CHAPTER 2

Materials and Methodology

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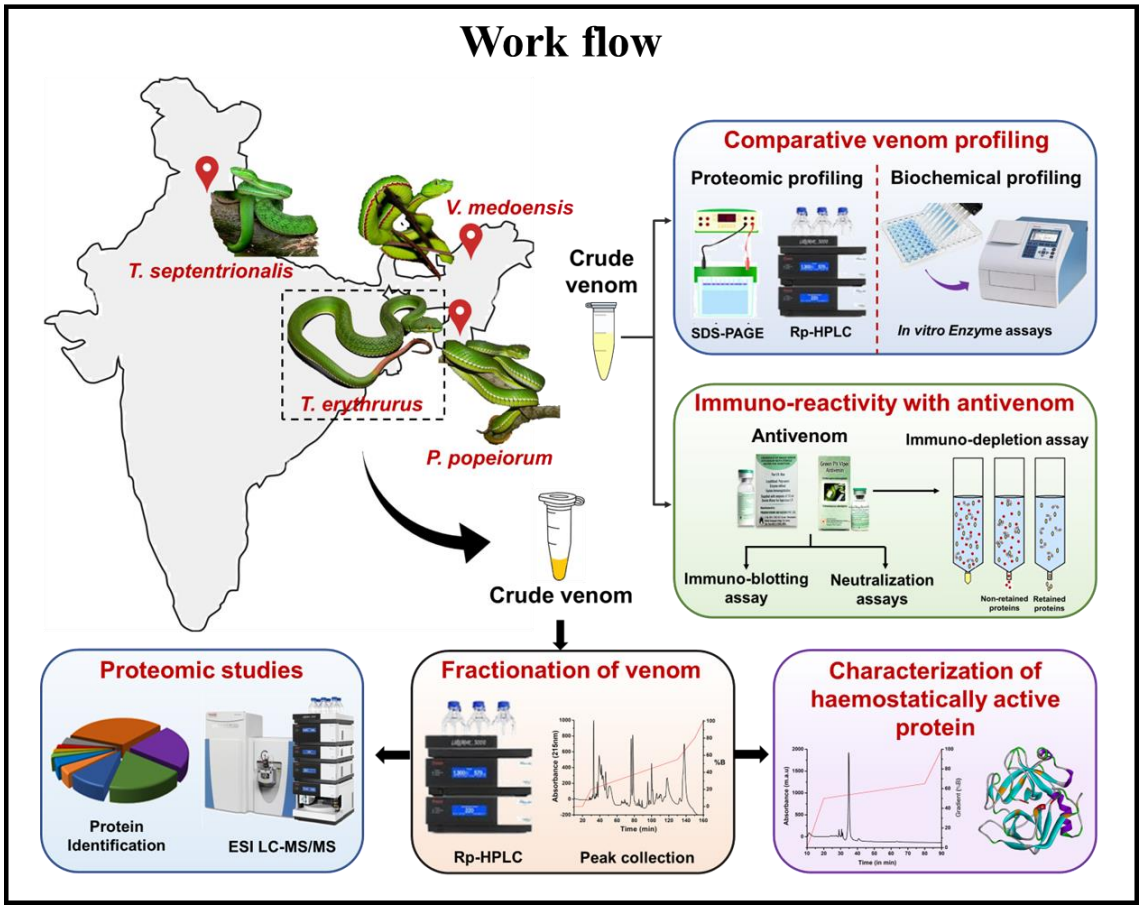

2.1. Materials

2.1.1. Venom collection

Crude venoms were collected from four species of green pit vipers from three different geographical locations of northern and north-eastern India (Figure 2.1). Venom of *Trimeresurus erythrurus* and *Popeia popeiorum* were collected from Aizawl, Mizoram. Crude venom of *Viridovipera medoensis* and *Trimeresurus septentrionalis* was collected from Leporiang, Arunachal Pradesh and Solan, Himachal Pradesh respectively. The permits for collection of venoms were obtained from Chief Conservator of Forests (Wildlife) and Chief Wildlife Warden of State, National Biodiversity Board of India (Appendix II). Crude venom of one individual per species was considered in the present

study. Russell's viper venom (*Daboia russelii*) was obtained from Irula Snake catchers Society, Tamil Nadu, India. Venoms collected from four adult female individuals of *T. erythrurus* from Aizawl, Mizoram were used for proteomics studies. All venoms were lyophilized and stored at -20˚C until further use. Lyophilized venom samples were resuspended in MilliQ water prior to use and the protein concentration was determined spectrophotometrically using Nanodrop 2000 (Thermo Fischer Scientific, USA) with an extinction coefficient of 1 mg/ml at A280nm.

