Chapter III

MATERIALS AND METHODS

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3.1 Materials

3.1.1 Venom and antivenom

Pooled and lyophilized crude Indian cobra (*Naja naja*) venom of eastern India (EI) origin was obtained from a licensed dealer, Calcutta Snake Park, Kolkata, India. The lyophilized venom was stored in a dessicator (~25 ºC) until further use. The polyvalent antivenom (PAV) raised against 'Big Four' snake (*Naja naja*, *Daboia russelii*, *Echis carinatus*, and *Bungarus caeruleus*) venoms was obtained from Bharat Serums and Vaccines Limited, Mumbai, India (batch no. A05315029, expiry date: January, 2019) and Premium Serum and Vaccines Pvt. Ltd., Maharashtra, India (batch no. 012015, expiry date: December, 2018). Monovalent antivenom (MAV) raised against *N. kaouthia* venom was purchased from VINS Bioproduct Limited, Hyderabad, India (batch no. 31AS11001, expiry date: April, 2015).

3.1.2 Chromatographic matrices, cell lines, and cell culture reagents

Sephadex G-50 (fine grade) was obtained from Pharmacia Fine Chemicals, Sweden. The NHS-activated Sepharose 4 Fast Flow matrix was purchased from GE Healthcare, Sweden. Pre-packed cation-exchange chromatography HiPrep FF 16/10 column was obtained from GE Healthcare, Sweden.

Human gliblostoma (U87MG), human breast adenocarcinoma (MCF-7), rat skeletal myoblasts (L6), human embryonic kidney (HEK-293), and rat pheochromocytoma (PC-12) cell lines were obtained from American Type Culture Collection (ATCC), USA. Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from HiMedia Laboratories, USA; Penicillin-Streptomycin Solution was obtained from Gibco™, Thermo Fisher Scientific, USA; Fluorescein isothiocyanate (FITC) was procured from Sigma-Aldrich, USA; while all other cell culture accessories were procured from Corning®, Sigma-Aldrich, USA.

3.1.3 Coagulation factors and fine chemicals

Argatroban monohydrate (A0487), thrombin from human plasma (T6884), fibrinogen from human plasma (F3879), chromogenic substrates (T1637 and F3301), low molecular weight heparin (~6 kDa) from porcine intestinal mucosa, L-α-phosphatidylcholine (PC) from egg yolk (P3556), L-α-phosphatidyl-L-serine (PS) from bovine brain (P7769), L-α-phosphatidylethanolamine (PE) from egg yolk (P7943), and other fine chemicals were purchased from Sigma-Aldrich, USA. Coagulation factor Xa was purchased from Calbiochem, Merck, USA. Sinapinic acid matrix and α-cyano-4-hydroxycinnamic acid matrix for MALDI-ToF-MS analysis were purchased from Sigma Aldrich, USA. Sequencing grade trypsin was purchased from Sigma-Aldrich, USA. C_{18} ZipTip pipette tips were purchased from Merck Millipore, USA. Pre-stained protein molecular markers were purchased from BioRad (16103777; 2–250 kDa) and Thermo Fisher Scientific (26616; 10-170 kDa or 26619; 10-250 kDa). Anti-rabbit IgG-HRP conjugate (A0545) and anti-horse IgG-HRP conjugate (A9292) were procured from Sigma Aldrich, USA. Anti-vimentin antibody (ab92547, Abcam, USA) was a generous gift from Dr. Biplab Bose, Indian Institute of Technology, Guwahati. Anti-rabbit IgG-Alexa fluor 488 was purchased from Invitrogen, Thermo Fisher Scientific, Waltham, USA.

3.1.4 Analytical grade chemicals and kits

All other analytical grade reagents were procured from Sigma-Aldrich, USA. Secretory phospholipase A_2 ($sPLA_2$) assay kit was purchased from Cayman Chemical, USA. All diagnostic kits used for serum analysis such as total protein, glucose, uric acid, creatinine, cholesterol, urea, ALP, CK, SGOT, and SGPT were purchased from Tulip Diagnostics Ltd., India and Crest Biosystem, Goa. The coagulation assays kits such as PT, APTT, TT, and Fibro-TEK were purchased from r2 Hemostasis Diagnostics, Uttar Pradesh, India.

3.1.5 Raising polyclonal antibodies against purified PLA_2 isolated from *N. naja* venom

Polyclonal antibodies (whole IgG) in rabbits were raised against purified PLA_2 (Nn-PLA_2-I) by outsourcing (BioBharati Pvt. Ltd., Kolkata, India). Briefly, two New

Zealand White rabbits (both sexes) were sub-cutaneously injected with 150 µg of purified PLA_2 mixed with Freund's complete adjuvant (FCA) (1:1). Five booster doses, each of 80 µg PLA_2 mixed with Freund's incomplete adjuvant (FIA) (1:1), were administered at intervals of 14 days. After the last booster dose, the animals were bled and the crude anti-sera containing anti-PLA_2 polyclonal antibodies were isolated. The polyclonal antibodies were purified by affinity purification using a protein A/G bead column [1].

3.1.6 Animals and housing conditions

Wistar strain albino rats of either sex, weighing around 120-150 g were used for *in vivo* experiments. The animals were bred in the Central Animal House Facility of Defense Research Laboratory, Solmara, Tezpur in pathogen free and normal environmental conditions pertaining to the climate of Assam and North-eastern India (temperature 33-35 ºC, relative humidity ≥ 75%) with proper feeding. General conditions of captivity were maintained and animals were kept in social groups before and after the experiments. All animal experiments were performed in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines (1981) and were approved by the Institutional Animal Ethical Committees of Tezpur University (Approval no: DoRDPro/TUAEC/10–56/14/Res-09) and Defense Research Laboratory (Approval no: DRL/AEC/09/2015).

3.2 Methods

3.2.1 Purification of an anticoagulant protein and its complex from eastern India *N. naja* venom

3.2.1.1 Cation-exchange chromatography of *N. naja* venom

Crude EI *N. naja* venom (NnV) (10 mg dry weight) was dissolved in 0.5 ml of 20 mM Tris-HCl buffer, pH 7.4 (buffer A), and fractionated through a HiPrep CM FF 16/10 cation-exchange column (pre-equilibrated with buffer A) coupled with an AKTA purifier FPLC system (GE Healthcare, Sweden). After washing the column with two column volumes of equilibration buffer, proteins bound to column were eluted with a linear gradient of 1.0 M NaCl dissolved in buffer A at a flow rate of 1.0 ml/min. The

elution of protein was observed at 280 nm, and a 1.5 ml fraction was collected in each tube. The protein content (section 3.2.5.1), PLA_2 activity (section 3.2.5.2), and anticoagulant activity (section 3.2.10.2.2) of each protein peak was screened.

3.2.1.2 Size-exclusion chromatography of the most anticoagulant fraction

The fractions showing the highest PLA_2 activity were pooled, desalted in PD 10 column (GE Healthcare, Sweden), concentrated (CentriVap Benchtop Vacuum Concentrator, Labconco Corporation, USA), and then fractionated through a Sephadex G-50 gel filtration column (1×60 cm^2) pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.4. The proteins were eluted with the same buffer and the flow rate was maintained at 20 ml/h. Fractions of 1.0 ml were collected and their absorbance was measured at 280 nm using a spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Germany). Each peak was pooled and screened for PLA_2 activity (section 3.2.5.2), anticoagulant activity (section 3.2.10.2.2), and protein content (section 3.2.5.1).

3.2.2 Determination of homogeneity, purity, and molecular weight of the anticoagulant phospholipase A_2

3.2.2.1 Reversed-phase HPLC of the anticoagulant PLA_2

To determine the homogeneity of the preparation, 10.0 µg of the purified PLA_2 was subjected to RP-HPLC analysis in a Dionex Acclaim C_{18} (2.1×150 mm, 3 µm) column coupled to an Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Germany). The column was pre-equilibrated with 95% of solvent A (Type I water containing 0.1% tri-fluoroacetic acid, TFA) and 5% of solvent B (90% acetonitrile containing 0.1% TFA). The proteins were eluted with a multi-step gradient (5% B from 0 – 2 min, 5-45% solvent B from 2 – 3 min, 45 – 47% B from 3 – 12 min, 47 – 100% B from 12 – 14 min, 100% B from 14 – 15 min) of solvent B. The flow rate was maintained at 0.5 ml/min and absorbance was recorded at 215 and 280 nm.

