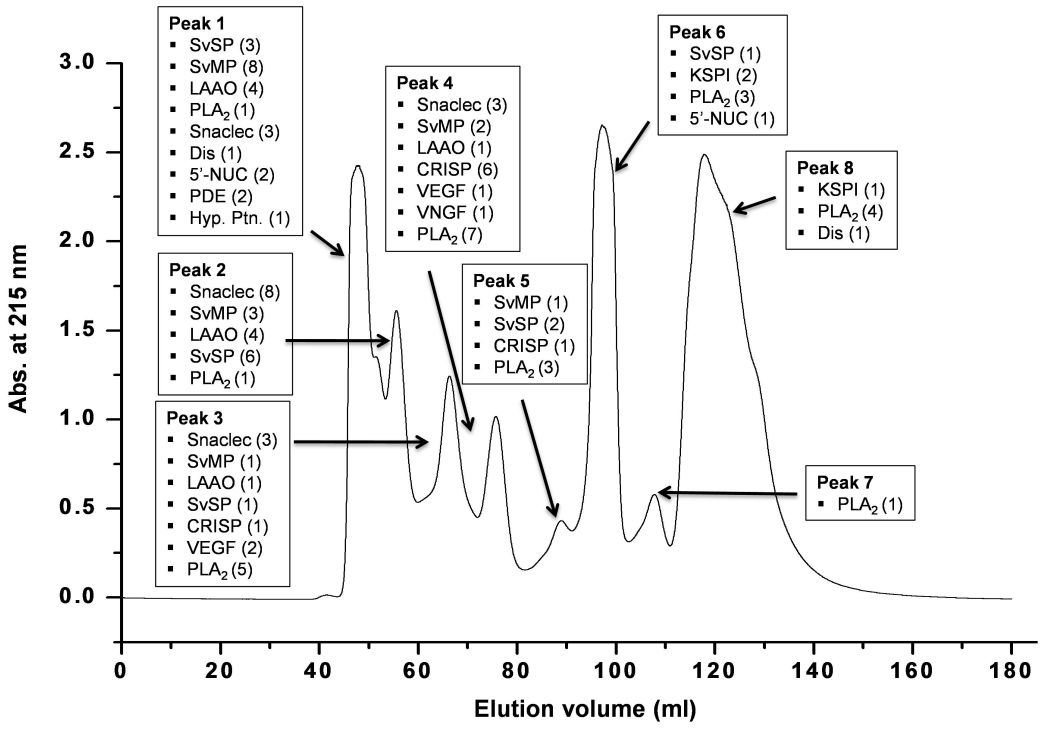


Figure 3.6: Schematic representation of the various snake venom protein families identified by tandem mass spectrometry in each gel filtration peaks of crude *Daboia russelii* venom.

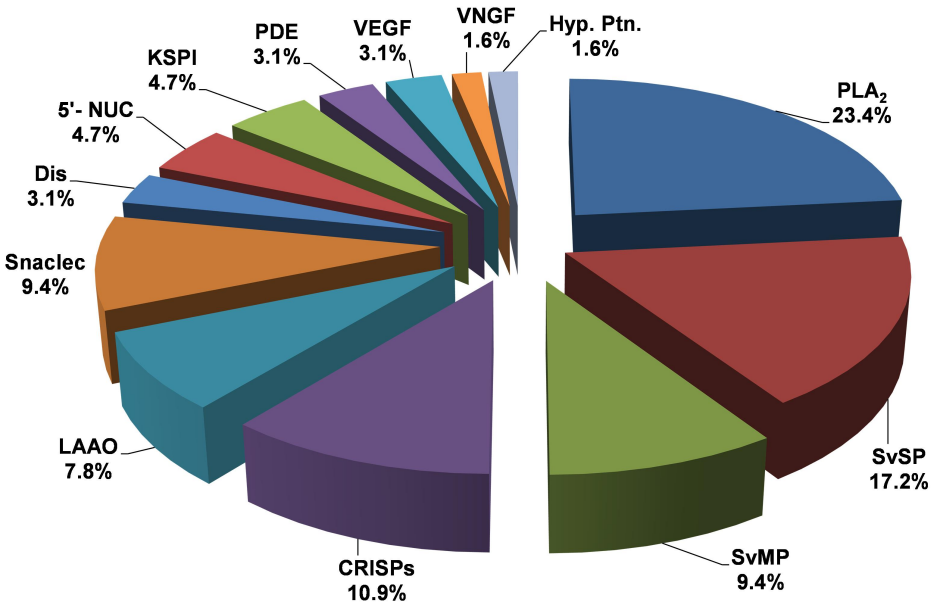


Figure 3.7: Relative distribution of the various snake venom protein families in Indian *Daboia russelii* venom identified by tandem mass spectrometry.

3.4.5 *In-vitro* anticoagulant assays

3.4.5.1 Recalcification time

The crude venom exhibited procoagulant effect on the PP plasma in a dose dependent manner (Figure 3.8). At a concentration of 0.033 µg/ml, the clotting time was reduced to 21.15 ± 0.85 s which is 4.52 fold lower than the normal clotting time (95.72 ± 3.33 s). However, with increasing concentration there was a gradual increase in clotting time which reached to saturation at 33.33 µg/ml.

3.4.5.2 Prothrombin time

The crude venom of *Daboia russelii* also displayed procoagulant effect on the prothrombin time of PP plasma in a dose dependent manner (Figure 3.9). The normal PT (NCT in this case) of the PP plasma was observed to be 18 ± 1.4 s. However, incubation of the PP plasma with crude venom reduced its clotting time from 18.53 ±

0.4 s at 0.01 µg/ml to 7.73 ± 0.5 s at 6.67 µg/ml which was 2.32 fold lower than the NCT of PT under the same experimental conditions.

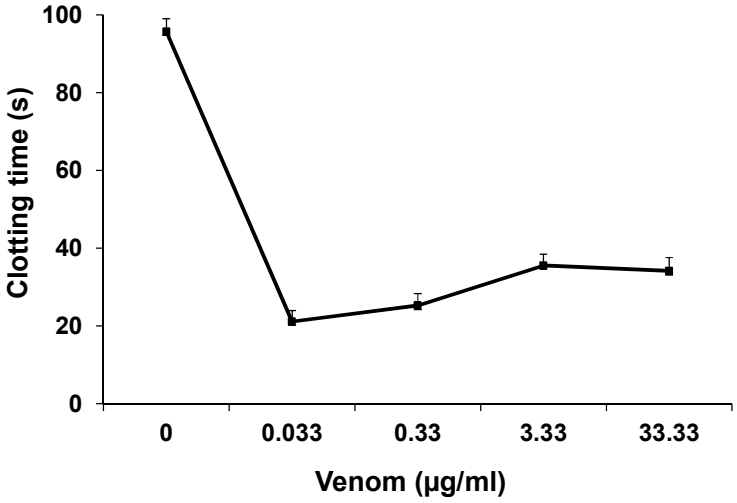


Figure 3.8: Recalcification time of crude *Daboia russelii* venom on PP plasma. Clotting was initiated by 50 mM of $CaCl_2$ and monitored using Coastat-1 Coagulation Analyser. The results are mean ± SD of three independent experiments.

