Chapter 2

Materials and methods

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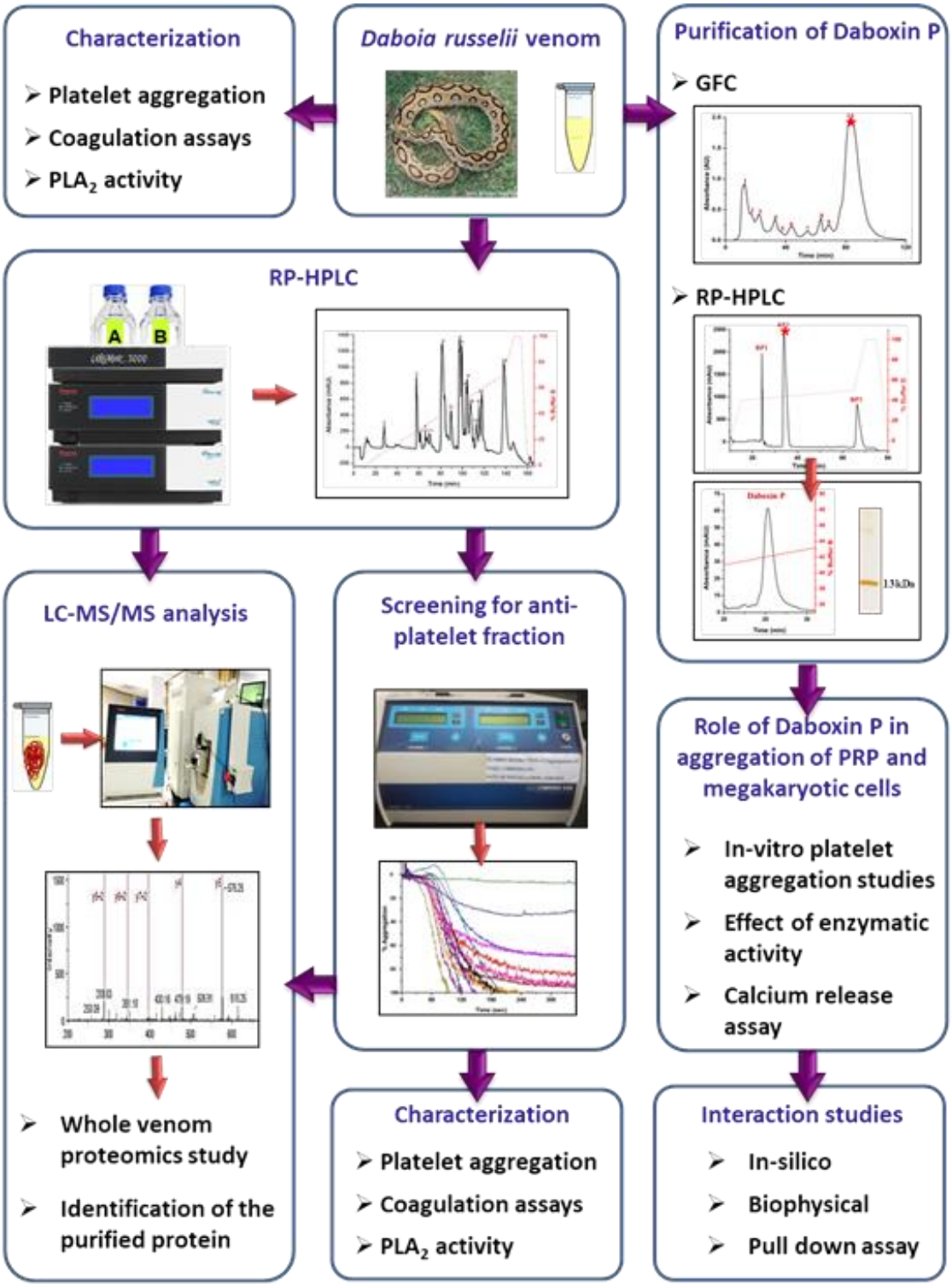
Work Flow



2.1 MATERIALS:

Crude venom: Lyophilised crude venom of *Daboia russelii* from Tanore, Rajshahi, Bangladesh (24°43'27"N 88°25'01.3"E) was generously gifted by Md Abu Reza, Professor, University of Rajshahi, Bangladesh. Collection/milking of venom was approved by the Indtitutional Animal, Medical Ethics, Biosafety and Biosecurity committee, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Lyophilised crude venom of *Daboia russelii* from Tamil Nadu, India was purchased from Irula Snake Catchers Society. Permission to purchase the venom for research purpose was obtained from Forest Range Officer, Wildlife Enforcement Range, Chennai-32