3.2.2.2 Analysis of purity and molecular mass of the anticoagulant PLA_2 by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the anticoagulant PLA_2 was determined by 12.5% SDS-PAGE with or without reduction of protein as described by Laemmli [2]. The 4% stacking and

12.5% resolving polyacrylamide gels were freshly prepared with the composition described in Table 3.1.

Table 3.1. Composition of stacking and resolving SDS-polyacrylamide gels.

S. no.	Component	Polyacrylamide Gels	
		4% (stacking)	**12.5% (resolving)**
1	Type I water	1.8 ml	3.3 ml
2	30% acrylamide-bisacrylamide solution	0.6 ml	4.0 ml
3	1.5 M Tris-HCl buffer, pH 8.8	-	2.5 ml
4	0.5 M Tris-HCl buffer, pH 6.8	2.5 ml	-
5	10% SDS solution	50 µl	100 µl
6	10% ammonium persulphate solution	50 µl	100 µl
7	TEMED (N,N,N',N'-tetramethylethane-1,2-diamine)	7 µl	10 µl

The resolving gel and stacking gel solutions were poured into a gel cassette and allowed to polymerize at room temperature. Freshly prepared running buffer containing 15.1 g/l of Tris base, 72 g/l of glycine, and 5 g/l of SDS was used for electrophoresis. The reduced and non-reduced protein samples for electrophoresis were freshly prepared by re-suspending 30 – 80 µg of concentrated/lyophilized protein samples in 5.0 µl of loading dye [0.1 % bromophenol blue (w/v), 10% SDS (w/v), and 10% glycerol in 0.05 M Tris-HCl, pH 6.8] with or without β-mercaptoethanol (30.0 µl/ml)-dithiothreitol (2.0 mM), respectively [2].

To allow the stacking of proteins, electrophoresis was initially carried out at 80 V, followed by 120 V (resolving voltage). Protein bands were visualized either by staining the gel with PhastGel Blue R stain (GE Healthcare, Sweden) or silver staining [3]. The gels were stained overnight (~16 h) with PhastGel Blue R stain and the protein bands were visualized after destaining with methanol: acetic acid: water (40:10:50). For silver staining, the gels were fixed in fixing solution (40% methanol (v/v), 10% acetic acid (v/v) in type I water) for 30 min. Thereafter, the gels were sensitized using 0.02%

$Na_2S_2O_3$ (w/v) in type I water followed by silver staining (0.2% $AgNO_3$ (w/v) solution in type I water) for 25 min. The gels were then developed with developing solution (3% Na_2CO_3 (w/v), 0.02% $Na_2S_2O_3$ (w/v), 0.02% of 0.5 µl/ml of formaldehyde in type I water) until the bands were clearly visible. Once the protein bands were clearly visible, 1.4% EDTA (w/v) solution was added to stop the further development of the gel.

The approximate molecular weight of the proteins were determined from a plot of log molecular weight (MW) of protein markers (10 – 170 kDa or 10 – 250 kDa or 2 – 250 kDa) vs. migration distance of the protein [4].

3.2.2.3 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-ToF-MS) analysis of the anticoagulant PLA_2

The molecular mass of the purified PLA_2 was determined by MALDI-ToF-MS analysis on a 4800 MALDI TOF/TOF™ Analyser (Applied Biosystems, USA) as described previously [4]. About 1.0 µg of purified protein in 0.1% TFA was mixed with 1.0 µl of α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml) or sinapinic acid matrix (10 mg/ml) and spotted onto an Opti-TOF-384 plate (ABSciex), dried, and analysed in a positive linear mode at an acceleration voltage of 25 kV and laser intensity of 3000. Molecular mass of the protein was determined in the ranges of 3000-12000 and 6000-18000 Da.

3.2.3 Biophysical characterization

3.2.3.1 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

3.2.3.1.1 In-solution and in-gel trypsin digestion of proteins

100 µg of the purified protein and/or protein fraction(s) dissolved in 10 mM ammonium bicarbonate was subjected to reduction by 10 mM DTT for 30 min at 56 ºC followed by alkylation with 55 mM iodoacetamide (IAA) for 30 min at dark. Thereafter, the protein was digested with sequencing grade trypsin (13 ng/µl in 10 mM ammonium bicarbonate containing 10% acetonitrile) at an enzyme: substrate ratio of 1:30 [5,6] for overnight (~16 h) at 37 ºC. The trypsin-digested peptides were desalted and concentrated using ZipTip C_{18} (Merck, USA) following the manufacturer's protocol.

In another set of experiment, the PhastGel Blue R stained protein SDS-PAGE band corresponding to the purified PLA_2 was excised using a sterile scalpel and transferred to sterile microfuge tubes containing 100% ACN. Thereafter, the protein was reduced and alkylated using 10 mM DTT and 55 mM IAA, respectively for 30 min. Thereafter, the gel pieces were dried and incubated with trypsin solution (~50 µl of a 13 ng/µl stock) for digestion at 37 ˚C for overnight (~16 h) [7]. The tryptic peptides were then desalted and concentrated using ZipTip C_{18} as mentioned above [8].

3.2.3.1.2 LC-MS/MS analysis of the tryptic peptides and identification of protein

The desalted and concentrated tryptic peptides were reconstituted in 0.1% formic acid and subjected to fractionation on a Zorbax - C_{18} column (Rapid Resolution HT 2.1 × 50 mm, 1.8 µm) coupled to an Agilent 1260 UHPLC system [8]. The flow rate was maintained at 300 nl/min. The peptides were separated with a mobile phase gradient of 11% solvent B for 5 min, 11 to 25% solvent B for 20 min, 25 to 53% solvent B for 16 min, 53 to 100% solvent B for 5 min, 100% solvent B for 4 min, and then 11% solvent B for 4 min. Solvent A and B were 0.1% formic acid and 80% acetonitrile (ACN) containing 0.1% formic acid, respectively.

The peptides eluted from the HPLC column were then fed into a Nanomate Triversa (Advion BioSciences, Ithaca, NY), equipped with an LC coupler and electrospray ionization (ESI) nanospray chip. The LC coupler connects the flow from the HPLC to the ESI chip, where the nano-ESI generated ions were transferred into an LTQ Orbitrap Discovery hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The ionization voltage was set to 1.7 kV. The raw data were acquired in a data-dependent acquisition (DDA) mode by Xcalibur software (Thermo Fisher Scientific, Bremen, Germany). One MS survey scan was followed by 5 MS/MS scans with exclusion duration of 120.0 s during DDA. Survey full-scan MS spectra (from m/z 300−2000 with lock mass set to 445.12 corresponding to polysiloxane) were acquired in Fourier Transform (FT) mode with a resolution of 30000 (full width at half-maximum). Subsequent fragmentation (MS/MS) was collision-induced dissociation (CID) with normalized collision energy set to 35% in linear ion trap mode. The following were the MS/MS triggering conditions: minimum signal intensity, 10,000; charge state, +2, +3; maximum injection time for MS/MS, 500 ms; and isolation width, 2 amu [9].

The raw MS/MS data was searched against the *Naja naja* (taxid 35670) protein entries of the non-redundant NCBI database using PEAKS 7.0 software (Bioinformatics Solutions Inc., Ontario, Canada). Carbamidomethylation of cysteine and the oxidation of methionine residues were set as fixed and variable modifications, respectively. Precursor and fragment mass error tolerances were set to 2.1 Da and 0.025 Da, respectively; up to two missed cleavages were allowed, and the false discovery rate was set to <1%. The minimum -10logP score for protein and peptide were set as ≥ 30 and ≥ 20, respectively. Identification of at least one unique peptide was considered to be the minimum pre-requisite for protein identification [5,8]. The tryptic peptide sequences of individual proteins were subjected for BLAST search in NCBI database of non-redundant protein sequences using BLASTP algorithm (http://blast.ncbi.nlm.nih.govlBlast.cgi).