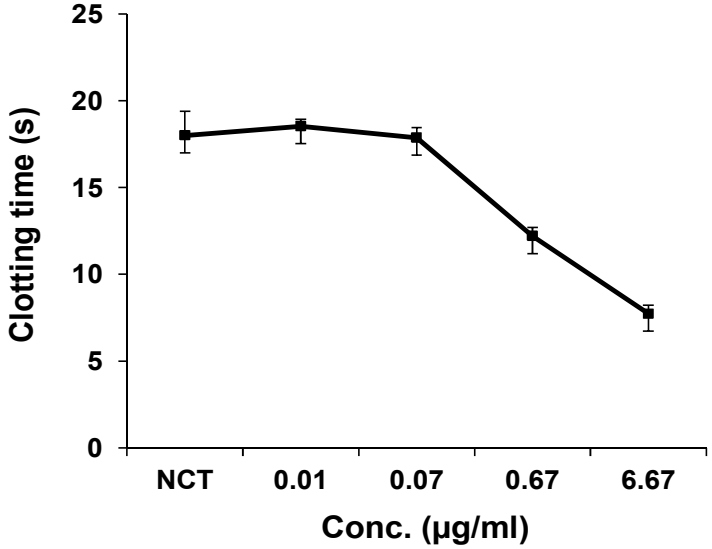


Figure 3.9: Prothrombin time of crude *Daboia russelii* venom on PP plasma. Clotting was initiated by addition of Uniplastin and clot formation was monitored using Coastat-1 Coagulation Analyser. The results are mean ± SD of three independent experiments.

3.4.5.3 Activated partial thromboplastin time

The crude venom of *Daboia russelii* also exhibited procoagulant effect on APTT of PP plasma in a dose dependent (Figure 3.10). The normal APTT (NCT in this case) of the PP plasma was found to be 42.21 ± 3.8 s however, with increasing concentration of the crude venom the clotting time was reduced from 36.76 ± 1.4 s at 0.01 µg/ml to 8.35 ± 0.4 s at 6.67 µg/ml.

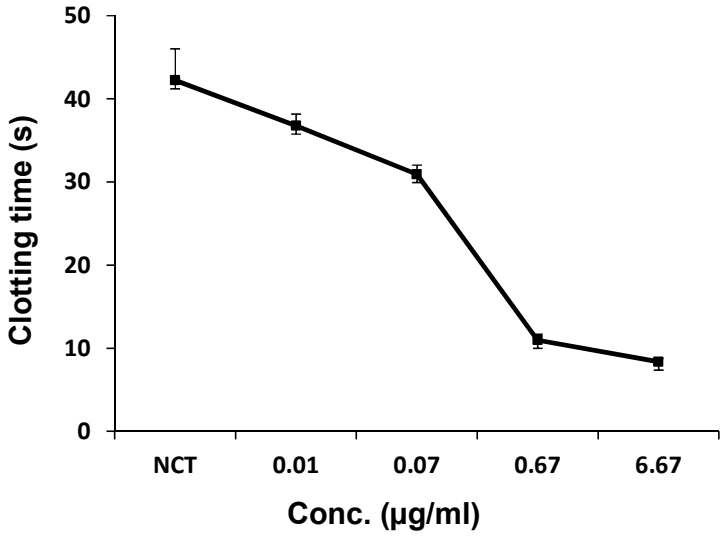


Figure 3.10: Effect of crude *Daboia russelii* venom on the activated partial thromboplastin time of PP plasma. Plasma **c**lotting was initiated by 25 mM of $CaCl_2$ and clot formation was monitored using Coastat-1 Coagulation Analyser. Results are mean ± SD of three independent experiments.

3.4.6 Fibrinogenolytic activity

The molecule of fibrinogen separates into three protein bands namely α (63.5 kDa), β (56 kDa) and γ (47 kDa) under reduced condition (Figure 3.11). It was observed that 1 µg of the crude venom of *Daboia russelii* could degrade the α-chain of fibrinogen completely followed by partial degradation of the β-chain. However, no effect was observed on its γ-chain.

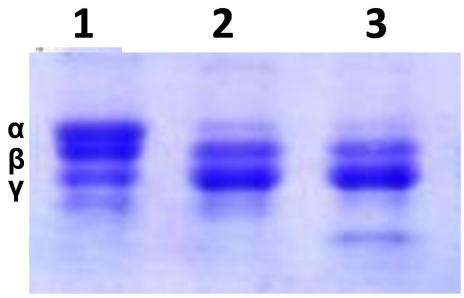


Figure 3.11: Fibrinogenolytic activity of crude venom of *Daboia russelii* on 12.5% glycine SDS-PAGE. Lane 1: fibrinogen treated with 20 mM Tris-Cl, pH 7.4 (considered as negative control), **Lane 2:** fibrinogen treated with thrombin (considered as positive control), **Lane 3:** fibrinogen treated with crude venom (1 µg) of *Daboia russelii*.

3.4.7 Haemolytic assays

3.4.7.1 Direct haemolytic assay

The crude venom exhibited direct haemolytic activity on erythrocytes in a dose dependent manner although the effect was not very prominent (Figure 3.12). The crude venom at 50 µg/ml showed up to 1.25 ± 0.09% of haemolysis.

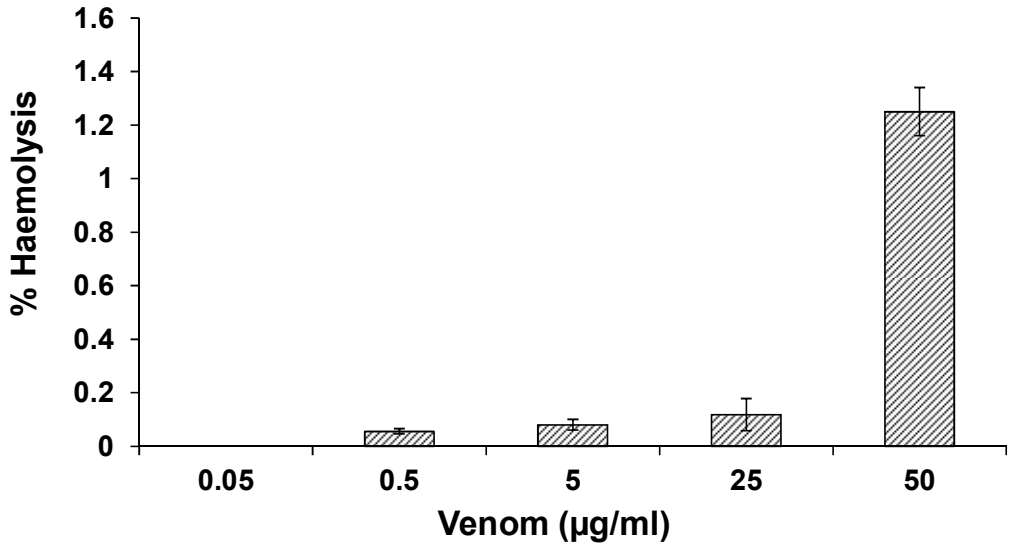


Figure 3.12: Direct haemolytic activity of crude *Daboia russelii* venom. 10% RBC suspension was treated with different concentrations of crude venom and incubated at 37°C for 1 h. The amount of haemolysis was quantified at 540 nm using UV-Vis MultiSkan Go spectrophotometer. Haemolysis caused by water was considered as 100%. Results are mean ± SD of three independent experiments.