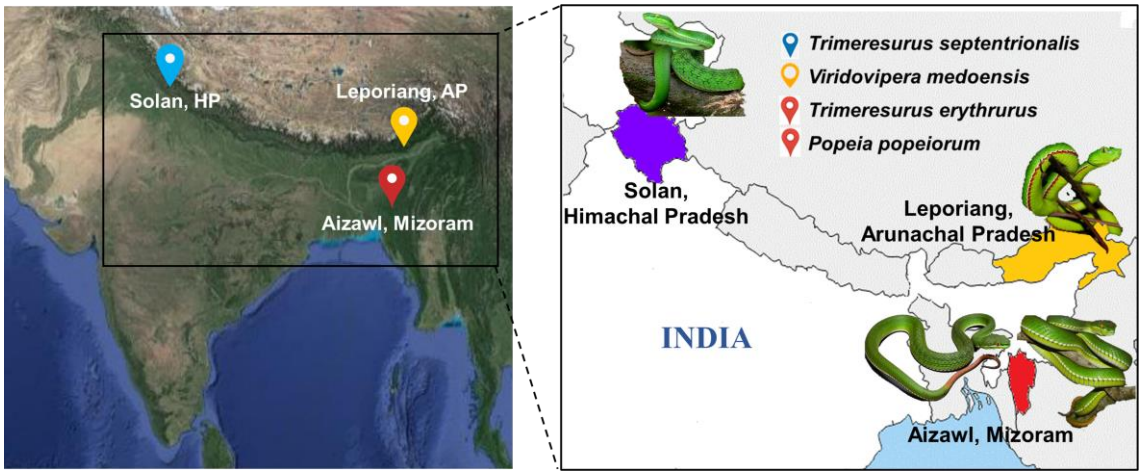

Figure 2.1. Map showing geographical locations of green pit vipers from which venom was collected.

2.1.2. Antivenoms

Indian snake Venom Antiserum (Premium Serum Polyvalent Antivenom, PSPAV) manufactured by Premium Serums and Vaccines Pvt. Ltd., Maharashtra, India (Batch no-212013; Exp- 08/2020) was used for cross reactivity assessment in the present study (Figure 2.2A). PSPAV is a commercially available antivenom in India which is procured from horses hyperimmunized with venoms of Big-Four snakes viz. *Naja naja*, *Daboia russelii*, *Bungarus caeruleus* and *Echis carinatus*. A monovalent Green Pit Viper Antivenin (GPVAV) raised against *Trimeresurus albolabris* venom manufactured by Queen Savoabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand (Batch no-TA00317; Exp- 07/2022) was also used for comparison (Figure 2.2B). The amount of $F(ab')_2$ fragments in the antivenoms was quantified using Nanodrop 2000 (Thermo Fischer Scientific, USA) with IgG mass extinction coefficient. Both the antivenoms were used within their shelf lives.

Figure 2.2. Commercially available Snake antiserum undertaken in the present study. (A) Premium serum polyvalent antivenom (PSPAV), **(B)** Green pit viper monovalent antivenom (GPVAV).

2.1.3. Chemicals and Reagents

Electrophoresis: Acrylamide, Bisacrylamide and β-mecaptoethanol (BME) was purchased from Sigma (Missouri, USA). Ammonium Persulphate (APS), Sodium dodecyl sulphate (SDS), Ethylene glycol, Glycerol, Coomassie brilliant blue R-250, Bromophenol blue, Acetic acid and Methanol were purchased from Merck (Darmstadt, Germany). Tetramethylethylenediamide (TEMED) was obtained from HiMedia (Mumbai, India). PageRuler plus pre-stained protein marker and Precision Plus Protein Standards were purchased from Thermo Scientific (Massachusetts, USA) and Bio-Rad (California, USA) respectively.

Western blotting: Immun-Blot® Polyvinylidene (PVDF) membrane for immunoblotting (pore size 0.2 µm) was purchased from Bio-Rad (California, US). 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/ nitro blue tetrazonium (NBT) solution and Anti-Horse IgG (whole molecule)- Alkaline phosphatase antibody was purchased from Sigma-Aldrich (St. Louis, US). Formaldehyde, methanol and Twin-20 was purchased from Merck (Darmstadt, Germany).

Chromatography: HPLC grade Acetonitrile and Trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany).

Mass spectrometry: Dithiothreitol (DTT), Iodoacetamide (IAA), formic acid and Ammonium bicarbonate (NH_4HCO_3) were purchased from Sigma (Missouri, USA). Calcium chloride ($CaCl_2$) was purchased from Merck (Darmstadt, Germany). Sequence grade trypsin was purchased from Promega (Wisconsin, US). Reconstituted trypsin stock was prepared in resuspension buffer (25 mM Ammonium Bicarbonate, 1 mM $CaCl_2$). All

the other reagents (Urea, IAA, DTT and $CaCl_2$) were prepared freshly in 50 mM Tris-HCl, pH 8.0.

***Assay kit and substrates*:** $sPLA_2$ assay kit was purchased from Cayman Chemical (Michigan, USA). Chromogenic substrate S2238 and S2251 was purchased from Chromogenix (USA).

Others: Tris-base, Tricine, Trisodium citrate, Sodium chloride, Sodium thiosulphate, sodium bicarbonate, Sodium bicarbonate ($NaHCO_3$) and Glycine were procured from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), bovine fibrinogen and Casein purified were purchased from HiMedia (Mumbai, India). Liquecelin and Uniplastin for coagulation assay were purchased from Tulip Diagnostics Pvt. Ltd. (Goa, India). Silver nitrate ($AgNO_3$) was purchased from Sigma (Missouri, USA). Cyanogen Bromide (CNBr) activated Sepharose™ was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden).