3.2.3.2 Determination of secondary structure

The secondary structure of the purified PLA_2 was determined by circular dichroism (CD) analysis using a Jasco 1715 Spectropolarimeter, Japan. Briefly, the far UV-CD spectra (190 – 250 nm) of protein solution (0.2 mg/ml in 20 mM potassium phosphate buffer, pH 7.0) in a quartz cuvette with path length of 0.1 cm were recorded at room temperature (~23 ºC). Yang's reference was set for the CD analysis. The bandwidth and response time were 1 run and 2 s, respectively. The final CD spectra (a cumulative of five scans) were corrected by subtraction of buffer (blank) and expressed in molar ellipticity (degrees cm^2/dmol) [10], using 113 as mean residue molecular mass [11]. CDPRO CLUSTER software was used to determine the secondary structure of isolated purified protein [12].

3.2.4 Determination of venom PLA_2 cognate complex(es) and identification of its components

3.2.4.1 Two-dimensional SDS-PAGE (2D SDS-PAGE) of *N. naja* venom

The 2D electrophoresis of EI *N. naja* venom (NnV) was done under both reduced and non-reduced conditions. Lyophilized NnV (1.0 mg) was dissolved in 20 mM Tris-HCl buffer, pH 7.4 and centrifuged at 10,000 rpm for 10 min. The supernatant was cleaned using 2-D Clean-Up kit (GE Healthcare, Sweden) and thereafter quantified

using 2-D Quant kit (GE Healthcare, Sweden) by following the manufacturer's protocol. Three hundred (300) µg of cleaned-up EI NnV was re-suspended in rehydration buffer with or without DTT for reduced and non-reduced electrophoresis, respectively. The re-suspended NnV was gently added to pH 3 – 10, 7 cm Immobiline DryStrip gel (GE Healthcare, Sweden) and rehydrated for 16 h (overnight). The Immobilin DryStrip gels were then subjected to iso-electric focusing (1D electrophoresis) on an Ettan IPGphor 3 Isoelectric Focusing System (GE Healthcare, Sweden) for 11,350 kVh (300 V for 4 h, 300 – 1000 V in 1 h, 1000 – 5000 V in 90 min, 5000 V for 1 h). After completion of 1D electrophoresis, the IPG strip containing reduced EI NnV was equilibrated with equilibration buffer containing 65 mM dithiothreitol (for reduction) and 135 mM IAA (for alkylation) for 10 min each at room temperature. For the non-reduced 2D SDS-PAGE, the DTT and IAA treatment steps were avoided. Thereafter, both the strips (containing reduced and non-reduced EI NnV) were subjected to 12.5% SDS-PAGE at 120 V, 40 mA. The proteins spots were visualized by staining the gel with PhastGel Blue R (GE Healthcare, Sweden) for overnight and destaining with methanol/acetic acid/water (40:10:50). The gels were scanned on an EPSON scanner using Silver Fast software and the protein spots were counted and analyzed by ImageMaster™ 2D Platinum v7.0 Software (GE Healthcare, Sweden).

3.2.4.2 Immunoblotting of 2D SDS-PAGE separated EI NnV

The EI NnV proteins separated by 2D SDS-PAGE were transferred from gels to Immobilon-P PVDF membranes by semi-dry gel transfer system at 78 mA (1.2 mA/cm^2) for 2 h. Thereafter, the membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for overnight at 4 ºC, and the blot was developed by incubating with rabbit anti-PLA_2 antibodies (BioBharati Pvt. Ltd., Kolkata, India) (1: 2000 dilutions) for 30 min and then HRP-conjugated goat anti-rabbit IgG (Sigma Aldrich, USA) (1: 5000 dilutions) for 30 min. The reaction was developed by adding 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for Membranes (Sigma-Aldrich, USA). The blots were scanned on an EPSON scanner (Epson Expression 11000XL, USA) using Silver Fast software. The experiment was repeated three times to assure the reproducibility [5,13].

3.2.4.3 Identification of components of PLA_2-cognate complex

3.2.4.3.1 RP-HPLC of PLA_2-cognate complex

The PLA_2 cognate complex [Nn(N)CM2, 150 µg] was subjected to RP-HPLC on a Dionex Acclaim C_{18} column (2.1 × 150 mm, 3.0 µm) pre-equilibrated with 95% solvent A (Type I water containing 0.1% TFA) and 5% solvent B (90% acetonitrile containing 0.1% TFA), coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Germany). The proteins were eluted using a multi-step gradient of solvent B with a flow rate of 0.5 ml/min. The eluted protein peaks were pooled and lyophilized, and the protein content of individual peak was determined (see section 3.2.5.1). Further, each major fraction (30 µg) was subjected to in-solution trypsin digestion (section 3.2.3.1.1) followed by LC-MS/MS analysis as described below.

3.2.4.3.2 In-sol trypsin digestion of Nn(N)CM2 and its RP-HPLC fractions followed by LC-MS/MS

Nn(N)CM2 obtained by cation-exchange chromatography of NnV (section 3.2.1.1) and/or its RP-HPLC (section 3.2.4.3.1) obtained fractions was subjected to in-solution trypsin digestion followed by LC-MS/MS analysis (section 3.2.3.1) to determine the composition of venom proteins in that fraction. The raw MS/MS data was searched against the *Naja naja* (taxid 35670) protein entries of the non-redundant NCBI database using PEAKS 7.0 software [14]. Carbamidomethylation of cysteine and the oxidation of methionine residues were set as fixed and variable modifications, respectively. Identification of at least one unique peptide per protein entry was considered to be the minimum pre-requisite for protein identification [5,8].

3.2.4.3.3 In-gel trypsin digestion of anti-PLA_2 antibody detected 2D SDS-PAGE spots followed by LC-MS/MS

The 2D gel spots, corresponding to that recognized by anti-PLA_2 antibodies in PVDF membrane, were excised and subjected to LC-MS/MS analysis for protein identification after in-gel trypsin digestion as described previously (section 3.2.3.1.1). Briefly, the tryptic peptides were desalted and concentrated by ZipTip C_{18} (Merck, USA) and separated on a Zorbax C_{18} column (Rapid Resolution HT 2.1 × 50

mm, 1.8 µm) coupled to an Agilent 1260 UHPLC system [15]. The column compartment temperature was set at 40 °C source and analyzed on an Agilent 6530 QTOF mass spectrometer. The MS and MS/MS spectra were acquired in the range of 100 to 2000 m/z, with a scan rate of 6 and 3 spectra/s for MS and MS/MS, respectively. The raw MS/MS data was searched against the *Naja naja* (taxid 35670) protein entries of the non-redundant NCBI database using Morpheus software [14]. Carbamidomethylation of cysteine and the oxidation of methionine residues were set as fixed and variable modifications, respectively. Precursor and fragment mass error tolerances were set to 2.1 Da and 0.025 Da, respectively; up to two missed cleavages were allowed, and the false discovery rate was set to <1%. Identification of at least one unique peptide per protein entry was considered to be the minimum pre-requisite for protein identification [5,8].