3.4.7.2 Indirect haemolytic assay

The crude venom exhibited indirect haemolysis on erythrocyte suspension in a dose dependent manner (Figure 3.13). At 0.05 µg, the percentage haemolysis was 1.5 ± 0.5% however, when the amount was increased to 50 µg, percentage haemolysis was observed to be 78 ± 1.5%.

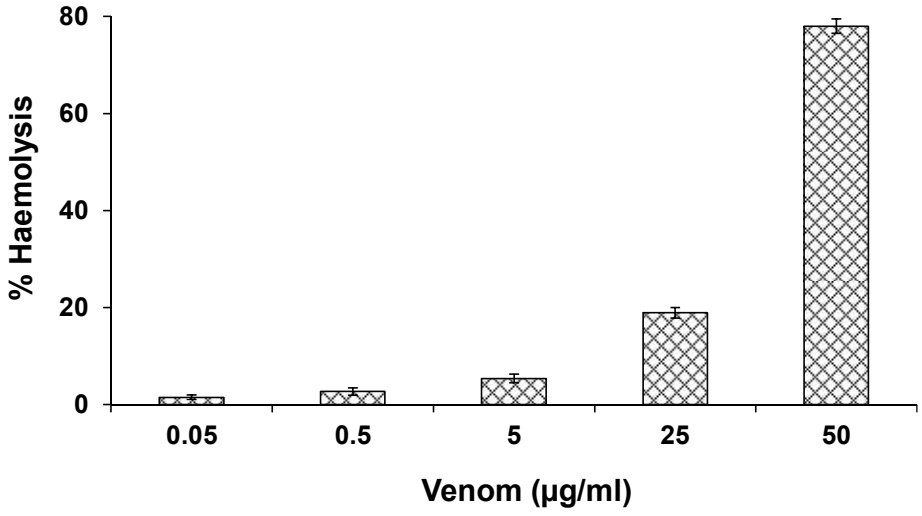


Figure 3.13: Indirect haemolytic activity of crude *Daboia russelii* venom. 10% RBC suspension was treated with different concentration of crude venom in the presence of egg yolk and incubated at 37°C for 1 h. The amount of haemolysis was quantified at 540 nm using UV-Vis MultiSkan Go spectrophotometer. Haemolysis caused by water was considered as 100%. Results are mean ± SD of three independent experiments.

3.4.8 PLA_2 activity

The crude venom exhibited PLA_2 activity on diheptanoyl thio-PC in a dose dependent manner (Figure 3.14). The specific activity of the crude venom in the presence of Ca^{2+} ions was found to be 48.89 ± 3.85 µmol/min/ml/mg while that of the venom was 278.16 µmol/min/ml/mg which was considered as the positive control.

3.4.9 Proteolytic activity

The crude venom exhibited proteolytic activity on casein in a dose dependent manner although; the activity was not very prominent (Figure 3.15). At 20 µg, the crude venom exhibited only 0.0198 ± 0.003 µg/min/ml of caseinolytic activity when compared with tyrosine standard curve.

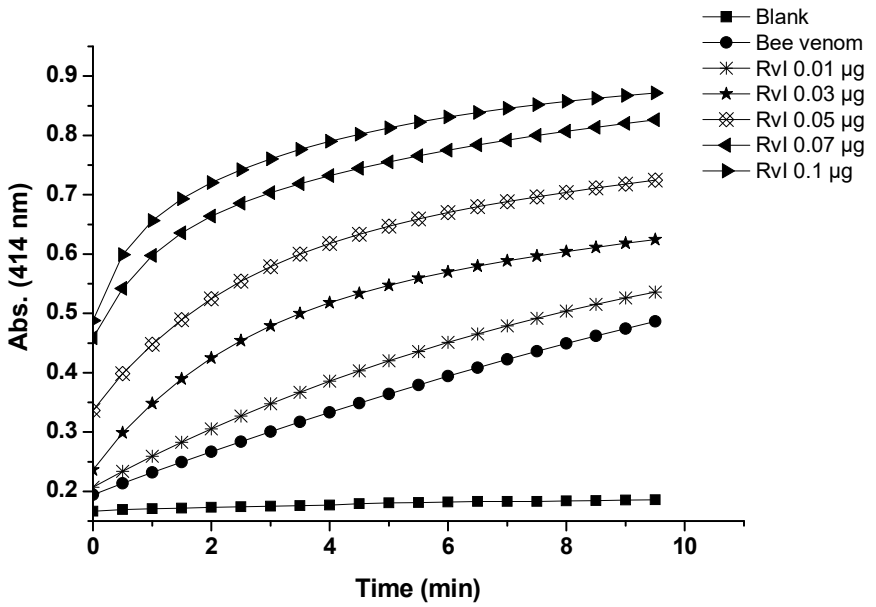


Figure 3.14: Progressive curve of PLA_2 activity of crude *Daboia russelii* venom using $sPLA_2$ assay kit. Different amount of crude venom were treated with diheptanoyl thio-PC and the amount of hydrolysis was quantified at 414 nm. Bee venom PLA_2 enzyme is considered as the positive control.RVI depicts Russell's viper Irula venom.

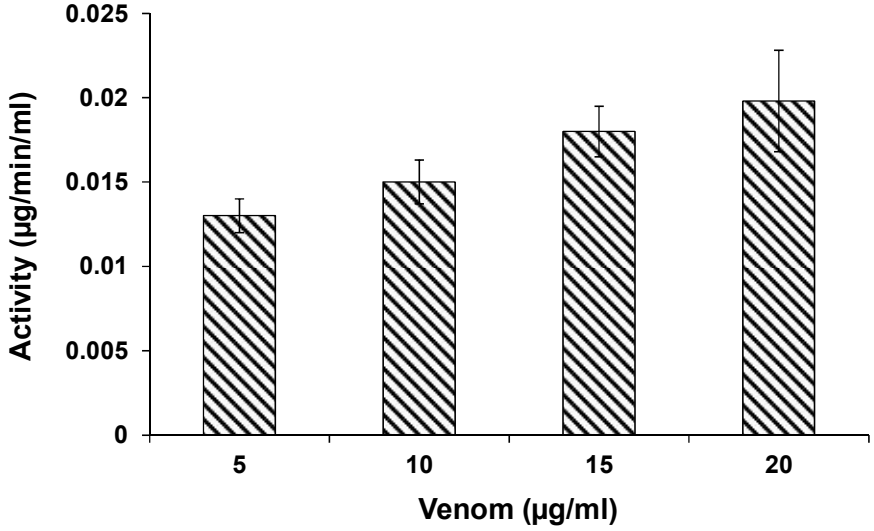


Figure 3.15: Proteolytic activity of crude *Daboia russelii* venom on casein. The amount of proteolysis was quantified at 660 nm using UV-Vis MultiSkan Go spectrophotometer. Results are mean ± SD of three independent experiments.