Thrombin, from bovine plasma was purchased from Merck (Darmstadt, Germany). Plasmin, from human plasma was purchased from Sigma (Missouri, USA). Tissue plasminogen activator (t-PA), from human was purchased from Calbiochem, Merck Millipore (Massachusetts, USA). Plasminogen from human was purchased from Roche (Basel, Switzerland). All the other chemicals used were obtained from either Merck Miliipore (Massachusetts, USA) or Sigma (Missouri, USA).

2.1.4. Columns

Symmetry™ C18 column with particle size 5 µm and 3.5 µm, pore size 300 Å and dimension 4.6 x 250 mm was procured from Waters Corporation (Milford, MA, USA) for reverse phase chromatography. Acclaim™ C18 column with particle size 3 µm, pore size 300 Å and dimension 2.1 x 150 mm was procured from Thermo Scientific (Massachusetts, USA) also for reverse phase chromatography. Pierce spin columns and pierce centrifuge concentrators was bought from Thermo Scientific (Massachusetts, USA).

2.2. Methodology

2.2.1. Profiling and fractionation of venom

2.2.1.1. Sodium Dodecyl sulphate -Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of crude venoms was performed on 10% Tris-tricine gel according to the method described by Laemmli, 1970 [174]. Briefly, 20 µg of each crude venom (*V. medoensis*, *P. popeiorum*, *T. erythrurus* and *T. septentrionalis*) was mixed with 5 µl of loading dye (0.5 M Tris-Cl, 6% Bromophenol blue, 30% glycerol) containing β-mercaptoethanol (βME) and heated at 95˚C for 5 min. Following heat treatment, samples were loaded on the gel along with protein ladder. Electrophoresis was carried out using Mini-PROTEAN^{TM} Tetra cell (Bio-Rad, CA, USA) with reservoir buffer (0.1M Tricine, 0.1 M Tris-base, 0.4 % SDS) at 80 V for stacking and 110 V for resolving gel. After electrophoretic run, the gel was stained with staining solution containing 0.2% Coomassie brilliant blue R250 in methanol, acetic acid and water (4:1:5) for 2 hours followed by destaining with methanol, acetic acid and water (4:1:5) to visualize protein bands, and gel image was taken with Chemidoc XRS imaging system (Bio-Rad, USA).

For the chromatographic peaks and purified proteins, 10% and 12.5% Tris-glycine gel was run respectively. Briefly, 2.5 µg of each peak/purified protein was loaded on gel in reduced or/and non-reduced condition along with standard protein ladder. Electrophoresis was carried out with reservoir buffer (192 mM glycine, 25 mM Tris-base, 10% SDS) in the similar condition. Post electrophoretic run, the gel was fixed in fixing solution containing 50% methanol, 10% acetic acid and 50 µl formaldehyde for 1 hour in shaking condition followed by washing 3 times with 50% ethanol, 20 minutes each. The gel was then precisely treated with hypo solution (0.02% sodium thiosulphate) for 1 min and subsequently washed 3 times with distilled water, 20 seconds each. Following washing, the gel was stained with 0.2% silver nitrate solution for 30 minutes in gentle shaking condition and washed rigorously with distilled water to remove extra stain. The gel was then developed using developing solution constituting 6% NaCl, 1 ml hypo solution and 50 µl formaldehyde till bands appeared. The reaction was stopped by 5% acetic acid solution.

2.2.1.2. Reverse phase High Pressure Liquid Chromatography (Rp-HPLC)

Profiling of crude venoms of Indian green pit vipers was done by reverse phase HPLC using Thermo Scientific UltiMate 3000 high performance gradient system equipped with DAD detector (Thermo Scientific, USA). Briefly, crude venoms (2 mg) were loaded on Symmetry C18 column with particle size 5 µm, pore size 300 Å and

dimension 150 × 4.6 mm (Waters, Milford, MA, USA) pre-equilibrated with 0.1% (v/v) Trifluoracetic acid (TFA). Elution was carried out at flow rate of 1 ml/min with a linear gradient of 20-55% acetonitrile containing 0.1% (v/v) TFA (Solution B) for 100 minutes followed by 55-80% (Solution B) for 20 minutes. Protein elution was monitored at 215 nm and peaks were collected manually.

For the proteomics studies, lyophilized crude venom from four individuals of *T. erythrurus* (1 mg) was reconstituted in ultrapure water and centrifuged to remove debris. Fractionation of was carried out using Symmetry C18 column with particle size 3.5 µm, pore size 300 Å and dimension 150 × 4.6 mm pre-equilibrated with 0.1% (v/v) TFA. The proteins were separated by a linear gradient of 20-65% acetonitrile containing 0.1% (v/v) TFA for 20-100 minutes followed by 65-100% for 100-110 minutes and elution was carried out at a flow rate of 0.8 ml/min at 215 nm. Fractions were collected manually and the concentration of protein in each peak (mg/ml) was measured using a Nanodrop 2000 (Thermo Scientific, USA). Relative area (in percentage) of each peak was calculated using the Chromeleon 6.8 software (Thermo Scientific, USA) and concentration of protein in each peak was equalized by volume adjustment.