3.2.4.3.4 Determination of relative abundance and stoichiometry of venom proteins in PLA_2 cognate complex

The relative abundances of LC-MS/MS identified NnV proteins of Nn(N)CM2 and 2D SDS-PAGE spots were calculated using MS2 (spectral count)-based label-free quantification techniques [5,16,17]. Normalization of MS2 spectral count (mean) was done by number of identified peptides using equation 3.1 [5,18,19].

$$Mean\ spectral\ count\ for\ protein\ X = \frac{\Sigma\ spectral\ count\ against\ MS1/MS2\ peptides\ of\ X}{number\ of\ identified\ peptides\ of\ X} \quad \text{-- (3.1)}$$

Thereafter, the relative abundance of a protein (X) in a particular chromatographic fraction (Y) was calculated using equation 3.2:

$$Relative\ abundance\ of\ X\ in\ chromatographic\ fraction\ or\ gel\ section\ Y = \frac{mean\ spectral\ count\ of\ X\ in\ Y}{total\ mean\ spectral\ count\ of\ all\ proteins\ in\ Y} \times protein\ yield\ (\%)\ or\ 100\% \quad \text{------ (3.2)}$$

Based on the percent relative abundance, the quantity of each protein (in moles) in 100 µg of Nn(N)CM2 was calculated using equation 3.3, and thereafter the stoichiometry of each protein in the complex was determined.

$$Protein\ X\ (in\ moles) = \frac{microgram\ of\ X\ in\ 100\ \mu g\ of\ cognate\ complex}{molecular\ mass\ of\ X\ (in\ Da)} \quad \text{------ (3.3)}$$

3.2.4.4 One dimensional (1D) SDS-PAGE to confirm complex formation by NnV PLA_2

To confirm the non-covalent interaction of purified PLA_2 with other venom toxins, 40 µg each of purified PLA_2, Nn(N)CM2, and NnV were separated by 12.5% SDS-PAGE under both reducing and non-reducing conditions at 120 V, 400 mA. The protein bands were visualized by staining the gel with PhastGel Blue R and distaining with methanol: glacial acetic acid: water (40: 10: 50). From densitometry analysis of the gel (ImageJ software, NIH, USA) and label-free quantification (section 3.2.4.3) the concentration of purified PLA_2 in Nn(N)CM2 fraction was determined.

3.2.5 Biochemical characterization

3.2.5.1 Estimation of protein content

The protein content of crude NnV or chromatography fractions or other protein samples was estimated by the method described by Lowry [20] or Bradford [21] using BSA (1.0 mg/ml) as a protein standard. The protein content of unknown samples was calculated from a standard protein calibration curve plotted by considering the absorbance at 660 nm (for Lowry method) [20] or 595 nm (for Bradford method) [21,22] vs amount of BSA (0.5 -10.0 µg).

3.2.5.2 Phospholipase A_2 activity assay

Phospholipase A_2 activity was assayed by the following two methods:

3.2.5.2.1 Turbidometric method

Phospholipase A_2 activity was assayed by the turbidometric method of Joubert and Taljaard [23] with slight modification as described previously [24,25]. Briefly, one egg yolk was suspended in 250 ml of 0.9% (w/v) NaCl containing 0.02% (w/v) sodium azide. Before PLA_2 assay, 1.0 ml of the suspension was mixed with 10.0 ml of 0.1 M Tris-HCl buffer, pH 8.0, and the absorbance of the resulting mixture was adjusted to 1.0 at 740 nm with the same buffer. For assaying the PLA_2 activity, different doses of crude venom / purified protein / peptide were mixed with 2.0 ml of the above reaction mixture, and the decrease in turbidity after 10 minutes was monitored at 740 nm against

a reagent blank. One unit of PLA_2 activity has been arbitrarily defined as a decrease in 0.01 absorbance at 740 nm after 10 min on incubation [24].

3.2.5.2.2 Secretory PLA_2 assay kit

Phospholipase A_2 activity of NnV / venom fractions / purified PLA_2 / peptides were validated by using the $sPLA_2$ assay kit (Cayman Chemical, USA) according to the manufacturer's protocol using diheptanoylthio-phosphatidylcholine as substrate [26]. The specific activity of PC hydrolysis by NnV / venom fractions / purified PLA_2 / peptides was expressed in micromoles of phosphatidylcholine hydrolyzed per min per mg of enzyme. Bee venom PLA_2 enzyme (0.001 μg/μl; supplied in the kit) was considered as a positive control for the experiment.

3.2.5.3 Substrate specificity and enzyme kinetics

For determination of phospholipids substrate specificity, a fixed concentration (25 nM) of purified PLA_2 was incubated with different phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) (final concentration 1 mM) at 37 ºC for the desired time periods. The PLA_2 activity was assayed by micro-titration of liberated fatty acids (FA) with 0.01 N NaOH, and the amount of FA liberated was determined from a standard curve of palmitic acid [25]. The Lineweaver-Burk plot was used to determine the kinetic parameters (K_m and V_{max}) of purified PLA_2 using different concentrations (0.5 – 3.0 mM) of substrate (PC), and then the values of 1/V were plotted as a function of 1/S.

3.2.5.4 Effect of pH and temperature on enzyme activity

To study the effect of pH on enzyme activity, 25 nmol of purified PLA_2 was incubated with 2.0 ml of egg-yolk suspension (as stated above) prepared with different buffers having pH 5.0 – 10.0. The different buffers used were 0.1 M sodium acetate buffer (pH 5.0 – 6.5), 0.1 M potassium phosphate buffer (pH 7.0 – 7.5), and 0.1 M Tris-HCl buffer (pH 8.0 – 10.0). The PLA_2 activity was determined for each pH against a reagent blank.

For determining the heat-denaturation of enzyme activity, 0.1 M PLA_2 solution (in 0.1 M Tris-HCl, pH 8.0) was incubated at 75 °C for different time intervals (10 – 60

min) and the catalytic (section 3.2.5.2) and anticoagulant activities (section 3.2.10.2.2) were assessed. The activity of the control (unheated enzyme) was considered as 100% activity, with other values compared to that.

3.2.5.5 Effect of chemical inhibitors and heparin on enzymatic and anticoagulant activities

For determining the effect of different inhibitors on catalytic (section 3.2.5.2) and anticoagulant (section 3.2.10.2.2) activities of purified PLA_2, 0.2 µM of the enzyme was pre-incubated with different inhibitors, viz. tosyl phenylalanyl chloromethyl ketone or TPCK (chymotrypsin-like serine protease inhibitor) (100 µM), N-α-p-tosyl-L-lysine chloromethyl ketone or TLCK (trypsin-like serine protease inhibitor) (100 µM), phenylmethanesulfonylfluoride or PMSF (broad spectrum serine protease inhibitor) (5 mM), *p*-bromophenacyl bromide or *p*-BPB (histidine alkylator) (2-5 mM), ethylenediaminetetraacetic acid or EDTA (metal chelator) (5 and 10 mM), dithiothretol or DTT (reduction of disulfide bond) (5 mM), and iodoacetamide or IAA (alkylation of cysteine residues) (2 mM) for 30 min at room temperature [11].

The purified PLA_2 was also pre-incubated with 20 mIU of heparin for 30 min at 37 °C. The catalytic (section 3.2.5.2) and anticoagulant (described below in section 3.2.10.2.2) activities of the modified enzyme were determined against appropriate controls in the corresponding assay systems. The activity of the unmodified enzyme (the control) was considered as 100% activity, with the other values compared to that.

3.2.5.6 Neutralization by commercial antivenom

The PLA_2 enzyme was pre-incubated for 30 min at 37 °C with antivenom (polyvalent or monovalent) at different protein to antivenom ratios ranging from 1:10 to 1:500 (protein: protein ratio). The mixtures were than assayed for their catalytic (section 3.2.5.2) and anticoagulant (described below in section 3.2.10.2.2) activities against appropriate controls by following standard protocols. The percent inhibition of enzyme/anticoagulant activity was calculated by comparing the activity to that of untreated enzyme (100% activity).

3.2.6. *In silico* interaction of PLA_2 with coagulation factor(s) docking analysis and peptide designing

3.2.6.1 Structure prediction of the purified PLA_2

The complete sequence of the purified PLA_2 obtained from LC-MS/MS analysis (section 3.2.3.1) was submitted to I-TASSER server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) for structure prediction [27]. The best structure model of the PLA_2 based on optimum Tm value and C-score was selected for docking analysis.

3.2.6.2 Docking analysis with thrombin and factor Xa

The best predicted model of the purified PLA_2 was docked with human thrombin (PDB ID 3RM2) and FXa (PDB ID 1C5M) using ClusPro 2.0 server (https://cluspro.bu.edu/) [28]. The best docking model was refined with Firedock server (http://bioinfo3d.cs.tau.ac.il/FireDock/) [29] and submitted to PDBSum online server (http://www.ebi.ac.uk/pdbsum) [30,31] for predicting the residue-to-residue interactions between the chains of PLA_2 enzyme and thrombin.