Columns: Zorbax 300SB C18 column from Agilent (CA, USA) was used to fractionate of crude venom for the proteomic analysis. Fractionation of crude venom for screening and identification of anti-platelet protein was performed on Jupiter C18 column from Phenomenex (CA, USA). For purification of Daboxin P, gel filtration column, Hiload ^{TM} 16/600 Superdex 75 column from GE Healthcare Life Sciences (Bucks, UK) and HPLC column, Acclaim^{TM} 300 from ThermoFisher Scientific (MA, USA) was used.

Chemicals and reagents: HPLC grade acetonitrile and trifluoroacetic acid were purchased from Merck Millipore (MA, USA). Sequence grade trypsin was purchased from Promega (Wisconsin).

Assay kit: $sPLA_2$ assay kit was purchased from Cayman Chemical (Michigan, USA).

Substrate: Chromogenic substrate, S2238 was purchased from Chromogenix.

Blood and plasma: For coagulation assays, Platelet Poor Plasma (PPP) was isolated from goat blood collected from local butcher. For platelet aggregation assays, Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) were isolated from blood collected from consenting healthy adult volunteers. Ethical permission was obtained from Tezpur University Ethics Committee (DoRD/TUEC/PROP/2022/01).

Agonists: The agonists for platelet aggregation, collagen, adenosine diphosphate (ADP), arachidonic acid (AA) and ristocetin were procured from Chrono-log Corporations (PA, USA) while bovine α-thrombin was purchased from Sigma-Aldrich (MO, USA).

Cells: K-562 cells were procured from National Centre for Cell Sciences (Pune, India).

Antivenom: Polyvalent antivenom was purchased from ViNS Bioproducts Limited (Hyderabad, India).

Others: All other reagents and chemicals used were of analytical grade and purchased from Merck Millipore (MA, USA), Sigma-Aldrich (MO, USA), Tulip Diagnostics Pvt. Ltd. (Goa, India), Himedia (Mumbai, India), Rankem (PA, USA), GE Healthcare Bio-Sciences (Uppsala, Sweden), Bio-Rad or ThermoFisher Scientific (MA, USA).

2.2 METHODOLOGY:

2.2.1 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE):

The venom of *Daboia russelii* was subjected to electrophoresis according to the method developed by Laemmli [104]. Briefly, 12.5% resolving poly acrylamide gel was prepared by adding 4.15 ml 30% poly acrylamide in a total 9.36 ml solution containing 0.4 ml glycerol, 2.5 ml 1.5M Tris Cl (pH 8.8), 0.1 ml 10% SDS, 0.1 ml 10% APS, 0.01 ml TEMED and 2.1 ml distilled water. Stacking poly acrylamide gel was prepared by adding 0.65 ml 30% poly acrylamide in a total 5 ml solution containing 0.2 ml glycerol, 1.25 ml 0.5M Tris Cl (pH 6.8), 0.05 ml 10% SDS, 0.05 ml 10% APS, 0.075 ml TEMED and 2.8 ml distilled water. 10 ml loading buffer was prepared by adding 0.6 g SDS, 3 ml glycerol, 0.5 M Tris Cl (pH 6.8) and bromophenol blue. For reducing condition, β-ME was added in the ratio of 1:9. 5 µl loading buffer was mixed with 15 µl sample (10µg) and loaded into wells. For reducing condition, the mixture was boiled for 3 minutes at 95°C before loading into the wells. The electrophoresis was performed at constant Volt (80 V) for stacking and 120 V for resolving. For detection of bands, coomassie brilliant blue staining was performed. The gel was stained with CBB-R250 dye (0.25% w/v) in methanol:water:acetic acid (50:40:10) and subsequently destained with destaining solution, viz., methanol:water:acetic acid (50:40:10) till the protein bands were visible.

For chromatographic fractions (anti-platelet fraction, gel filtration fractions and Daboxin P), 5 µg protein was subjected to 12.5% SDS-PAGE under both reducing and non-reducing conditions. For visualization of protein bands, the gel was subjected to silver staining. The gel was fixed in fixing solution containing 50% methanol, 10%

acetic acid and 50 µl formaldehyde for 1 hour and washed 3 times with 50% ethanol for 20 mins each. It was then treated with hypo solution (0.02% sodium thiosulfate) for 1 min precisely and the excess solution was washed off with distilled water three times for 20 seconds. Subsequently, the gel was treated with 0.2% silver nitrate solution for 30 minutes with gentle shaking followed by washing with distilled water thrice. Then the gel was developed using developing solution (6% sodium carbonate, 1 ml hypo solution and 50µl formaldehyde) till bands become visible. The reaction was subsequently stopped using 5% acetic acid solution.