For protein purification, 50 µg of Rp-HPLC peak was loaded on Acclaim^{TM}300 C18 column pre-equilibrated with 0.1% (v/v) TFA. The flow rate was set on 0.2 ml/min. The proteins were separated by a linear gradient of 50-65% acetonitrile containing 0.1% (v/v) TFA for 20-80 minutes and monitored at 215 nm. Purity of collected peak was checked on 12.5% tris-glycine SDS-PAGE as per described in section 2.2.1.

2.2.2. In-solution trypsin digestion and LC-MS/MS

Mass spectrometry of crude venom/purified protein was performed according to the method described by Kinter and Sherman [175]. The fractions collected from chromatography (~22.2 µg) were dried and reconstituted in Milli-Q water followed by addition of 50µl of 6M Urea and 2.5µl of 200mM DTT, and incubation at room temperature for 1 hour. Post incubation, 10µl of 200mM Iodoacetamide (IAA) was added to the mixture, vortexed and incubated again in dark with same condition. To consume any unreacted IAA, 10µl of 200mM DTT was added followed by another incubation in dark for 1 hour and addition of 387.5µl of 1mM $CaCl_2$ to reduce the urea concentration to ~0.6 M. Finally, 150 ng/µl of reconstituted trypsin solution (Promega) was added to a final ratio of 1:50 (w/w, trypsin: protein). The solution was vortexed and incubated at

37°C overnight. To stop the reaction, formic acid was added to the mixture and pH was adjusted to 3-4.

The digested peptides in the mixture were separated by desalting using Nanospray capillary column (PepMapTM RSLC C18, ThermoFisher Scientific, Waltham, MA, USA). Peptide mixtures were loaded on C-18 Zip tips pre-equilibrated with 0.1% TFA. The samples were aspirated and dispensed repeatedly ten times for effective binding of peptides to the C18 tips. The tips were washed in 0.1% TFA and peptides were eluted in 200µl of 70% acetonitrile, 0.1% TFA. The eluted fractions were vacuum-dried and reconstituted in 5% acetonitrile, 0.1% TFA and subjected to sequencing by Q-Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA). MS/MS mass tolerance was set to 10 ppm. Carbamidomethyl cysteine was set as a fixed modification, and oxidation of methionine and deamidation of Arginine and Glutamine were set as variable modifications. The raw files were viewed in Proteome Discoverer 2.2 (Thermofisher) using the Sequest program. The txid used to generate the protein list was 8764 (*Trimeresurus*). Double and triple charged peptides were selected and based on sequence similarity, peptide fragments were assigned to the proteins in the NCBI database. The matched proteins were filtered for at least two unique peptides and sequest score more than 2. The relative distribution of venom toxin families was calculated considering the total number of proteins identified by MS/MS as 100%.

$$\text{Relative distribution of toxin families} = \frac{\text{No. of protein belonging to one family}}{\text{Total no. of proteins obtained}} * 100$$

2.2.3. In vitro enzyme activity

2.2.3.1. Phospholipase A_2 (PLA_2) activity

The PLA_2 activity of crude venom of green pit vipers was determined by $sPLA_2$ assay kit according to manufacturer's instruction. Different doses of crude venoms (0.01-1 µg/ml) were mixed with assay buffer to a final volume of 7.5 µl followed by addition of 5 µl DNTB [5,5'-dithio-bis-(2-nitrobenzoic acid)]. The reaction was initiated by adding 100 µl of 1.66 mM of substrate (diheptanoyl thio- phosphatidylcholine). The rate of hydrolysis of substrate was quantified by measuring OD at 405 nm for 10 minutes using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). One unit of PLA_2 activity (U) was expressed as micromoles of substrate hydrolysed per minute. Crude Russell's viper venom (RVV) was used as control for comparative assessment.

2.2.3.2. Coagulation activity

Effect of crude venoms on coagulation time was assessed by calculating Recalcification time (RT), Prothrombin time (PT) and Activated partial thromboplastin time (APTT) using platelet poor plasma. Crude Russell's viper (*Daboia russelii*) venom (RVV) was used as positive control for comparative assessment in each of these clotting experiments.

Preparation of platelet poor plasma (PPP): Fresh goat blood was collected from butcher shop in 3.8% Trisodium citrate in 9:1 (whole blood: anticoagulant) ratio. Platelet poor plasma (PPP) was separated from whole blood by centrifugation at 5000 rpm for 20 minutes at 4ºC. The pale yellowish supernatant was collected as platelet poor plasma in microcentrifuge tubes and stored at -20ºC.

Recalcification time (RT): RT was determined using Platelet Poor Plasma (PPP) according to the methods described by Deka and team [45]. Different doses of crude venoms (0.1-20 µg/ml) were preincubated with 50 µl of PPP for 2 minutes at 37˚C. Formation of clot was initiated by addition of 50 mM $CaCl_2$ and change in OD was monitored at 405 nm every 10 sec for 900 seconds using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). The clotting time of PPP without venom was taken as Normal clotting time (NCT).