3.2.6.3 Designing of anticoagulant peptides and their synthesis

Based on the *in silico* analysis of PLA_2-thrombin interaction and pharmacological site of cobra venom PLA_2 [32,33], a set of twelve peptides (ACR1 – 12) were designed. The peptides were synthesized using 9-fluorenylmethyloxycarbonyl (Fmoc) based solid phase peptide synthesis (SPPS) [34] by outsourcing (GenPro Biotech, India). The purity of the preparation (99% purity) was confirmed by RP-HPLC (section 3.2.2.1) and MALDI-ToF-MS analyses (section 3.2.2.3).

3.2.6.4 Physico-chemical characterization and *in silico* antigenicity determination of the synthetic peptides

The sequences of the peptides were submitted to Expasy web server (https://www.expasy.org/) to determine the physico-chemical properties of the peptides. The peptide sequences were also submitted to EMBOSS Antigenic explorer

(http://www.bioinformatics.nl/cgi-bin/emboss/antigenic) to determine the antigenicity of the peptide sequences [35].

3.2.6.5 Structure prediction of the 7-mer anticoagulant peptide

The sequence of the 7-mer peptide (E^1-K^2-I^3-S^4-G^5-G^6-W^7) was submitted to PEPFOLD-3 Peptide Structure Prediction online web server (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) [36]. The structure with the lowest model energy was considered for docking studies.

3.2.6.6 Docking of 7-mer peptide with thrombin and FXa

The best peptide model with the lowest energy was docked with human thrombin (PDB ID: 3RM2) and FXa (PDB ID: 1C5M) using CABS Dock web server (http://biocomp.chem.uw.edu.pl/CABSdock) [37,38]. The contact map analysis was also performed using the same server. The Ligplot analysis to determine the residue-to-residue interaction was performed using PDBSum server (http://www.ebi.ac.uk/pdbsum) [31].

3.2.7 Preparation of peptide solutions

All the peptides, except ACR9 and ACR12, were dissolved in 1X PBS (8 mM $Na_2HPO_4 \cdot 7H_2O$, 137 mM NaCl, 2.7 mM KCl, and 2 mM KH_2PO_4), pH 7.4 to prepare a 10 mg/ml stock solution of the peptides. However, ACR9 and ACR12 (1.0 mg) were first dissolved in 5 µl of dimethyl sulfoxide (DMSO) and then 95 µl of 1X PBS, pH 7.4 was added to obtain a 10 mg/ml peptide solution. Before each assay, the stock solutions were diluted to 1:100 in 1X PBS, pH 7.4 (final concentration of DMSO was 0.05%).

3.2.8 *In vitro* effect of NnV PLA_2 and synthetic peptide on coagulation factors (thrombin, factor Xa, and prothrombin)

3.2.8.1 Effect on thrombin

3.2.8.1.1 Effect on amidolytic activity of thrombin

The effect of purified PLA_2/synthetic peptide on the amidolytic activity of thrombin was assayed using the chromogenic substrate of thrombin T1637 [N-(*p*-

Tosyl)-Gly-Pro-Arg-*p*-nitroanilide acetate] [39,40]. Graded concentrations of purified PLA_2 / synthetic peptide were incubated with a fixed amount of thrombin (167 nM) at 37 °C for 30 min. Thereafter, the reaction was initiated by adding 2 µl of 10 mM T1637 (final concentration 0.2 mM). The final volume of the reaction mixture was maintained at 100 µl. Thrombin cleaves its chromogenic substrate at the site after Arg residue thereby releasing the *p*-nitroaniline (*p*-NA) group which imparts a bright yellow colour to the reaction mixture. The amount of *p*-NA released was determined by measuring the absorbance of the reaction mixture at 405 nm [39] in a 96-well plate reader (Multiskan GO, Thermo Fisher Scientific, USA). As a control, only thrombin (without PLA_2/peptide) was also run in parallel. The activity of control thrombin was considered to be 100%, and the other values were compared with that. The IC_{50} value of thrombin inhibition was determined from the Hill's equation by plotting the log [inhibitor concentration] vs variable response (A_{405} nm of *p*-NA release post 20 min of incubation) using GraphPad Prism 5.0 software [41].

3.2.8.1.2 Effect on fibrinogen clotting activity of thrombin

Graded concentrations of PLA_2 / peptide were pre-incubated with thrombin (3 µl of 10 NIH U/ml) for 30 min at 37 °C. A control was also set up in which thrombin was incubated with 1X PBS, pH 7.4 under identical conditions. The reaction was started by adding 40.0 µl of fibrinogen (2.5 mg/ml), and the time of clot formation was recorded by visual inspection [39].

To measure fast or slow inhibition, 0.5 µM of the PLA_2 was pre-incubated with thrombin (3 µl of 10 NIH U/ml) from 5 – 30 min at 37 °C prior to the addition of human fibrinogen, and the clotting time was recorded as stated above.

3.2.8.1.3 Kinetics of thrombin inhibition

To determine the *Ki* value for the inhibition of amidolytic activity of thrombin by the purified PLA_2 or synthetic peptide, a fixed concentration of thrombin (3 µl of 10 NIH U/ml, 167.0 nM) was pre-incubated with two different concentrations of purified PLA_2 (0.15 µM and 0.30 µM) or synthetic peptide (1.0 µM and 2.0 µM) at 37 °C for 30 min. A control was run in parallel where thrombin was incubated with a buffer instead of PLA_2 / synthetic peptide under identical experimental conditions. The reaction was

initiated by adding graded concentrations (0.1 – 0.8 mM for PLA_2 or 0.1 – 0.4 mM for peptide) of chromogenic substrate for thrombin (T1637). Thereafter, the release of *p*-NA was determined, as stated above (section 3.2.8.1.1). For kinetic analysis, the reaction rate (V) was plotted against substrate concentration (S) at each concentration of inhibitor, and the data was fitted to a hyperbolic Michaelis-Menten model using GraphPad Prism 5.0 software. The inhibitory constant (*Ki*) was determined using the mixed model (Equations 3.4-3.6) for enzyme inhibition and using the same software.

$$VmaxApp = \frac{Vmax}{(1+\frac{I}{\alpha Ki})} \quad \text{----- (3.4)}$$

$$KmApp = \frac{Km(1+\frac{I}{Ki})}{(1+\frac{I}{\alpha Ki})} \quad \text{----- (3.5)}$$

$$Y = \frac{VmaxApp*X}{(KmApp+X)} \quad \text{----- (3.6)}$$

In the above equations (3.3) and (3.4), I indicate the inhibitor (PLA_2 / peptide) concentration, and *VmaxApp* and *Vmax* represent maximum velocity in the presence and absence of the inhibitor, respectively. The *Km(App)* and *Km* in equation (3.5) denote the Michaelis constant in the presence and absence of inhibitor, respectively. The α is a constant.

3.2.8.1.4 Effect of inhibitors on thrombin inhibition

3.2.8.1.4.1 Effect of heparin and/or antithrombin on inhibition of amidolytic activity of thrombin by PLA_2 and/or peptide

The effect of heparin, antithrombin-III (AT-III) and heparin / AT-III complex on the thrombin inhibition property of purified PLA_2 ($NnPLA_2$-I) was determined as described previously [40]. A fixed dose of PLA_2 (0.5 µM) or peptide (1.0 µM) was pre-incubated with different doses of heparin (0.5 IU and 1.0 IU), AT-III (2.5 µM and 5.0 µM), and heparin / AT-III (0.5 IU heparin and 2.5 µM AT-III; and 1.0 IU heparin and 5.0 µM AT-III) for 30 min at 37 °C in a 96-well microplate. This was followed by the addition of thrombin (3 µl of 10 NIH U/ml) and T1637 (chromogenic substrate for thrombin). The kinetics of substrate hydrolysis was determined by measuring the release of *p*-NA at 405 nm for 10 min in a microplate reader (Multiskan GO, Thermo Fisher

Scientific, USA) (section 3.2.8.1.1). A control was run in parallel where thrombin was pre-incubated with 1X PBS, pH 7.4 under identical conditions before assaying its amidolytic activity.