3.4.10 Edema inducing activity

The crude venom of *Daboia russelii* induced edema formation in the tested mice (Figure 3.16). It was observed that 15 µg of the venom could induce 208.3% of edema in the injected leg of the experimental mice. On the other hand, the mice injected with the vehicle control did not exhibit any edema formation.

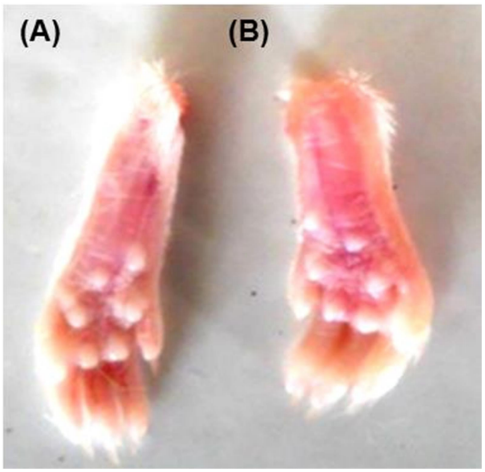


Figure 3.16: Edema inducing activity of crude *Daboia russelii* venom. (A): PBS treated paw **(B):** Crude venom (15 µg) treated paw. The feet of the treated mice were removed from the ankle joints and were weighted.

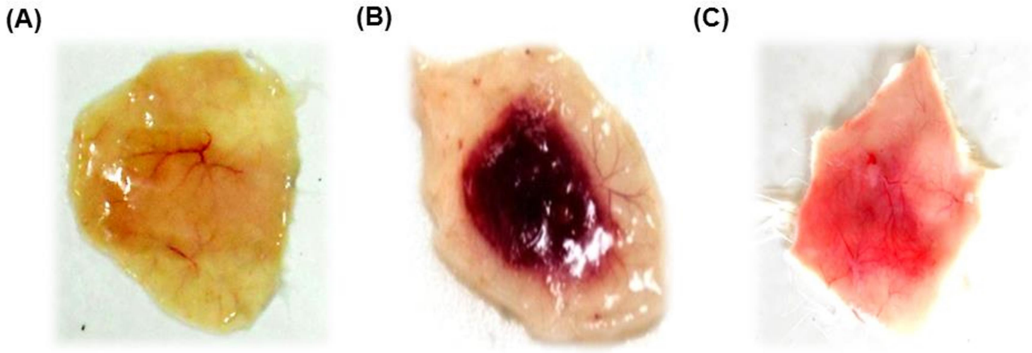


Figure 3.17: Haemorrhagic activity of crude *Daboia russelii* venom. (A): mice injected with PBS, **(B):** mice treated with 3 µg of *Echis carinatus* venom, **(C):** mice treated with 15 µg of crude *Daboia russelii* venom. The dorsal skin of the treated mice was removed after sacrifice and the inner surface of the skin was observed for any haemorrhagic effect.

3.4.11 Haemorrhagic activity

The crude venom displayed mild haemorrhagic effect on the dorsal skin of tested mice (Figure 3.17). The haemorrhagic effect induced by 15 µg of the *Daboia russelii* venom was mild compared to the effect induced by 3 µg of *Echis carinatus* venom. The spot induced by *E. carinatus* was very intense with a diameter of 2.5 cm while *Daboia russelii* venom induced a very mild spot with a diameter of 2.0 cm. The PBS injected mice, however, did not show any haemorrhagic effect.

3.4.12 Cytotoxicity

The crude venom of *Daboia russelii* showed cytotoxic effect on both the normal (HEK-293) and cancerous (MCF-7) mammalian cell lines in a dose dependent manner (Figures 3.18 to 3.21). It was observed that up to 1.7 µg/ml of the crude venom there was no significant effect on the morphology and viability of both the cell lines. However, at 2 µg/ml visible change in the morphological pattern of the cells were observed and the cell viability was reduced to 78.57 ± 2.86% for HEK-293 cells and 82.97 ± 2.16% for MCF-7 cells. The cells treated beyond 2 µg/ml of the crude venom, gradually lost their capability of cell to cell adherence and turned round in shape. At 3 µg/ml of crude venom, only 10.95% of HEK-293 cells were viable and beyond this concentration there was no change in percentage cell viability (Figure 3.19 A). However, for MCF-7 cells the cell viability was reduced to 14.97 ± 2.15% at 3 µg/ml of the crude venom and there was a gradual decrease in percentage cell viability of cells up to 5 µg/ml (11.95 ± 2.9%) (Figure 3.21 A). The IC50 of the crude venom for HEK-293 cells and MCF-7 cells were calculated to be 2.24 µg/ml and 2.47 µg/ml respectively (Figure 3.19 B & 3.21 B).

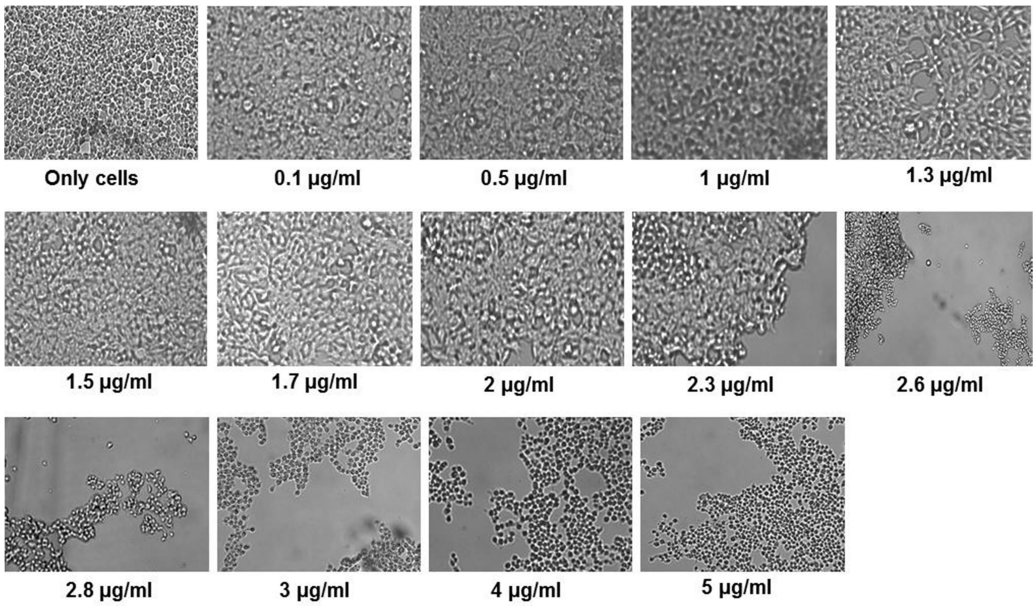


Figure 3.18: Microscopic images of HEK-293 cell lines after treatment with crude venom of *Daboia russelii*. Images were photographed at 10X magnification under Inverted microscope (Axio Vert A1., Zeiss) after 24 h of venom treatment.