2.2.2 Fractionation of crude *Daboia russelii* venom:

For proteomics studies, lyophilized crude *Daboia russelii* venom (1mg) reconstituted in miliQ water was fractionated using Zorbax 300SB C18 column (4.6x150 mm, 3.5µ, 300A^{o}). The column was pre-equilibrated with miliQ water containing 0.1% trifluoro acetic acid (TFA) and fractionated using 100% acetonitrile (MeCN) containing 0.1% TFA. The fractionation was carried out with a multi-step gradient of 15-45% and 45-70% of acetonitrile maintained in 7-37 minutes and 37-82 minutes respectively with a flow rate was maintained at 0.8 ml/min. Total run time was set for 95 minutes. The protein peaks monitored at 215 nm were collected manually and vacuum dried.

For screening of anti-platelet proteins, lyophilized crude *Daboia russelii* venom (2mg) was fractionated using Jupiter C18 column (4.6 x 250mm, 3µ, 300A^{o}) with gradient of 5-70 % of MeCN maintained in 15 -140 minutes. The flow rate was maintained at 0.6ml/min and total run time was set for 166 minutes.

For purification of Daboxin P, a two-step chromatographic approach consisting of gel filtration chromatography (GFC) and reverse phase- HPLC (RP-HPLC) was used. Lyophilized crude *Daboia russelii* venom (10mg) was fractionated using Hiload^{TM} 16/600 Superdex 75 prep grade column pre-equilibrated with 50 mM Tris-Cl (pH 7.4). Fractionation was carried out at a flow rate of 1 ml/min under an isocratic condition with the same buffer and monitored at 215 nm. The fractions were collected manually. The gel filtration peak 10, P10 (1mg) was subjected to RP-HPLC using Acclaim C18 column. The flow rate was maintained at 1ml/min and a gradient of 40-50% MeCN in 15-65 minutes was used for the separation of proteins. The total time of run was set for

90 minutes. The RP-HPLC fraction 2 (RP2) was rechromatographed using the same conditions to ensure its purity.

2.2.3 LC-MS/MS analysis:

2.2.3.1 In-solution digestion method:

For proteomics analysis of crude Daboia russelii venom, in-solution trypsin digestion was performed. The dried HPLC fractions were reconstituted in Milli Q water to a final concentration of 1ug/ul and subjected to reduction with 50µl of 6M Urea and 2.5µl of 200mM DTT followed by alkylation with 200mM Iodoacetamide (IAA). Both the reduction and alkylation steps were carried out at room temperature in dark for 1 hour. Excess unreacted IAA was consumed by addition of another 200mM DTT and the concentration of urea was reduced by addition of a buffer containing 50mM Tris-HCl and 1mM $CaCl_2$. Subsequently, each fraction was treated with trypsin in a ratio of 1:50 (w/w, trypsin: protein) at 37℃ overnight; the reactions were later quenched by adding formic acid. The trypsin digested samples were desalted using C18 Zip tips (ThermoFisher Scientific, USA) pre-equilibrated with 0.1% TFA by repeated aspiration and dispension at least ten times for effective binding of peptides to the tips. The samples were eluted in 400µl of buffer containing 70% MeCN and 0.1% TFA and vacuum dried. The samples were reconstituted in 5% MeCN and 0.1% TFA before loading in a Nanospray capillary column (PepMap™ RSLC C18, Thermo Fisher Scientific, Waltham, MA, USA) (75µm x 150mm, 3µ, 100A^{o}). The analytical separation was carried out using a gradient of 20-45% MeCN at a flow rate of 300nl/min and simultaneously subjected to MS/MS analysis in Q-Exactive HF (Thermo Fisher Scientific, Waltham, MA, USA). The spectra were analyzed using Proteome Discoverer 2.2 using Sequest program against the NCBI protein database of *Daboia* genus (NCBI Taxid: 42188). Carbamidomethyl cysteine was set as a fixed modification and oxidation of methionine and deamidation of Arginine, and Glutamine were set as variable modification. MS/MS mass tolerance of 10 ppm was allowed. Double and triple charged peptides were selected and each peptide fragment was assigned to a protein in the NCBI database based on m/z ratio and sequence similarity. Assignment of the proteins was validated based on presence of alteast 2 unique peptides and more than 2 sequest score. The relative abundance of the snake venom protein families present in

the venom was calculated by considering total number of proteins identified as 100% [105].