3.2.8.1.4.2 Effect of heparin on inhibition of fibrinogen clotting activity of thrombin by PLA_2

To study the effect of heparin on inhibition of fibrinogen clotting activity of thrombin by PLA_2, 1.0 µM of purified PLA_2 ($NnPLA_2$-I) was pre-incubated with 20 mIU of heparin and 1X PBS, pH 7.4 (the control) for 30 min at 37 °C. Then, thrombin (3 µl of 10 NIH U/ml, 326.0 nM) was added to this reaction mixture and re-incubated for an additional 30 min at 37 °C. In another set of experiments, only heparin (20 mIU) was incubated with thrombin under identical conditions. To these mixtures, 40 µl of fibrinogen (2.5 mg/ml) was added, and the clotting time was recorded with a coagulometer or by visual inspection (section 3.2.8.1.2). To determine the IC_{50} value of heparin on the thrombin inhibition activity of purified PLA_2, different doses of heparin (5 – 40 mIU) were pre-incubated with 1.0 µM of purified PLA_2 for 30 min at 37 °C. The mixture was then re-incubated with thrombin (326.0 nM) for 30 min at 37 °C, and then the fibrinogen clotting activity of thrombin was determined, as stated above (section 3.2.8.1.2). For each experiment, a control was run in parallel. The IC_{50} value of heparin on PLA_2 was also determined from the thrombin inhibition curve.

3.2.8.1.4.3 Effect of *p*-BPB on inhibition of amidolytic activity of thrombin by PLA_2

The effect of histidine modification on thrombin inhibitory activity of purified PLA_2 was also determined. Briefly, 1.0 µM of PLA_2 was treated with 2.0 mM *p*-BPB for 30 min at 37 ºC. Thereafter, 3 µl of 10 NIH U/ml of thrombin (167.0 nM) was added to the reaction mixture and incubated for 30 min at 37 ºC. Then, the amidolytic activity of thrombin was assessed by addition of T1637 to the reaction mixture and the absorbance of *p*-NA release was monitored by a plate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, USA) (section 3.2.8.1.1).

3.2.8.2 Effect on factor Xa

3.2.8.2.1 Effect on amidolytic activity of FXa

To measure the inhibitory effect of PLA_2 / peptide on amidolytic activity of factor Xa, the procedures described by Saikia et al., [25] were followed. Briefly, different concentrations of purified PLA_2 / synthetic peptide was pre-incubated with 0.1 µg of FXa (22 nM) isolated from human plasma (Calbiochem, Merck, USA) for 60 min at 37 °C. Thereafter, 2.0 µl of 10 mM F3301 or CH_3COO-D-CHA-Gly-Arg-pNA-AcOH (chromogenic substrate of FXa, 0.2 mM final concentration) for FXa was added to the reaction mixture. The release of *p*-NA was monitored for 20 min at intervals of 30 s at 405 nm in a plate reader (Multiskan GO, Thermo Fisher Scientific, USA). A control was run in parallel where FXa was pre-incubated with PBS only (100% activity) and other values were compared to that. The IC_{50} value of FXa inhibition was determined from the Hill's equation by plotting the log [inhibitor concentration] vs variable response ($A_{405\ nm}$ of *p*-NA release post 20 min of incubation) using GraphPad Prism 5.0 software [41].

3.2.8.2.2 Effect on prothrombin activation by FXa

3.0 µM of PLA_2 / 1.0 and 2.0 µM of synthetic peptide were pre-incubated with 0.1 µg of Factor Xa for 30 min at 37 °C. Thereafter, 12 µg of human prothrombin (PTH) was added, and the reaction mixture was incubated at 37 °C for 60 min. Thereafter, PTH activation by FXa in presence or absence (control) of PLA_2/peptide was determined by 12.5% SDS-PAGE analysis of the reaction products (PTH activated products) under reducing conditions. The proteins were stained with PhastGel Blue R stain and destained with methanol, acetic acid, and water (40:10:50). The protein bands were subjected to densitometry scanning with the help of ImageJ Software (NIH, USA). The formation of thrombin from prothrombin was reconfirmed by LC-MS/MS analysis of the 36 kDa protein band (section 3.2.3.1) and searching the data against *Homo sapiens* (taxid: 9606) database.

3.2.8.2.3 Kinetics of FXa inhibition

To determine the Ki value for the inhibition of amidolytic activity of FXa by the synthetic peptide, a fixed concentration of thrombin (1 µl of 0.1 mg/ml, 22 nM) was pre-incubated with two different concentrations of the synthetic peptide (1.0 µM and 2.0 µM) at 37 °C for 30 min. A control was run in parallel where thrombin was incubated with a buffer instead of synthetic peptide under identical experimental conditions. The reaction was initiated by adding graded concentrations (0.1 – 0.4 mM) of chromogenic substrate for FXa (F3301). Thereafter, the release of p-NA was determined, as stated above (section 3.2.8.2.1). The reaction rate (V) was plotted against substrate concentration (S) at each concentration of inhibitor, and the data was fitted to a hyperbolic Michaelis-Menten model using GraphPad Prism 5.0 software. The inhibitory constant (Ki) was determined using the uncompetitive model (Eq 3.7 – 3.9) for enzyme inhibition and using the same software.

$$VmaxApp = \frac{Vmax}{(1+\frac{I}{\alpha Ki})} \quad \text{----- (3.7)}$$

$$KmApp = \frac{Km}{(1+\frac{I}{\alpha Ki})} \quad \text{----- (3.8)}$$

$$Y = \frac{VmaxApp*X}{(KmApp+X)} \quad \text{----- (3.9)}$$

In the above equations I indicate the inhibitor (peptide) concentration, and $VmaxApp$ and $Vmax$ represent maximum velocity in the presence and absence of the inhibitor, respectively. The $KmApp$ and Km denote the Michaelis-Menten constant in the presence and absence of inhibitor, respectively. The α is a constant.

3.2.8.3 Effect of 7-mer peptide on prothrombin: An amidolytic assay to determine thrombin formation

7-mer peptide (5.0 µM) was pre-incubated with prothrombin (0.1 µM) at 37 ºC for 30 min and thereafter incubated with FXa (22 nM) for 30 min. Then, 0.2 mM of T1637 (chromogenic substrate for thrombin) was added to the reaction mixture and thrombin formation from prothrombin was measured by recording the absorbance at 405 nm (as described in section 3.2.8.1.1) [42]. Prothrombin incubated with FXa was

used as positive control and its activity was considered to be 100% while other values were compared to that.

3.2.9 Protein-protein interaction studies

3.2.9.1 Spectrofluorometry assay of interaction of purified PLA_2 / peptide with phospholipid and coagulation factors

3.2.9.1.1 Interaction with PC

0.1 µM of the purified PLA_2 was added to 1.0 mM PC in either the absence or presence of 2.0 mM Ca^{2+}, our previously described procedure was followed [25]. The excitation wavelength used was 280 nm with a slit width of 10 nm, while the emission spectrum was observed from 300 nm to 400 nm range [25] using a fluorescence spectrometer (LS55, Perkin Elmer). The increase in λmax of PC in presence of PLA_2 was calculated and the one-site specific binding curve was plotted using GraphPad Prism 5.0 software [25]. All the spectrofluorometry binding assays were done in triplicate to ensure reproducibility.