RT was also determined for different doses (0.01-10 µg/ml) of purified protein using same protocol. Clotting of crude venom (10 µg/ml) was taken as control.

Prothrombin time (PT): PT was measured using Uniplastin (PT reagent) according to the instructions of manufacturer. Different doses of crude venoms (0.1-20 µg/ml) were preincubated with 50 µl of PPP for 2 minutes at 37˚C. Clot formation was initiated by addition of 50 µl of Uniplastin and change in OD was monitored at 405 nm every 2 sec for 120 seconds using a MultiSkanGO Spectrophotometer (Thermo Scientific, USA). The clotting time of PPP without venom was taken as Normal clotting time (NCT).

Activated partial thromboplastin time (APTT): APTT was measured using Liquecelin (APTT reagent) according to the instructions of manufacturer. Different doses of crude venoms (0.1-20 µg/ml) were preincubated with 50 µl of PPP and 50 µl of liquecelin for 3 minutes at 37˚C. Clot formation was initiated by addition of 50 µl of 25 mM $CaCl_2$ and change in OD was monitored at 405 nm every 2 sec for 120 seconds using a

MultiSkanGO Spectrophotometer (Thermo Scientific, USA). The clotting time of PPP without venom was taken as Normal clotting time (NCT).

2.2.3.3. Thrombin-like activity assay

Thrombin-like activity assay of crude venoms was assessed on chromogenic substrate S2238 specific for thrombin. Briefly, different doses of crude venom (0.1-20 µg/ml) were incubated with assay buffer (20 mM Tris-Cl, 150 mM NaCl and 1% BSA, pH 7.4) to a final volume of 100 µl for 2 min at 37˚C. The reaction was initiated by adding 50 µl of S2238 (final concentration 100 µM) and the release of coloured product *p*-nitroaniline (*p*-NA) was monitored by measuring OD at 405 nm for 15 minutes using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). Thrombin (final concentration 0.5 nM) was taken as control. One unit of thrombin-like activity (U) was expressed as micro moles of substrate hydrolysed per minute.

Screening of Rp-HPLC fractions were done on the basis of their thrombin-like activity and the assay was performed at each step of purification. Thrombin-like activity of purified protein was assessed for an increasing dose of 0.01-10 µg/ml considering the activity of crude venom of *T. erythrurus* as control.

2.2.3.4. Plasmin-like activity assay

Screening of Rp-HPLC fractions was also performed by assessing plasmin-like activity using chromogenic substrate S2251. Briefly, each fraction (10 µg/ml) was incubated with 50 µl assay buffer (20 mM Tris-Cl and 150 mM NaCl, pH 7.5) to a final volume of 100 µl for 2 min at 37˚C. Post incubation, the reaction was initiated by adding 50 µl of substrate S2251 (final concentration 200 µM) and the release of coloured product *p*-nitroaniline (*p*-NA) was monitored by measuring OD at 405 nm for 15 minutes using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). Plasmin (final concentration 10 nM) was taken as positive control. One unit of plasmin-like activity (U) was expressed as micro moles of substrate hydrolysed per minute.

Plasmin-like activity of purified protein was assessed in the similar way for an increasing dose of 0.01-10 µg/ml considering the activity of crude venom of *T. erythrurus* as positive control.

2.2.3.5. Caseinolytic activity

Proteolytic activity of crude venoms was studied using casein as substrates [64]. Different doses of crude venoms (1-50 µg/ml) were preincubated with 1 ml of 1% casein for 90 min at 37˚C. The reaction was stopped by addition of 500 µl of 10% ice cold trichloroacetic acid (TCA) followed by incubation at 4˚C for 20 min. The mixtures were then centrifuged at 5000 rpm for 10 min and the supernatant was subjected to protein estimation by Lowry's method using tyrosine standard curve. One unit of caseinolytic activity (U) was defined as number of mole equivalent of tyrosine formed per min per ml. Crude Russell's viper venom (RVV) was used as a positive control for comparative assessment.

2.2.3.6. Haemolytic activity

Haemolytic assay was performed using goat red blood cells according to the method previously described [176] including modification by Doley and Mukherjee [177].

Preparation of red blood cells (RBCs) suspension: Fresh goat blood collected in 3.8% Trisodium citrate was centrifuged at 5000 rpm for 20 minutes at 4ºC to collect the supernatant as platelet poor plasma. The pellet containing red blood cells (RBCs) was washed with 0.9% NaCl by centrifugation at 5000 rpm for 15 minutes at 4ºC. The process was repeated three times and the washed pallet was resuspended in 0.9% NaCl in a way to make 10% RBC suspension.

Direct haemolytic assay: For direct haemolytic assay, different doses of crude venoms (0.1-20 µg/ml) were incubated with 150 µl of 10% RBC to a final volume of 2 ml, adjusted by 0.9% NaCl for 1 hour at 37˚C. Following incubation, the reaction mixture was centrifuged at 10000 rpm for 10 min and OD of supernatant was measured at 540 nm using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). The percentage of haemolysis was calculated considering haemolysis caused by distilled water as 100%. The RBCs incubated with only 0.9% NaCl was taken as negative control. Haemolytic activity of Russell's viper venom (RVV) was used as a positive control for comparison.