Relative abundance of the protein families = (Number of proteins belonging to one family/ Total number of proteins) x 100%.

2.2.3.2 In-gel digestion method:

For identification of the anti-platelet fraction (P9), in-gel trpsin digestion was performed. P9 (10 µg) was subjected to 12.5% SDS-PAGE under reducing condition as discussed earlier in section 2.2.1. For visualization of protein bands, the gel was subjected to coomassie brilliant blue staining. Subsequently, the protein band was excised and subjected to digestion by trypsin. In-gel trypsin digestion of coomasie stained gel bands obtained from reducing SDS-PAGE was carried out according to Babele et al., 2019 [106] with slight modifications. The protein bands were excised from SDS-PAGE gels, repeatedly washed with 50% acetonitrile for 1 h at room temperature and dried. The dried gel pieces (100ng) were treated with trypsin (Promega, USA) in 50 mM NH_4HCO_3 at 37°C overnight and the peptides were extracted with 60% acetonitrile and 0.1% formic acid.

The trypsin digested samples were subsequently desalted offline by C18 column prior to loading them on Trap (ChromXP C-18-CL-3 µm, 120Å, 300 µm x 0.5 mm) and analytical micro column (3C18 CL, 300 µm x 15 mm, 3 µm, 120Å). LC-MS/MS was performed using a micro gradient pump for peptide separation which were analysed using an Eksigent nano LC-Ultra® 2D System, connected on-line with a Triple TOF 5600 mass spectrometer (Sciex) as described by Babele et al., [107]. The peptides obtained were identified via database searching against the NCBI *Daboia russelli* protein database (July 2022, 125 entries), with ProteinPilot 5.0 software. The peptide fragments obtained were aligned with Daboxin P (C0HK16.1), Dabocetin alpha (ADK22821.1) and Dabocetin beta (ADK22822.1) online using the blastp algorithm.

Similarly, presence of Daboxin P in RP2 was confirmed by in-gel trypsin digestion followed by LC-MS/MS analysis. The peptide fragments obtained were aligned with Daboxin P (C0HK16.1) online using the blastp algorithm.

2.2.4 Multiple sequence alignment and phylogenetic analysis:

The amino acid sequences of the PLA_2 enzymes identified in the proteome were retrieved from NCBI database by blastp (blast.ncbi.nlm.nih.gov) search. For multiple sequence alignment, these sequences were aligned using DNAMAN 4.1.5.1 (Lynnon BioSoft). Subsequently, the phylogenetic relationship of the PLA_2 enzymes was studied using the Mega 11 software [108]. The retrieved sequences were aligned using ClustalW with default parameters and the phylogenetic tree was constructed using Neighbour joining algorithm. A bootstrap value of 1000 replicates was considered for the analysis.

2.2.5 Biochemical characterizations:

2.2.5.1 Coagulation assays:

Preparation of platelet poor plasma (PPP): For the coagulation assays, goat platelet PPP was prepared by centrifuging citrated whole goat blood at 900g for 20 minutes and collecting the clear yellowish supernatant.

Recalcification time: Recalcification time was determined according to method developed by Langdell and colleagues with slight modifications [109]. Different doses of crude *Daboia russelii* venom and the anti-platelet fraction (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml) were pre-incubated with 50 µl of goat PPP for 2 minutes at 37ºC and the plasma clot formation was initiated by addition of 25 µl of 50 mM $CaCl_2$. The change in absorbance was monitored at 405 nm every 15 seconds for 900 seconds using a MultiSkanGO spectrophotometer (Thermo Scientific, USA). For each set of experiment, clotting time of PPP with 20 mM Tris-Cl buffer (pH 7.4) was considered as the normal clotting time (NCT).

Prothrombin time: Prothrombin time was measured using Uniplastin according to the manufacturer's protocol. Different doses of crude *Daboia russelii* venom and the anti-platelet fraction (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml) were pre-incubated with 50 µl of goat PPP for 2 minutes at 37ºC. Subsequently, 50 µl of Uniplastin was added to initiate clot formation which was monitored at 405 nm every 2 seconds for 120 seconds using a MultiSkanGO spectrophotometer (Thermo Scientific, USA). For each set of experiment, clotting time of PPP with 20 mM Tris-Cl buffer (pH 7.4) was considered as NCT.