3.2.9.1.2 Interaction with thrombin and FXa

Gradient concentrations of the purified PLA_2 or synthetic peptide was incubated with thrombin (40 nM for PLA_2 or 0.3 µM for peptide) / FXa (0.1 µM) / 1X PBS, pH 7.4 containing 0.05% DMSO / only 1X PBS, pH 7.4 (control) for 5 min at room temperature. The reaction mixture was excited at 280 nm and its emission spectrum was monitored in the range of 300 and 500 nm using a fluorescence spectrometer (LS55, Perkin Elmer) or Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Denmark). The slit length was maintained at 10 nm or 12 nm for the two instruments, respectively. The increase in λmax of thrombin / FXa in presence of PLA_2 / peptide was calculated and the one-site specific binding curve was plotted using GraphPad Prism 5.0 software [10,25]. All the spectrofluorometry binding assays were done in triplicate to ensure reproducibility.

3.2.9.2 Surface plasmon resonance to determine the binding with thrombin

The binding affinity of 7-mer anticoagulant peptide with thrombin was analyzed using Biacore X100 (GE Healthcare, Freiburg, Germany) as described by Thakur et al. [40], with slight modifications. Briefly, thrombin (0.4 mg/ml) was immobilized on a CM5 sensor chip using the manufacturer's protocol (~8000 RU). Thrombin binding was measured in multi-cycle mode (~25 ºC; flow rate of 80 µl/min) against increasing concentrations of the peptide (2.5 – 15.0 µM) dissolved in 1X PBS, pH 7.4 containing 0.5% DMSO. Between each cycle, the chip was regenerated with glycine, pH 2.5. The dissociation constant (K_D) was calculated by plotting the Response Unit (RU) at steady state (Req) versus concentrations of peptide by fitting the data to the following equation (Eq 3.10):

$$Req = [A]Rmax/([A]+K_D) \quad \text{----- (3.10)}$$

Where, [A] refers to concentration of the synthetic 7-mer peptide.

3.2.10 Pharmacological characterization

3.2.10.1 Whole blood clotting assay

To study the whole blood clotting time, goat blood obtained from a slaughter house was collected in 3.8% tri-sodium citrate (9:1). Different concentrations of the 7-mer peptide or argatroban (positive control) were pre-incubated with 300 µl of PPP for 3 min at 37 °C, and clotting was initiated by adding 40 µl of 250 mM $CaCl_2$. For control, equivalent volume of 1X PBS, pH 7.4 was used. The time required to form a stable blood clot was measured using a stopwatch. One unit of anticoagulant activity has been defined as peptide-induced 1 s increase in clotting time of the control whole blood.

3.2.10.2 Plasma clotting assays

3.2.10.2.1 Screening of anticoagulant activity of peptides

The anticoagulant activity of the synthetic peptides (0.25 – 1.0 µM) was assayed against mammalian (goat) platelet poor plasma (PPP) as described in section 3.2.10.2.2. Briefly, 0.25 – 1.0 µM of all peptides / 0.25 – 1.0 µM purified PLA_2 / 0.25 – 1.0 µM heparin (positive control) / 1X PBS, pH 7.4 (control) / 1X PBS, pH 7.4 containing

0.05% DMSO (control for peptides ACR9 and ACR12) was incubated with 300 µl of PPP for 3 min at 37 ºC and the clotting time was determined (section 3.2.10.2.2).

3.2.10.2.2 Plasma re-calcification assay

To study the plasma clotting activity, goat blood obtained from a slaughter house was collected in 3.8% tri-sodium citrate in 9:1 ratio. The platelet poor plasma (PPP) was prepared by centrifuging the blood twice at 5000 rpm for 20 min at 4 °C. Different concentrations of venom fraction / purified PLA_2 (40 to 200 nM in a total volume of 20 µl) were pre-incubated with 300 µl of PPP for 3 min at 37 °C, and clotting was initiated by adding 40 µl of 250 mM $CaCl_2$ [24,43]. For control, instead of PLA_2, the same volume of 1X PBS, pH 7.4 was used. The anticoagulant potency of the purified PLA_2 was compared with equivalent doses of commercial drugs heparin and warfarin.

In another set of experiments, graded concentrations (1.0 – 10.0 µM) of 7-mer peptide / argatroban (positive control) / 1X PBS, pH 7.4 containing 0.05% DMSO (control) were incubated with 300 µl of PPP or whole blood for 3 min at 37 ºC. The re-calcification time against PPP was measured as described above. One unit of anticoagulant activity has been defined as crude venom / purified PLA_2 / custom peptide-induced 1s increase in clotting time of the control PPP [24,43].

3.2.10.2.3 Effect of time on re-calcification of PPP

To determine the correlation between the kinetics of plasma phospholipids hydrolysis and that of the anticoagulant activity, a fixed amount of purified PLA_2 or synthetic peptide was incubated with 300 µl of plasma from 3 – 20 min at 37 °C. Then, 40 µl of 250 mM $CaCl_2$ was added and the clotting time was recorded [25].

3.2.10.2.4 Prothrombin time (PT) assay

The prothrombin time (PT) of goat PPP was tested by using a commercial kit following the manufacturer's protocol [40,44]. Briefly, 0.1 ml of PPP was pre-warmed in a test-tube by placing it in a water bath for 3 to 5 min at 37 °C. Then gradient doses of the purified PLA_2 / synthetic peptide showing anticoagulant activity (ACR9) was incubated with the pre-warmed PPP for 3 min at 37 °C. Thereafter, 0.2 ml of PT reagent

(pre-warmed at 37 °C) was added forcefully to the reaction mixture and the tube was gently shaken to mix the contents. The tube was observed for any appearance of visible fibrin thread / clot formation and the time of visible clot formation was recorded with the help of a stop watch. For control, equivalent volume of PPP was incubated with 1X PBS, pH 7.4, and the coagulation time was determined in a similar manner. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP as compared to clotting time of normal PPP (control) under identical assay conditions [40,44].

3.2.10.2.5 Activated partial thromboplastin time (APTT) assay

The activated partial thromboplastin time (APTT) of goat PPP was also tested by using a commercial kit following the manufacturer's protocol [39,40,44]. Briefly, 0.1 ml of PPP was pre-warmed in a test-tube by placing it in a water bath for 3 to 5 min at 37 °C. Then gradient doses of purified PLA_2 / synthetic peptide showing anticoagulant activity (ACR9) were incubated with the pre-warmed PPP for 3 min at 37 °C. Thereafter, 0.1 ml of APTT reagent (pre-warmed at 37 °C) was added gradually to the reaction mixture and the tube was gently shaken to mix the contents. The reaction mixture was further incubated for 3 min at 37 ºC and thereafter 0.1 ml of 25 mM $CaCl_2$ solution was added to the mixture. The tube was then observed for any appearance of visible fibrin thread / clot formation and the time of visible clot formation was recorded with the help of a stop watch. For control, equivalent volume of PPP was incubated with 1X PBS, pH 7.4, and the coagulation time was determined in a similar manner. One unit of anticoagulant activity has been defined as 1 s increase in the clotting time of PPP as compared to clotting time of normal PPP (control) under identical assay conditions.

3.2.10.2.6 Thrombin time (TT) assay

The thrombin time (TT) of goat PPP was also tested by using a commercial kit following the manufacturer's protocol [45]. Briefly, 0.1 ml of PPP was pre-warmed in a test-tube by placing it in a water bath for 3 to 5 min at 37 °C. Then gradient doses of the purified PLA_2 / synthetic peptide showing anticoagulant activity (ACR9) were incubated with the pre-warmed PPP for 3 min 37 °C. Thereafter, 0.2 ml of TT reagent (pre-warmed at 37 °C) was added gradually to the reaction mixture and the tube was

gently shaken to mix the contents. The tube was then observed for any appearance of visible fibrin thread/clot formation and the time of visible clot formation was recorded with the help of a stop watch. For control, equivalent volume of PPP was incubated with 1X PBS, pH 7.4, and the coagulation time was determined in a similar manner. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP as compared to clotting time of normal PPP (control) under identical assay conditions.

3.2.10.2.7 Determination of fatty acid release from plasma

To determine the fatty acid release due to plasma phospholipid hydrolysis by the purified PLA_2, the enzyme was incubated with plasma under identical experimental conditions (30 min at 37 °C) and the liberated fatty acids were titrated with 0.01 N NaOH as described in section 3.2.5.3. The amount of FA liberated was determined from a standard curve of palmitic acid [25].