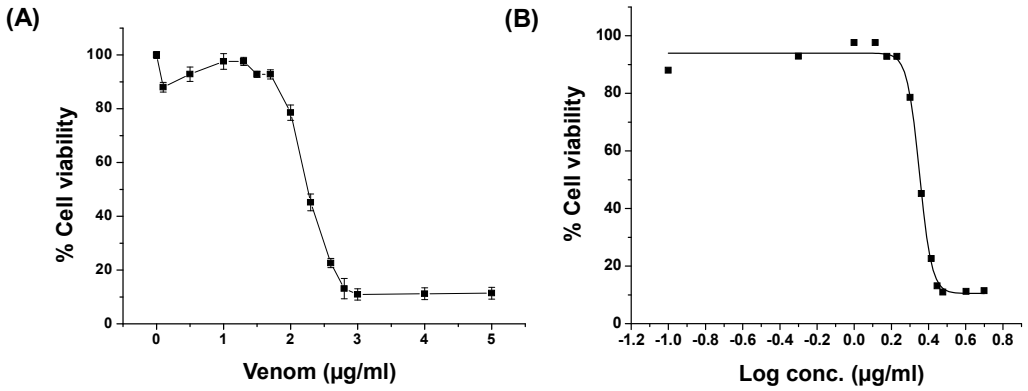


Figure 3.19: Cytotoxic effect of crude venom of *Daboia russelii* on HEK-293 cell lines. (A): Percentage cell viability of HEK-293 after treatment with crude venom for 24 h at 37^{o}C. Cytotoxic effect of the crude venom was determined using MTT assay. **(B):** IC50 curve. The curve was plotted using non-linear curve fit using Origin (OriginLab).

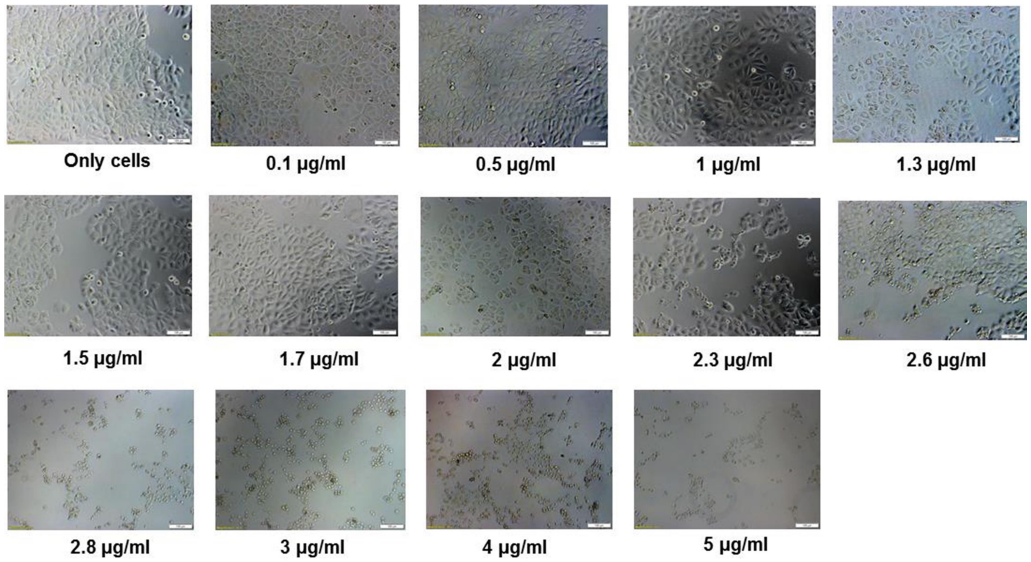


Figure 3.20: Microscopic images of MCF-7 cell lines after treatment with crude venom of *Daboia russelii*. Images were photographed at 10X magnification under Inverted microscope (Axio Vert A1., Zeiss) after 24 h of venom treatment.

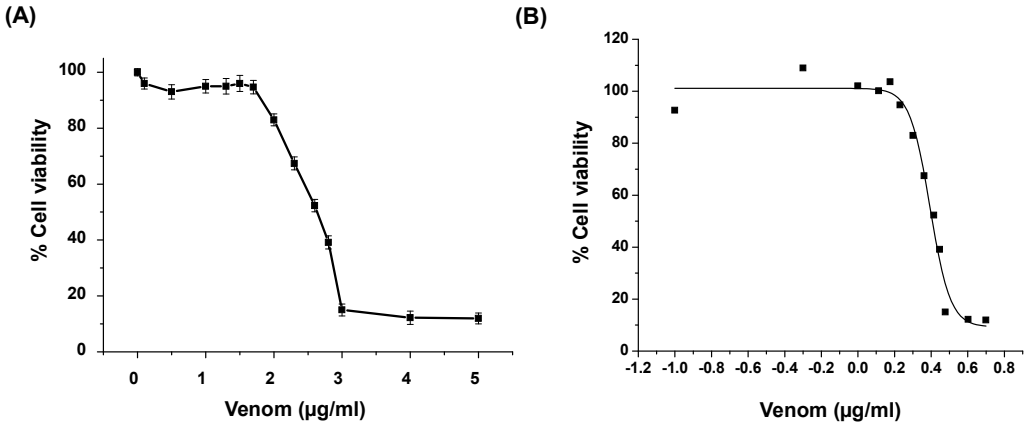


Figure 3.21: Cytotoxic effect of crude venom of *Daboia russelii* on MCF-7 cell lines. (A): Percentage cell viability of MCF-7 after treatment with crude venom for 24 h at 37^{o}C. Cytotoxic effect of the crude venom was determined using MTT assay. **(B):** IC50 curve. The curve was plotted using non-linear curve fit using Origin (OriginLab).

3.4.13 Recalcification time of the gel filtration fractions of the crude *Daboia russelii* venom

The recalcification time of the crude venom fractions using PP plasma revealed different clotting effects (Figure 3.22). It was observed that 3.33 µg/ml of peaks P1 to P5 shortened the clotting time with respect to NCT (115 ± 4.2 s) while same amount of peaks P6 to P8 prolonged the clotting time of plasma. This reveals the procoagulant effect of peaks P1 to P5 while the anticoagulant effect of P6 to P8. P1 exhibited strongest procoagulant activity with a clotting time of 24 ± 3.98 s while P6 showed highest anticoagulant activity delaying the clot formation beyond 600 ± 2.3 s.