Activated partial thromboplastin time: Activated partial thromboplastin time was determined using Liquecelin according to the manufacturer's instructions. Briefly, different doses of the crude *Daboia russelii* venom and the anti-platelet fraction (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml) were pre-incubated with 50 µl goat PPP and 50 µl APTT reagent for 3 min at 37°C. Subsequently, 50 µl of 25 mM $CaCl_2$ was added to trigger the clotting time of plasma which was monitored at 405 nm every 2 seconds for 300 seconds using a MultiSkanGO spectrophotometer (Thermo Scientific, USA). For each set of experiment, clotting time of PPP with 20 mM Tris-Cl buffer (pH 7.4) was considered as NCT.

2.2.5.2 Phospholipase A_2 (PLA_2) activity:

Turbidometric method: The PLA_2 activity was determined using turbidometric method as described by Joubert and Taljaard and as modified by Doley and Mukherjee [110,111]. Briefly, different doses of crude *Daboia russelii* venom and the anti-platelet fraction (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml), added in a final volume of 60 µl with 20 mM Tris was mixed with 140 µl of egg yolk substrate (one egg yolk suspended in 250 ml of 0.9% NaCl) and the decrease of absorbance at 740 nm at 15 seconds interval over a period of 10 minutes was measured using MultiSkanGO multiplate reader (Thermo Scientific, MA, USA). 20 mM Tris-Cl, was considered as blank. One unit of PLA_2 activity is defined as a decrease in 0.01 OD in 10 minutes.

$sPLA_2$ assay: The PLA_2 activity of the alkylated Daboxin P was determined using $sPLA_2$ assay kit (Cayman, MI, USA) according to the manufacturer's protocol using diheptanoylthio-phosphatidylcholine as substrate. Briefly, 1 µg /ml of alkylated and non-alkylated Daboxin P, vehicle control and crude *Daboia russelii* venom were added to reaction mixture containing DTNB [5,5'- dithio-bis-(2-nitrobenzoic acid)] and the reaction volume was adjusted to 20 µl with 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) was added to initiate the hydrolytic reaction. The rate of hydrolysis was quantified at 405 nm for 10 min MultiSkanGO spectrophotometer (Thermo Scientific, USA). Reaction mixture containing DTNB and assay buffer was taken as blank. Enzyme activity (U) is expressed in micromoles of substrate hydrolysed per min/µg of enzyme (µM/min/µg). The results are mean ± SD of three independent experiments.

2.2.5.3 Caesinolytic activity:

The caesinolytic activity of the crude venom was analysed according to the method developed by Ouyang and Teng with slight modification by Mukherjee and co-workers [112,113]. Briefly, different doses of crude venom (0.001 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 20 µg/ml and 50 µg/ml) were 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 by the method developed by Lowry using tyrosine standard curve [114]. Enzyme activity was measured as amount of protein released per minute (µg/min). The results are mean ± SD of three independent experiments.

2.2.5.4 Haemolytic activity:

Preparation of Red Blood Cells (RBC) suspension: Washed RBC suspension was prepared by centrifuging citrated whole goat blood at 2800 g for 20 minutes. The pellet containing RBCs was then washed repeatedly by 0.9% NaCl.

Direct haemolysis: For direct haemolytic activity, 150 µl of 10% RBC was incubated with different doses of crude *Daboia russelii* venom and the anti-platelet fraction (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml) and the volume adjusted to 2ml with 0.9% NaCl. The reaction mixture was incubated at 37°C for 1 hr by centrifugation at 10,000 g for 10 mins and the absorbance of supernatant was measured at 540 nm using a MultiSkanGO spectrophotometer (Thermo Scientific, USA). RBC suspension incubated with filtered distilled water was taken as positive control while RBC suspension with 0.9% NaCl was taken as negative control. The percentage of haemolysis was calculated considering haemolysis caused by distilled water as 100%.

Indirect haemolysis: Indirect haemolytic activity was determined similar to direct haemolytic activity, but with the addition of 20 µl of egg yolk suspension (one egg yolk suspended in 250 ml of 0.9% NaCl) to the reaction mixture containing RBCs and different doses of crude *Daboia russelii* venom and the anti-platelet fraction (0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml).