Indirect haemolytic assay: Indirect haemolytic activity of crude venom was assessed using egg yolk suspension as described previously by Deka et al 2017. Egg yolk suspension was freshly prepared by mixing yolk of one egg with 250 ml 0.9% NaCl.

Different doses of crude venom (0.1-20 µg/ml) were mixed with 150 µl of 10% RBC suspension and 20 µl of egg yolk suspension in a final volume of 2 ml adjusted with 0.9% NaCl. The reaction mixture was then incubated for 1 hour at 37˚C followed by centrifugation at 10000 rpm for 10 min to collect supernatant. The absorbance of supernatant was measured at 540 nm using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). The percentage of haemolysis was calculated considering haemolysis caused by distilled water as 100%. RBCs treated with 0.9% NaCl was taken as negative control. Haemolytic activity of Russell's viper venom (RVV) was used as a positive control for comparison.

Haemolytic activity of purified was performed for an increasing dose of 0.001-1 µg/ml considering the activity of crude venom as positive control.

2.2.3.7. Platelet aggregation activity

Platelet aggregation activity was performed using platelet rich plasma (PRP) and platelet poor plasma (PPP) separated from human blood (ethical clearance no DoRD/TUEC/PROP/2022/01 dated 21/03/2023, enclosed in Appendix II).

Preparation of platelet rich plasma (PRP): For preparation of PRP, whole blood was collected from healthy adult volunteer in tubes containing 3.8% tri-sodium citrate and allowed to stand vertically for 10 mins. PRP was separated out by centrifuging the standing whole blood tubes at 160g for 6 mins. The yellowish supernatant containing PRP was collected by pipetting out and used immediately within 4 hours. After collecting PRP, the tubes were centrifuged again at 5000 rpm for 20 minutes, and the pale-yellow coloured clear supernatant was collected as PPP.

Platelet aggregation study: The effect of crude venom on platelet aggregation was studied according to method developed by Born using Lumi-Aggregometer (CHRONO-LOG Corporation, USA) [178]. Briefly, 500 µl of PRP was pre-incubated in glass cuvettes for 3 minutes at 37ºC followed by addition of 0.5 µg/ml, 1 µg/ml and 2 µg/ml of crude venom separately and percentage of aggregation was observed. The maximum platelet aggregation was recorded within 6 minutes with continuous stirring at 37ºC. The light transmittance was calibrated with PPP. Platelet aggregation of PRP induced by 1.34 nM thrombin and 1 µg (in reaction) collagen was taken as positive control and aggregation of only PRP was taken as blank.

Percentage of platelet aggregation was also evaluated for PPP treated with different doses of purified protein (1- 5 µg) in the similar way.

2.2.3.8. Plasminogen activation activity

Plasminogen activation assay was performed according to the method used by Park et al. [179]. Briefly, different doses of purified protein (0.1-10 µg/ml) were incubated with 0.01U of plasminogen in a total volume of 100 µl of assay buffer (20 mM Tris-Cl buffer and 150nM NaCl, pH 7.5) for 10 minutes at 37˚C. Following incubation, 20 µl aliquot was taken in a microtiter plate and mixed with 180 µl of plasmin-specific substrate S2251 (1 mM stock). The release of coloured product *p*-nitroaniline (*p*-NA) was monitored by measuring OD at 405 nm for 15 minutes using a MultiSkan GO Spectrophotometer (Thermo Scientific, USA). Ten nanomolar of tissue- plasminogen activator (t-PA) was taken as positive control. One unit of plasminogen activation (U) was expressed as micro moles of substrate hydrolysed per minute.

2.2.3.9. Fibrino(gen)olytic activity

SDS-PAGE method: The fibrinogenolytic activity of crude venoms was estimated using bovine plasma fibrinogen as per the method described by Ouyang and Teng [180]. Briefly, different doses of crude venoms (0.1-10 µg) were preincubated with 50 µl fibrinogen (2 mg/ml) and 50 µl assay buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.4) for 1 hr at 37˚C. Following incubation, the resultant products were subjected to SDS-PAGE using 10% Tris-glycine gel in reducing condition as mentioned in section 2.2.1. After the electrophoretic run, gel was stained with Coomassie Brilliant Blue and analysed for band degradation. Fibrinogen without venom was considered as negative control. Fibrinogen incubated with 10 u/ml Thrombin was taken as positive control.

The fibrinogenolytic activity of purified protein was also estimated by electrophoretic method in both dose dependent and time dependent manner. Different doses of purified protein (0.1-10 µg) were incubated with fibrinogen for 1 hour at 37˚C. Following incubation, the resultant products were subjected to SDS-PAGE using 12.5% Tris-glycine gel in reducing condition. For time dependent analysis, 10 µg of purified protein was incubated with fibrinogen in similar condition as mentioned above. Aliquots were taken at different time intervals ranging from 0 minute to 48 hours and subjected to electrophoresis. Fibrinogen without venom was considered as negative control.

Fibrinogen treated with Thrombin (10 u/ml), plasmin (1 µg) and crude venom (5 µg) was considered as positive control.