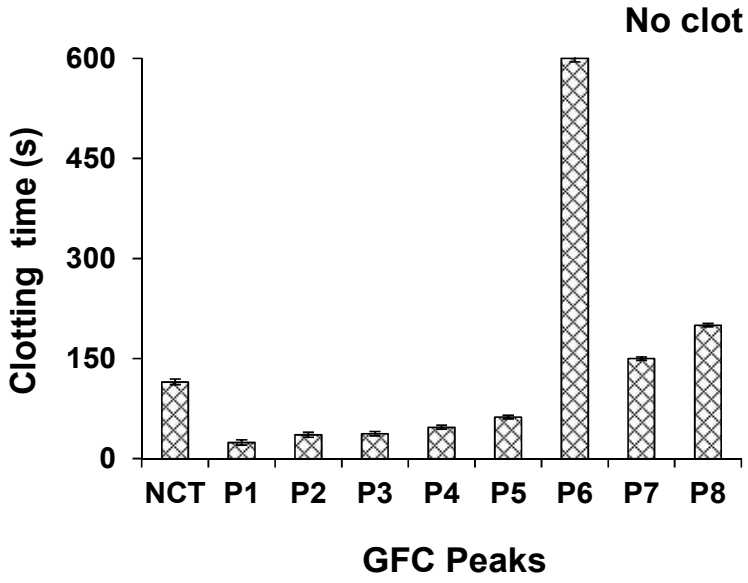


Figure 3.22: Recalcification time of the gel filtration peaks (3.33 µg/ml) of crude *Daboia russelii* venom on PP plasma. Clotting was initiated by 50 mM of $CaCl_2$ and monitored on Coastat-1 Coagulation Analyser. Results are mean ± SD of three independent experiments.

3.4.14 PLA_2 activity of the gel filtration fractions of the crude *Daboia russelii* venom

The gel filtration peaks of *Daboia russelii* venom showed different level of PLA_2 activity. It was observed that 0.04 µg/ml of peaks P1 to P5 did not show PLA_2 activity while the same concentration of peaks P6 to P8 displayed PLA_2 activity

(Figure 3.23). Peak P6 exhibited significant level of (p-value < 0.01) hydrolytic activity (93.69 ± 1.2 µmoles/min) compared to the other two peaks.

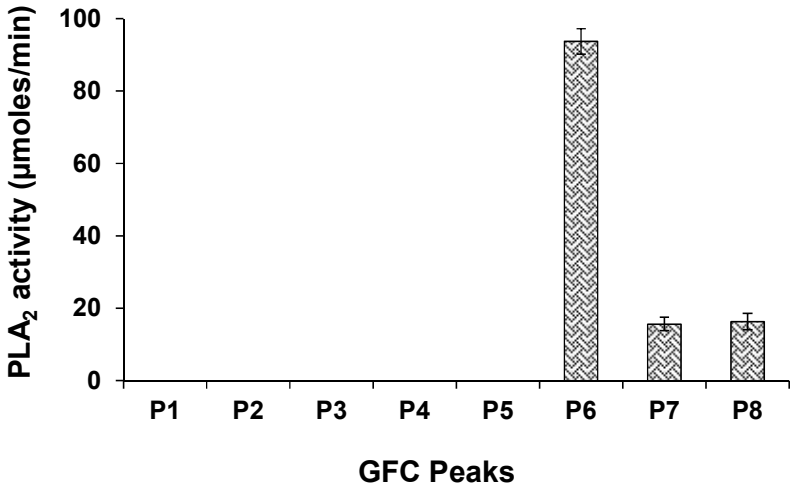


Figure 3.23: PLA_2 activity of the gel filtration peaks (0.04 µg/ml) of crude *Daboia russelii* venom using $sPLA_2$ assay kit. The amount of diheptonoyl thio-PC hydrolysis was quantified at 414 nm using UV-Vis MultiSkan Go spectrophotometer.

3.5 DISCUSSION

The complex mixture of proteins and polypeptides in snake venom are responsible for the manifestation of various pathophysiological signs and symptoms in prey or victims. Thus, it is important to explicate these venom components to unveil the complex proteome profile and identify the major as well as the minor proteins both qualitatively and quantitatively. In addition to this, comparison of the proteome profile with the various pharmacological experiments will provide a better overview of the potentiality of these venom proteins on the physiological systems of the victim. Hence, elucidation of the proteome profile might provide better strategies to design more specific and effective antivenom and manage snakebite problem more effectively.

The electrophoretic profile of the crude venom and its 8 gel filtration fractions revealed the presence of high and low molecular mass proteins ranging from 170 to 8 kDa. Faint protein bands at 100 kDa suggest the presence of high molecular weight

proteins like 5'-NUC and PDE in minor quantities while sharp protein bands near 55 and 15 kDa indicate the relative abundance of proteases and PLA_2 enzymes in the crude venom. The SDS-PAGE profile of P1 displayed prominent protein bands near 100, 70 and 55 kDa suggesting the abundance of high molecular weight proteins like 5'-NUC, PDE, LAAO and SvMP in it. Interestingly, bands below 15 kDa were also observed which propose the presence of low molecular weight proteins like PLA_2 enzymes, disintegrins or KSPI. A distinct band near 40 kDa in P2 proposes the abundance of SvSP in it while P3, P4 and P5 displayed the presence of relatively lower molecular weight proteins. Nonetheless, gel filtration peak P6 showed the presence of a relatively high intensity protein band at ~14 kDa suggesting the predominance of low molecular weight proteins like PLA_2 enzymes in it. On the other hand, P7 and P8 demonstrated traces of low molecular weight proteins. Noteworthy that the electrophoretic profile of all the gel filtration peaks have shown bands near 15 kDa of different intensities suggesting the presence of PLA_2 enzymes in them. The existence of low molecular weight proteins in the initial peaks suggest the interaction of these proteins with some high molecular weight proteins to form complexes, which needs to be analysed by further proteomic studies.

Thorough analysis of the peptide fragments obtained from tandem mass spectrometry of the trypsinized gel filtration fractions of the crude *Daboia russelii* venom provided a comprehensive overview of different proteins present in the crude venom. Analysis revealed the presence of 63 different proteins which were categorized into 12 snake venom protein families based on sequence homology.