2.2.5.5 Amidolytic Activity: Thrombin like amidolytic activity of the anti-platelet fraction was assessed for doses 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml in buffer containing 20 mM Tris pH7.4, 100 mM NaCl and 1 mg/ml bovine serum albumin. Each dose was pre-incubated for 2 min followed by addition of substrate S2238 (200 mM). The release of colored product *p*-nitroaniline (p-NA) was monitored at 405 nm for 900 secs at an interval of 10 sec using a MultiSkanGO spectrophotometer (Thermo Scientific, USA). Reaction containing buffer, thrombin and substrate was taken as control.

2.2.5.6 Inhibition of amidolytic of thrombin: Inhibition of amidolytic activity of thrombin by the anti-platelet fraction was assessed for doses 0.01µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml in buffer containing 20 mM Tris pH7.4, 100 mM NaCl and 1 mg/ml bovine serum albumin. Each dose was pre-incubated with 0.5 nM thrombin for 30 min followed by addition of substrate S2238 (200 mM). The release of colored product *p*-nitroaniline (p-NA) was monitored at 405 nm for 900 secs at an interval of 10 sec using a MultiSkanGO spectrophotometer (Thermo Scientific, USA). Reaction containing buffer, thrombin and substrate was taken as control.

The effect of Daboxin P on amidolytic activity of thrombin was studied for doses, 5 nM, 100 nM, 150 nM and 200 nM.

2.2.5.7 Platelet aggregation assays:

Preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP): For preparation of PRP, fresh blood from healthy and consenting adults was collected in tubes containing 3.8% tri-sodium citrate (9:1, blood: tri-sodium citrate). It was allowed to stand vertically for 10 mins and PRP was prepared by centrifuging the whole blood tubes at 160g for 6 mins. The yellowish supernatant containing PRP was collected by pipetting out and used immediately within 4 hours. For separation of PPP, the blood left after PRP separation was subjected to another centrifugation step at 900g for 20 minutes. The clear supernatant was collected by pipetting out and used immediately.

Preparation of washed platelets: Fresh platelet-rich plasma was washed with HEPES buffer by two subsequent centrifugation steps at 100g and 800g for 20 minutes each and collecting the supernatant and pellet respectively. The pellet was then resuspended in Tyrode's buffer for further experiments.

In-vitro platelet aggregation assay: In-vitro platelet aggregation was studied according to the method developed by Born [115] using Lumi- Aggregometer (CHRONO-LOG Corporation, PA, USA). Briefly, 1μg/ml of the crude venom was added to PRP and platelet aggregation rate was recorded within 6 minutes with continuous stirring at 37^{o}C. The light transmittance was calibrated with PPP. The dose-dependent effect of *Daboia russelii* venom on platelet aggregation induced by collagen was also studied. Different doses of the venom (0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml), were pre-incubated with 500 μl of PRP in glass cuvettes for 3 minutes at 37^{o}C. Following this, platelet aggregation was induced by addition of agonist, i.e. collagen (2 μg/ml). Platelet aggregation of only PRP induced by collagen was taken as positive control and that without addition of any agonist or venom was taken as negative control.

For identification of anti-platelet fractions, the RP-HPLC fractions of *Daboia russelii* venom were screened for their anti-platelet activity. Briefly, 1 μg of each of the RP-HPLC fractions were pre-incubated with 500 μl of PRP in glass cuvettes for 3 minutes at 37^{o}C. Subsequently, platelet aggregation was induced by addition of agonist, i.e. collagen (2 μg/ml). Percentage inhibition of only PRP induced by agonist was taken as control and considered to be 0%.

The fraction with the most anti-platelet activity was observed to be P9. The effect of P9 on aggregation of platelets induced by different agonists was studied. Briefly, 1 μg of P9 was incubated with 500 μl of PRP in glass cuvettes for 3 minutes at 37^{o}C. Following this, platelet aggregation was initiated by addition of different agonists, namely, adenosine diphosphate (ADP) (10 μM), arachidonic acid (AA), (0.5 mM), ristocetin (1 mg/ml) collagen (2 μg/ml) and thrombin (1.34 nM). Dose dependent experiments were performed to study the effect of P9 on aggregation induced by collagen and thrombin. Briefly, different doses of P9 (0.01 μg/ml, 0.1 μg/ml, 1 μg/ml and 10 μg/ml) were pre-incubated with 500 μl of PRP in glass cuvettes for 3 minutes at 37^{o}C. Subsequently, platelet aggregation was induced by addition of collagen (2 μg/ml) and 1.34 nM of thrombin.