Rp-HPLC method: The fibrinogenolytic activity of purified protein was also evaluated chromatographic method for the analysis of cleavage pattern. Firstly, 10 µg of purified protein was mixed with 100 µg of bovine fibrinogen in 50 mM Tris-Cl, 150 mM NaCl, pH 7.4 and incubated at 37ºC for 1 hour. Fibrinogen only and fibrinogen treated with thrombin and plasmin was taken as negative and positive controls for the experiment. Following incubation, the tubes were cooled in a refrigerator for 5 minutes and subjected to reverse phase-HPLC using acclaim C18 column with a linear gradient of 20-65% acetonitrile with 0.01% TFA.

2.2.3.10. Fibrinolytic activity

Fibrinolytic activity of purified protein was assessed according to the method described by Astrup and Mullertz [181] and slightly modified by Kaur et al in 2019 [182]. Firstly, fibrin plate was prepared by mixing fibrinogen (5 mg/ml) and thrombin (3 U/ml) in 1% agarose (w/v). The plate was allowed to stand at room temperature till fibrin polymerization. Wells were punctured post polymerization and different doses (1-15 µg) of protein and plasmin was added to the wells. Assay buffer without any treatment was taken as blank. The plate was allowed to stand at room temperature for 2 hour and then incubated overnight at 37˚C. The presence of zone of clearance around the wells confirmed fibrinolytic activity.

2.2.3.11. Plasma clot formation/dissolution activity

The coagulation pattern of platelet poor plasma (PPP) upon treatment with crude venoms and purified protein was observed by manual examination of clot formation and dissolution. Firstly, 50 µl of platelet poor plasma isolated from human blood was mixed with 25 µl of 20 mM Tris-Cl, pH 7.4 on a clean glass slide. Clot formation was initiated upon treatment with $CaCl_2$, thrombin, crude *Daboia russelii* venom, crude *Trimeresurus erythrurus* venom and purified protein separately. Formation of clot was observed by appearance of fine thread-like structure on the needle. For each set of experiment, clot formation time, clot dissolution time and nature of clot was recorded. Platelet poor plasma only was taken as negative control.

2.2.4. Immuno-reactivity assays

2.2.4.1. Immuno-blotting study

The immunoreactivity of crude venoms and purified protein towards commercially available antivenom was examined using western blotting [183]. Crude venoms (20 µg) were subjected to 10% Tris-Tricine SDS-PAGE in reduced condition and electrophoresis was carried out at 120 V using Mini PROTEAN (Bio-Rad, CA, USA). For purified protein, 8 µg of sample was run on 12.5% Tris-glycine gel in reducing condition. Separated venom proteins in gel matrix were electro-transferred to pre-soaked PVDF membrane (activated by methanol) at 100 volts for 90 min. The membrane was immersed in blocking solution (1% BSA in TBST) for 1 hour at room temperature with gentle shaking followed by washing thoroughly with wash buffer (TBST). The blocked membrane was then incubated overnight at 4℃ with 1:500 (v/v) dilutions of polyvalent antivenom- PSVAV (10 mg/ml) and monovalent antivenom- GPVAV (10 mg/ml). The membrane was then washed with wash buffer and incubated with 1:1000 (v/v) dilution of alkaline phosphatase conjugated anti-horse IgG for 2 hours with gentle shaking. The membrane was washed again rigorously with wash buffer and developed using pre-mixed 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) liquid substrate till colour appeared. The reaction was stopped with 1% acetic acid.

2.2.4.2. Neutralization of biochemical activities

Neutralization studies were performed for PLA_2 activity, procoagulant activity, thrombin-like activity and fibrinogenolytic activity of crude venoms by PSPAV and GPVAV. Fixed doses of crude venoms (0.1 µg/ml for PLA_2 activity assay, 10 µg/ml for coagulation and thrombin-like activity assay, 5 µg for fibrinogenolytic activity) were incubated with PSPAV and GPVAV separately along with assay buffers at 37˚C for 1 hour in a venom: antivenom ratio of 1:100 (w/w). Following incubation, the mixtures were subjected to respective assays as previously described in section 2.2.2.1, 2.2.2.2, 2.2.2.3 and 2.2.2.9. Venom incubated with assay buffer without antivenom was used as control for all sets of experiments. Percentage inhibition was calculated taking enzyme activity of control as 100%.

$$\text{Inhibition percentage} = \left(\frac{\text{Residual enzyme activity}}{\text{Enzyme activity of control}}\right) \times 100$$

Immuno-reactivity of purified protein with Indian polyvalent antivenom was assessed by neutralization of thrombin-like activity and plasmin-like activity. Purified

protein (10 µg) was incubated with assay buffer containing Indian polyvalent antivenom in a venom: antivenom ratio of 1:0, 1:1, 1:10, 1:100 and 0:1 ratio (w/w) at 37˚C for 1 hour. Post incubation, the mixtures were subjected to thrombin-like activity and plasmin-like activity assays as described in section 2.2.2.3. and 2.2.2.4 and residual activity was calculated.