PLA_2 enzymes were found to be the most abundant enzymatic protein families in this venom proteome with 15 isoforms. They constituted 23.4% of the total proteome of the crude venom. The members of this protein family are known to induce wide range of pharmacological effects including myotoxicity, neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathic, haemorrhage, edema, tissue damage and convulsion during envenomation (46). PLA_2 enzymes have been reported to exist either as monomers or as complexes, vipoxin and viperotoxin F are such complexes of viperid venom (423). Vipoxin from the venom of *Vipera ammodytes meridionalis*, is a heterodimeric complex with a PLA_2-like natural inhibitor (Chain A)

and a highly toxic basic enzymatically-active PLA_2 (Chain B) (424). In this proteome, five peptide fragments including three unique peptides similar to chain A of vipoxin were observed (Accession 1408314, Table 3.2). One of the peptide fragments homologous to this acidic subunit with the substitution of His48 to Gln48 at the active site was also found. However, no peptide homologous to toxic basic PLA_2 (Chain B) was observed in the proteome. Similarly, RV-4 (enzymatically active) and RV-7 (non-toxic acidic subunit) are the two subunits of viperotoxin F, the major toxic component of *Vipera russelii formosensis* (425). Six peptide fragments similar to RV-7 subunit (accession. No. 400714) from *D. russelii siamensis* were identified in the venom, while the active form, RV-4 was absent. The absence of Chain B of vipoxin and RV-4 subunit of viperotoxin F indicates the absence of functional vipoxin and vipertoxin F homologus in *Daboia r. russelli* venom from South India. However, *Daboia russelli siamensis* from Myanmar is reported to have both the subunits of these toxin complexes in the venom (396). Interestingly, peptide fragments similar to daboiatoxin, a major PLA_2 toxin in *Daboia russelii siamensis* from Myanmar which shows myotoxic, neurotoxic and cytotoxic activity was not observed in this proteome (426). Absence of daboiatoxin in the venom might be due to the difference in the geographical origin of *Daboia russelii* and validate their classification as distinct subspecies. It would be interesting to analyse and validate whether the inactive forms of PLA_2 enzymes interacting with some other PLA_2 components of the venom from South India and impose distinct synergistic functional characteristics. VRV-PL-VIIIa, a basic PLA_2 enzyme reported as the major toxin in *Daboia russelii* venom was found in this venom (387). VRV-PL-VIIIa exhibits diverse pharmacological effects like neurotoxicity, myonecrosis, *in-vivo* organ damaging activity and anticoagulant effect (427). In the present analysis we have observed six peptide fragments of this toxin in gel filtration peak 5, 6 and 8. Peptide fragments homologous to ammodytin I1 (C) (Accession No. 50874332, Table 3.2) a pre-synaptic neurotoxic PLA_2 from the venom of *Vipera berus berus* were also observed (428).

The PLA_2 enzymes of *D. russelii* are classified into N-type and S-type based on the N-terminal residues, asparagine or serine (429). The N-type PLA_2 is reported to be present in *D. r. siamensis*, *D. r. russelii* and *D. r. formosensis* venoms while the S-type is found in *D. r. pulchella* venom (429). However, in the present venom

proteome, both the S-type and N-type PLA_2 enzymes were observed along with peptide fragments homologous to PLA_2 with (13936543, 50874332 and 223635543) histidine at the N-terminus (Appendix-I). This suggests the lack of correlation between different types of PLA_2 enzymes (classified based on N-terminal amino acid residue) and subspecies of Russell's viper.

The crude venom of *Daboia russelii* was found to exhibit potent anticoagulant, PLA_2, indirect haemolytic and edema inducing activities which could be attributed to the presence of these large number of PLA_2 isoenzymes. A noteworthy observation is that, although all the gel filtration fractions revealed the presence of one or more isoforms of PLA_2 enzymes, but at 0.01 µg, only P6, P7 and P8 exhibited PLA_2 activity while P1-P5 did not show any hydrolytic activity. This could be due to the involvement of these PLA_2 isoforms with other venom proteins in these peaks (P1-P5) or the presence of some inhibitors which might have affected the hydrolytic activity of these enzymes in these peaks.

SVSPs were found to be the 2nd most relatively abundant enzymatic components constituting 17.2 % of the proteome with 11 isoforms. Homolog of elegaxobin (Accession No. 90116798) from *Protobothrops elegans* and RVV-Vα from *D. siamensis* were observed (48,430). RVV-V converts coagulation factor V to Va (co-factor in the prothrombinase complex) by cleaving at the Arg1545-Ser1546 bond (48). In addition to this, three isoforms of β-fibrinogenase similar to proteins reported from *D. siamensis, Macrovipera lebetina* and *Gloydius blomhoffii* were also observed in this proteome. The observed procoagulant and fibrinogenolytic activities of the crude venom of *Daboia russelii* in the present study could be attributed to these enzymes in the proteome.

SVMPs formed the 3rd highest enzymatic component constituting 9.4% of the total proteome. In general, SVMPs are classified into three broad classes, namely PI, PII (a, b, c, d) and PIII (a, b, c and d) based on the difference in the domain structure (50,431,432). During envenomation SVMPs cause both local and systemic injuries which include pathological effects like myonecrosis, haemorrhage, edema formation and blistering (433). Viperidae venoms are a rich source of these proteins which

induce the most profound damaging effects to the basement membrane of the blood vessel capillaries leading to extravasation of endothelial cells including excess bleeding (317,433). Hence, insufficient blood supply and loss of basement membrane integrity might hamper skeletal muscle regeneration (434,435). Thus, the manifestation of haemorrhagic spot in the dorsal skin of the experimental mice in the present study suggests the effect of these SVMPs. Six isoforms of RVV-X (coagulation factor X activating enzyme) were observed in this proteome (Table 3.2 and 3.3). RVV-X belongs to the PIIId family of metalloprotease with a heavy chain and two light chains (chain A and B) (186). The two light chains are connected to each other via an inter-chain disulphide bond between Cys79 of chain A and Cys77 of chain B. While the light chain is connected to the heavy chain via another disulphide bond between Cys133 of chain A and Cys339 of heavy chain (436). It converts coagulation factor X to Xa by cleaving at the Arg51-Ile52 of FX leading to severe coagulopathy in victim/prey (437).

Hence, the presence of a large number of these SVMPs and SVSPs in this venom proteome could be responsible for the observed procoagulant effects on the various aspects of the blood coagulation cascade. It is reported that the thrombus formed by these enzymes in victims or prey gets readily dissolved by plasmin due to the absence of proper cross-linked fibrins leading to excess blood loss at the site of injury (49). Moreover, the consumption of the blood clotting factors in a short span of time renders the body of the clotting factors soon leading to consumptive coagulopathy in victims which is one of the most common pathological effects of Russell's viper envenomation (49). Nonetheless, although a number of proteases were identified, but the proteolytic activity of the crude venom on casein was considerably lower which could be due to non-specificity of these enzymes towards casein as substrate.

LAAO were observed to be the 4th highest enzymatic components of this crude venom with 5 isoforms constituting 7.8% of the venom proteome. These enzymes (57-68 kDa, monomeric form) are homodimeric high molecular weight proteins (438). They catalyse the oxidation of hydrophobic L-amino acids releasing α-keto acid, ammonia and H_2O_2 (51,52). The liberated H_2O_2 causes various pathologies, such as edema formation, ADP or collagen induced platelet aggregation inhibition or activation,

apoptosis, antibacterial effect, antiparasitic, anticoagulant, haemolytic and haemorrhagic effects (51,52,439). LAAO from *A. h. blomhoffii* delays the activated partial thromboplastin time (intrinsic pathway of blood coagulation) especially by targeting factor IX (223). Five isoforms of LAAO identified in the proteome were found to be similar with protein sequences previously reported (Table 3.2). Most of these proteins are reported to inhibit ADP and collagen-induced platelet aggregation (52,440). Thus, LAAO together with SVSPs, SVMPs and PLA_2 might act synergistically on the haemostatic system of the prey or victim and exhibit plethora of pharmacological effects.