2.2.6 Effect of Daboxin P on platelet aggregation:

2.2.6.1 Aggregation of PRP:

Briefly, 1µg of Daboxin P (163 nM) was pre-incubated with 500µl of platelet-rich plasma in glass cuvettes for 3 minutes at 37^{o}C. Following this platelet aggregation was induced by the addition of agonists, i.e. collagen (2µg/ml), adenosine diphosphate (ADP) (20 µM), arachidonic acid (AA) (1 mM) or thrombin (0.15 IU/ml). For dose-dependent effect of Daboxin P on collagen-mediated platelet aggregation, different doses, i.e. 10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM and 450 nM of protein were added to platelet-rich plasma (adjusted to 2x10^{8} cells/ml) before the addition of collagen as the agonist. In another set of experiments, the said doses were pre-incubated with collagen and added to platelet-rich plasma to study the platelet aggregation. For dose-dependent effect of Daboxin P on thrombin-mediated platelet aggregation, the different doses studied were 10 nM, 50 nM, 100 nM, 150 nM and 200 nM. The effect of Daboxin P on inhibition of thrombin-mediated platelet aggregation was also studied in washed platelets by replacing platelet-rich plasma with washed platelets and platelet poor plasma with resuspension buffer. The results are represented as mean ± SD of six independent experiments with platelets collected from three healthy individuals.

Anti-Aggregation Dose 50 (AD_{50}): To determine the AD_{50} dose of Daboxin P, the percentage inhibition of platelet aggregation induced by 0.15 IU/ml thrombin caused by different doses of Daboxin P (0.1 nM – 1000 nM) was determined as described in the previous section and AD_{50} was calculated using AAT Bioquest [116].

2.2.6.2 Aggregation studies of K-562 cells:

K-562 cells were subjected to megakaryocytic differentiation induced with 50nM phorbol 12-myristate 13-acetate (PMA) for 24 hrs and then incubated in serum-free media for 12 hrs before the experiments were performed. The effect of Daboxin P on the aggregation of megakaryotic cells induced by various agonists was studied as described by Chan et.al., 2018 [117]. Briefly, 100 µl of differentiated K-562 cells suspension was pre-incubated with 163nM Daboxin P and were added to 96 well plates pre-prepared with agonists, i.e. adenosine diphosphate (ADP) (10 µM), arachidonic acid (AA) (0.5 mM), collagen (2 µg/ml) or thrombin (0.15 IU/ml) and incubated for another 5 minutes. Subsequently, the absorbance was recorded on a multimode plate reader (Synergy HTX., Multimode reader) at 600 nm. Aggregation of cells in absence of any

agonist was taken as negative control and that induced by the respective agonists was taken as positive control.

To study the effect of Daboxin P on thrombin-mediated aggregation of megakaryotic cells, 163 nM of Daboxin P was pre-incubated with differentiated K-562 cell suspension for 5 minutes and added to wells pre-treated with 0.15 IU/ml thrombin and absorbance was recorded at 600 nm. In a separate set of experiments, Daboxin P pre-incubated with thrombin for 10 minutes was added to the differentiated cells and absorbance at 600 nm was recorded. The aggregation of cells induced by thrombin was taken as 100%.

2.2.7 Effect of Daboxin P on thrombin mediated calcium mobilization:

The effect of Daboxin P on thrombin mediated calcium mobilization was studied according to Ishii et. al. with slight modifications [118]. Briefly, 2 μM Fura-2AM was incubated with PRP for 1 hour at 37℃ and centrifuged at 800g for 10 mins. The platelet pellet was then suspended in calcium-free buffer (adjusted to $2x10^8$ cells/ml). The extracellular calcium was adjusted to 1 mM $CaC1_2$. Subsequently, the fluorescent intensities were recorded at excitation wavelengths of 340nm and 380nm and at emission wavelength 510 nm using Varioskan Lux spectrophotometer (Thermo Scientific, USA) in resting phase and following addition of agonist (0.15 U/ml thrombin) .To study the effect of Daboxin P, different doses (10 nM, 50 nM, 100 nM, 150 nM and 200 nM) were added to the platelets before addition of thrombin. In a separate set of experiments, the stated doses of Daboxin P, pre-incubated with thrombin for 10 mins at 37℃ were used to stimulate the platelets. The maximum fluorescence signal was obtained after lysing the platelets by addition of 0.1% Triton X-100 at the end of each experiment and then 8mM EGTA was added to obtain the minimum fluorescent signals. The Ca^{2+} levels were calculated as previously described [119,120]. The effect of Daboxin P on thrombin mediated calcium mobilization was also studied on differentiated K-562 cells using Fura-4AM.