2.2.4.3. Immunodepletion study

Immunodepletion study was performed to evaluate the immunocapturing capability of premium serum polyvalent antivenom (PSPAV) towards venom toxins of green pit vipers using the method described previously [63, 184]. Firstly, 0.35 gram of CNBr activated Sepharose 4B matrix was swelled in 25 ml of 1mM ice-cold HCl for 1 hour with gentle shaking, followed by washing with another 25 ml of ice cold HCl and 2 matrix volumes (2 ml) of coupling buffer (0.2M $NaHCO_3$, 0.5M NaCl, pH 8.3) respectively. Coupling of antivenom with matrix was achieved by incubating premium serum antivenom (PSPAV) dissolved in 2 matrix volumes coupling buffer (30 mg) with activated matrix overnight at 4 ºC with gentle shaking. Antivenom coupling yield, quantified by measuring the concentration before and after incubation, using Nanodrop 2000 (ThermoFishcher Scientific, USA) was estimated to be 16 mg/ml beads. The uncoupled active groups were blocked with 2 matrix volumes of 0.1 M Tris-Cl, pH 8.5 at room temperature for 4 hours. Affinity columns were prepared by packing 250 µl matrix in pierce centrifuge columns containing 4 mg antivenom coupled to matrix. The columns were alternatively washed with 500 µl of low pH buffer (0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0) and 500 µl high pH buffer (0.1 M Tris-Cl, pH 8.5). The process was repeated for 6 times followed by equilibration of columns with 5 matrix volume of binding buffer (PBS; 20 mM phosphate buffer, 135 mM NaCl, pH 7.4).

Following affinity column preparation, 50 µg of crude green pit viper venom dissolved in 125 µl of PBS was incubated with matrix for 1 hour at 25 ºC with gentle shaking (venom: antivenom ratio was maintained at 80:1). Post incubation, non-retained fractions were collected in 5 matrix volume of PBS and retained fractions were eluted out in 0.1 M glycine-HCl, pH 2.0 and immediately neutralized with 125 µl 1M Tris-HCl, pH 9.0. The collected fractions were concentrated using pierce centrifuge concentrators and reconstituted in Milli Q water. Fractions were then subjected to Rp-HPLC using Thermo Scientific UltiMate 3000 high performance gradient system equipped with DAD

detector. The fractions were loaded on Acclaim C18 column pre-equilibrated with Milli Q water containing 0.1% TFA. The separation was carried out at a flow rate of 0.2 ml/minute with a gradient of 20-55% acetonitrile for 50 minutes followed by 55-80% acetonitrile for 10 minutes and eluted protein was detected at 215 nm.

2.2.5. Clinical case study

Clinical features of green pit viper bites were recorded from a retrospective observational study conducted in Demow Community Health Centre, Sivasagar District, Assam, India (27.128056652348704 N, 94.7396135802723 E) between 2010 and 2021. The permission for data review and publication was obtained from Institutional Head and Office of the Joint Director of Health, Sivasagar District, Assam, India (Permission Number 834, dated 2/2/2022, Appendix II). Data of 100 patients with confirmed green pit viper bite, treated conservatively without antivenom, was considered for the study. Clinical data was retrieved from the bed-head tickets of all admitted patients with due consent. Identification of the snake as green pit vipers was done by photographs and/or the dead body of the snake responsible for the bite, brought to the health centre by relatives of patients.

2.2.6. Computational studies

2.2.6.1. Multiple sequence alignment

Proteins showing high similarity with purified protein were identified by online blastp algorithm (http://blast.ncbi.nlm.nih.gov) and their FASTA sequence were retrieved from NCBI database. Multiple sequence alignment of FASTA sequences viz. GPV-TL1 (accession no. A7LAC6) and chitibrisin (accession no. P0DJF6) from *T. albolabris*, stejnobin (accession no. Q8AY81) and stejnofibrase 1 (accession no. AAN52348.1) from *V. stejnegeri* was performed using online Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). Amino acid sequence of commercially available therapeutic thrombin-like enzymes batroxobin (accession no. P04971.1) from *Bothrops atrox* and ancrod (accession no. P47797.1) from *Calloselasma rhodostoma* along with heavy chain of human alpha-thrombin (accession no. 494462) were also aligned. Residues were highlighted in different colours manually.

2.2.6.2. Homology modelling and structure validation

Since all the fragments of purified protein could not be retrieved from MS/MS and the identified fragments showed 100% homology with albofibrase, structure prediction was performed for albofibarse. The primary sequence of albofibrase was retrieved from UniProt database (https://www.uniprot.org/). Tertiary structures of albofibrase was modeled using homology modeling approach in ExPASy SWISS-MODEL server (https://swissmodel.expasy.org/) [185, 186]. Homology modeling of albofibrase was done using the structure of defibrase, a thrombin-like enzyme from *Gloydius saxatilis*as template (PDB ID: 3S691_A) as it has 81.97% identity with albofibrase. The modeled structure was visualized in Biovia Discovery Studio 2021 R2 software package. The accuracy of the modeled structures was validated by generating Ramachandran Plot at PROCHECK program of SAVES v6.0 server (https://servicesn.mbi.ucla.edu/PROCHECK/). For comparison, structure of human alpha-thrombin (PDB ID: P00734) was superimposed with the predicted structure of albofibrase in Chimera 1.16 (https://www.cgl.ucsf.edu/).