5'-NUC formed the 5th highest enzymatic family of the crude venom proteome forming 4.7% with 3 isoforms. They belong to the metallophosphatase superfamily of proteins (441). Presence of homologous peptides suggest their presence in the venom and they might be involved in the release of adenosine (purines) which cause hypotension and paralysis leading to prey immobilization and digestion (54,79).

PDEs were the 6th highest enzymatic family with 2 isoforms forming 3.1% of the total proteome of the crude *Daboia russelii* venom. These enzymes are the high molecular weight (> 90 kDa) proteins with endonucleases activity on double and single stranded RNA and DNA releasing 5'-mononucleotides (53). In this proteome, analogous sequence of PDEs reported previously from *Macrovipera lebetina* (Accession No. 586829527) and *Protobothrops flavoviridis* (Accession No. 538259853) were identified (Table 3.2) (441). Pharmacologically, PDEs inhibit platelet aggregation, decrease the mean arterial pressure and hinder locomotion (442).

CRISP were found to be the largest group of non-enzymatic proteins (10.9%) in the *D. russelii* venom with 7 isoforms. Several peptides similar to Da-CRPa Dr-CRPK, Ch-CRPKa, Cv-CRP, Dr-CRPB, Pg-CRP and a prepro CRISP from crotalinae and viperinae subfamily were identified (Table 3.2). They are reported to inhibit cyclic nucleotide gated ion channels in photoreceptor and olfactory cells potassium activated smooth muscle contraction and vascular smooth muscle contraction (68-70).

Snaclecs formed the 2nd highest non-enzymatic family of the venom proteome with 6 isoforms constituting 9.4% of the venom proteome. Peptide sequence homologous to α and β subunits of dabocetin documented earlier from subspecies of *D. russelii* were identified (Table 3.2). α dabocetin is reported to bind to glycoprotein Ib of platelet to induce inhibitory effect on ristocetin-induced platelet aggregation (443). Similarly, peptide sequences analogous to α and β subunits of P31 (Accession No. 300490484) and α subunit of P68 (Accession No. 300490470), crotocetin-1 (Accession No. 82129809) and snaclec A14 (Accession No. 218526485) were also observed. Moreover, β subunit of alboaggregin-A (Accession No. 3023231) reported earlier from *Trimeresurus albolabris* is a strong activator of platelet, binding via GPIb α and GP VI of platelets was also identified in this proteome (444-446). The abundance of snaclecs in this proteome might be the cause of enhanced coagulopathic disorders along with SVMPS, SVSPs and PLA_2 enzymes in the envenomed victims.

KSPI are the low molecular weight proteins having 50-60 amino acid residues with a conserved Kunitz motif typical to bovine pancreatic trypsin inhibitor (BPTI) (62,63). Upon envenomation, these proteins are reported to inhibit trypsin and chymotrypsin along with potassium and calcium ion channels as well as exhibit diverse pharmacological effects on the victim like fibrinolysis and anticoagulation (64,65,447-449). The present study revealed the presence of three isoforms of KSPI having sequence similarity with trypsin inhibitor precursor-4 and -5 reported earlier from *D. siamensis* and KSPI- 4 from *D. russelii* which constituted 4.7 % of the total proteome (Table 3.2) (447).

Disintegrin formed 3.1% of the total proteome with 2 isoforms. These polypeptides are rich in cysteine residues liberated by the proteolytic cleavage of multidomains of metalloproteases in the venom (74). They are found in the viperidae family and are known to cause inhibition of integrin receptors (74). Two peptide fragments homologous to adinbitor (Accession No. 50365991), a disintegrin with RGD motif from *Gloydius brevicaudus* were observed in this venom. Disintegrin with RGD motif are reported to inhibit angiogenesis and platelet aggregation. Sequences similar to disintegrin CV with RTS integrin inhibitory motif studied previously from *Cerastes vipera* were also observed in this venom proteome (75). These observed peptides are

found to have sequence similarity with Russellistatin reported from *D. russelii* (450). Disintegrin CV is reported to inhibit cell adhesion and migration via collagen I and II, anti-angiogenic and interact with α1 and β1 of integrin (75). The integrin receptor inhibitory loop with RGD motif is antagonist to β1and β3 ($\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$) integrins while the inhibitory loop with RTS is specific to α1β1 (74,451).

VEGF constituted 3.1% of the proteome with 2 isoforms. Peptide sequences similar to Vegf toxin and Vegf-A reported from viperinae family were identified (78,452). These proteins are reported to cause nitric oxide induced hypotension, angiogenesis, capillary permeability, cell proliferation, migration and anti-apoptosis (78).

VNGF formed the lowest non-enzymatic family with 1 isoform constituting 1.6% of the proteome. Peptide sequence similar to VNGF-2 reported earlier from *Daboia russelii* of Taiwan was observed. VNGFs are reported to cause apoptosis, vascular permeability and wound healing (77,453).

Apart from these well characterized protein families, peptide sequences similar to a hypothetical protein reported in the transcriptome of *Crotalus adamanteus* (Accession No. 387016758) were also observed in the present study (454). It will be interesting to understand the role of this protein during envenomation by Russell's viper venom.

The crude venom of *Daboia russelii* was found to be cytotoxic to both the normal and cancerous mammalian cell lines when tested using MTT based colorimetric method. Out of the 8 gel filtration fractions of the crude *Daboia russelii* venom, P6 exhibited the highest anticoagulant and PLA_2 activity. Moreover, it constituted 32% of the total protein of the crude venom and displayed a sharp protein band on SDS-PAGE. This suggests that P6 consists of one of the major protein of this crude venom whose purification and characterization would be important to understand its role in the overall toxicity of the crude venom.

Thus, analysis of the proteome of Indian *D. russelii* by using tandem mass spectrometry has provided a painstaking understanding of the potential protein components in this venom. The proteome profile correlates well with the clinical

manifestations of Russell's viper envenomation. The protein profile shows clear distinction with *D. r. siamensis* from Myanmar although they belong to the same species. The absence of daboiatoxin (the major lethal component of Myanmar viper), CRISPs, VNGF, KSPI, 5'-NUC and PDE in *D .r. siamensis* clearly indicate the variation of venom components in these subspecies. This study reports the presence of 5'-NUC, CRISPs, KSPI, disintegrins, VNGF and VEGF in the Indian *D. russelii* venom for the first time. Such proteomic studies on the venoms of Indian *D. russelii* from other geographical regions need to be carried out for the better understanding of the venom variation. This will help in designing the better quality, regiospecific antivenoms which might reduce the occurrence of anaphylactic reactions in the victims and help in better management of snakebite cases.