2.2.8 Alkylation of Daboxin P:

The alkylation of the active site histidine residue of Daboxin P was carried out according to the method developed by Maduwage et. al. with slight modifications [121] Briefly, 50μg of Daboxin P treated with 5μl of the dissolved p-BPB (100 μg in 30μl of

100% ethanol) was incubated at room temperature for 20 hours and desalted by dialysing in 20mM of Tris-Cl pH 7.4 at 4^{o}C for 3 times. Alkylation of Daboxin P was confirmed by $sPLA_2$ activity test. Subsequently, the effect of alkylated Daboxin P (100 nM) on aggregation of platelet-rich plasma and washed platelets as well as on increase in intracellular calcium concentration was studied.

2.2.9 Interaction studies of Daboxin P and thrombin:

2.2.9.1 In-silico approach:

The pdb files of Daboxin P (C0HK16), thrombin (P00734), the extracellular fragments of PAR1 (3LU9) and PAR4 (3QDZ) were retrieved from UniProt database [37,91,122,123] Interactions between Daboxin P – Thrombin, Thrombin – extracellular fragment of PAR1 and Thrombin – extracellular fragment of PAR4 were studied in-silico. Molecular docking was done in ClusPro 2.0 server [124]. Tertiary structures of the proteins and fragments were given as input and the docked structure of the respective complexes along with the binding free energy was received as output. The complexes were visualized in Biovia Discovery Studio 2017 R2 software [125]. The contact maps of the interacting residues were generated in PDBsum server [126].

2.2.9.2 Fluorescence emission spectroscopy:

Briefly, thrombin (2 µM) was pre-incubated for 20 min with Daboxin P in ratios 1:1, 1:2, 1:3, 1:4 and 1:5 for 20 min at 37℃ in a total reaction mixture of 100 µl. The emission spectra of the individual components i.e., Daboxin P, thrombin and the Daboxin P-thrombin mixture were recorded from 300 to 600 nm with an excitation wavelength of 280 nm using a Varioskan Lux spectrophotometer (Thermo Scientific, USA). The emission spectrum of the mixture was compared with that of the individual components to observe for any changes in the fluorescence emission after incubation.

2.2.9.3 Affinity column chromatography:

Briefly, 20 µg of Daboxin P was covalently linked to Cyanogen bromide (CNBr) activated sepharose matrix in the presence of coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3). Non-specific binding was avoided by treating the matrix with blocking buffer (0.2 M glycine) with gentle shaking at room temperature. The blocking solution was then removed by repeated washes alternatively with acidic buffer (0.1 M acetate buffer, pH 4.0) and basic buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3).

Subsequently, thrombin (4 μg) was added to the matrix immobilized with Daboxin P and incubated for 1 h at room temperature with mild shaking. The unbound thrombin was removed by an extensive wash with coupling buffer and the bound thrombin was eluted with elution buffer (20 mM HCl and 1 M NaCl). The elution of bound thrombin was confirmed by analysing the flow-through and eluents on 12.5% glycine SDS-PAGE under non-reducing conditions and visualized by silver staining.

2.2.10 Inhibition of fibrinogenolytic activity of thrombin by Daboxin P:

In order to study the effect of Daboxin P on fibrinogenolytic activity of thrombin, Daboxin P was incubated with thrombin for 1 hour at 37ºC followed by incubation with fibrinogen under same conditions. Thrombin incubated with fibrinogen for 1 hour at 37ºC and only thrombin was taken as controls. Subsequently, the reactions were terminated by addition of BME and subjected to SDS-PAGE under reducing conditions.

2.2.11 Neutralization of anti-platelet activity of Daboxin P with polyvalent antivenom:

In order to determine the effectiveness of the polyvalent antivenom (PAV), 0.1μg Daboxin P was incubated with PAV in the ratios 1:1, 1:10, 1:100 and 1:1000 for 1 hour at 37ºC. Subsequently, platelet aggregation was induced by thrombin.

2.2.12 Statistical analysis:

Statistical significance was calculated by Paired t-test. For in vitro platelet aggregation assays, $N=6$, for all other analysis, $N=3$ and p value <0.05 was considered statistically significant.