3.2.10.3 Platelet modulating activity

3.2.10.3.1 Isolation of platelet rich plasma and washed platelets

Platelet rich plasma (PRP) and subsequently washed platelets were prepared from citrated mammalian (human or goat) blood by following the procedures described by Bednar et al. [46]. Briefly, citrated goat or human blood (fresh) was centrifuged at 350 g for 10 min at 4 ºC to isolate the PRP. The supernatant containing PRP was carefully aspirated out and platelets were isolated by centrifuging the PRP at 650 × g for 10.0 min. The pellet, containing platelets, was washed twice in Tyrode buffer (5.0 mM HEPES, 13.0 mM NaCl, 2.7 mM KCl, 12.0 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 1.0 mM $MgCl_2$, 0.1% glucose and 0.25% BSA). Finally, the pellet was re-suspended in the same buffer and the volume was adjusted with Tyrode buffer to give an absorbance of 0.15 at 650 nm.

3.2.10.3.2 Effect on PRP or washed platelets

Different concentrations (3 – 240 nM) of purified PLA_2 were added to 100 µl of pre-warmed (at 37 °C for 5 min) PRP or washed platelets suspension either in presence or absence of 2 mM Ca^{2+} in a 96-well microplate and the contents were mixed for 5 s in a microplate reader (Multiskan GO, Thermo Scientific, Waltham, USA). The

absorbance was then recorded continuously at 540 nm for 300 s at an interval of 15 s. As a control, the absorbance of PPP as well as PRP was also recorded under identical conditions. The absorbance value of PPP was subtracted from the experimental readings to determine the absorbance only due to platelets in PRP. Percent platelet aggregation after 300 s of incubation of platelets with different concentrations of PLA_2 was calculated by the following formula [47]:

% platelet aggregation = (A_{540} of the platelet suspension/PRP before the addition of agonist – A_{540} of the platelet suspension after the addition of agonist) ÷ (A_{540} of the platelet suspension/PRP before the addition of agonist – A_{540} of the PPP/Tyrode buffer) × 100. ----- (3.11)

Where, A_{540} denotes absorbance value at 540 nm.

3.2.10.3.3 Effect of phospholipids on PLA_2 induced platelet deaggregation

Washed platelets were pre-incubated with 0.1 µg/ml of PC or PS or 10 µl of PPP for 5 min before addition of 60 nM of purified PLA_2 (NnPLA_2-I) to the washed platelet suspension. Thereafter, the platelet modulating activity was determined as described above (section 3.2.10.3.2).

3.2.10.3.4 Effect of heparin, *p*-BPB, and antivenom on platelet modulating property

Purified PLA_2 (60 nM) was pre-incubated with 20 mIU of low molecular weight (<8 kDa) heparin or 2 mM *p*-BPB for 30 min at room temperature (~23 °C) and then excess of reagents were removed by 3 kD cut-off membrane filtration. As a control, the PLA_2 was incubated with Tyrode buffer under identical conditions. The platelet modulating activity of PLA_2 (native or modified) was assayed as above. The activity of unmodified (native) PLA_2 was considered as base value (100% activity) and other values were compared to that. The neutralization of platelet modulating activity of PLA_2 by polyvalent and monovalent antivenom was studied by pre-incubating a fixed concentration of PLA_2 (0.5 µM) with different concentrations of PAV or MAV for 30 min at room temperature (~23 °C) and then assayed for platelet modulating activity (as described in section 3.2.10.3.2) against appropriate control.

3.2.10.3.5 Effect on collagen-induced platelet aggregation

PRP (100 µl, 1 × 10^6 cells/ml) was pre-incubated with different concentrations of PLA_2 (2 – 10 nM) for 5 min prior to addition of collagen type IV (1.0 µg/ml). The platelet aggregation was assayed as described in section 3.2.10.3.2. The aggregation induced by the same dose of collagen was considered as 100% activity and other values were compared with that. The IC_{50} value was calculated from the regression analysis of inhibition curve [40].

3.2.10.3.6 Effect on thrombin-induced platelet aggregation

The thrombin-inhibitory activity was assayed on PRP prepared from citrated mammalian (goat) blood. The *in vitro* platelet aggregation inhibition property was investigated by pre-incubating different concentrations (0.5 – 5.0 µM) of EI NnV purified PLA_2 with human thrombin (0.2 µg/ml) for 10 min. A control reaction was also set up where 1X PBS, pH 7.4 instead of purified PLA_2, was used. The platelet aggregation was monitored in a CHRONO-LOG® WBA Model 592 Dual Channel aggregometer (Chrono-Log, Havertown, USA) for 10 min. The PLA_2 induced thrombin inhibition, if any, was determined by calculating the percent platelet aggregation after 10 min by using the formula 3.11 described in section 3.2.10.3.2.

3.2.10.3.7 Platelet binding assay

The ability of native or heparin / *p*-BPB-modified NnV PLA_2 to bind with washed platelet was studied by ELISA method. The 96-well microtitre plate coated with 1 × 10^6 platelets were washed for three times, each for 5 min duration, with 1X PBS containing 0.05% Tween-20 (washing buffer) to remove the unbound platelets [48]. The wells were then blocked with 5% fat-free milk, washed for three times with washing buffer, and then incubated with 5.0 µg/ml of purified PLA_2 (native or unmodified) for 30 min at room temperature. The unbound PLA_2 was removed by washing three times with wash buffer followed by incubation with 1:500 dilutions of monovalent equine antivenom (1 mg/ml) against *N. kaouthia* for 2 h at room temperature, and again washed with the wash buffer. Rabbit anti-horse IgG conjugated with horseradish peroxidase (HRP) (1:2000) was used as the secondary antibody to detect the bound primary antibodies. After 2 h of incubation, the substrate (1X TMB / H_2O_2) was added and the

plate was incubated at room temperature in dark. The reaction was terminated by adding 50 µl of 2.0 M H_2SO_4 and the absorbance of the reaction mixture was measured at 492 nm against appropriate blanks in a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, USA). The binding of native (unmodified) PLA_2 with platelets was considered as 100% binding and other values were compared to that.

3.2.10.4 Direct and indirect hemolytic activity assay

Hemolytic activity (both direct and indirect) of the purified PLA_2 / venom fraction / custom peptide against the washed erythrocytes (5%) obtained from citrated goat blood was measured, as stated previously [24]. Goat blood collected in 3.8% tri-sodium citrate was centrifuged at 5000 rpm for 20 min at 4 ºC. The pellet, containing erythrocytes was washed twice with 0.9% NaCl solution (saline) prepared in type I water. Finally the washed erythrocytes were re-suspended in 1X PBS, pH 7.4 to obtain a final erythrocytes concentration of 5% (v/v). Different concentrations of the purified PLA_2 / venom fraction (Nn(N)CM2 / anticoagulant custom peptide were incubated with 2.0 ml of 5% erythrocytes suspension for 90 min at 37 ºC. For indirect hemolytic activity assay, different concentrations of purified PLA_2 were pre-incubated with 10 µl of 1.0 mM PC/egg-yolk suspension for 10 min at room temperature (~25 ºC). Thereafter, 2.0 ml of erythrocyte suspension was added to the reaction mixture and it was incubated for 90 min at 37 ºC. After incubation, the erythrocyte suspensions were centrifuged at 10,000 rpm for 15 min and the absorbance of the supernatant was recorded at 405 nm using a plate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, USA) [24,49]. For blank and positive control, equivalent volumes of 1X PBS, pH 7.4 and 0.1% triton X-100 was used. Hemolytic activity, if any, was expressed as % hemolysis compared to hemolysis induced by 0.1% triton X-100 (100%).

3.2.10.5 Antibacterial activity assay

Antibacterial activity of the purified PLA_2 or Nn(N)CM2 or 7-mer peptide against gram positive *Bacillus subtilis* (MTCC no. 441) and gram negative *Escherichia coli* (MTCC no. 443) was assayed by our previously described procedure [49,50]. Briefly, mid-logarithmic phase bacterial culture ($A_{630\ nm}$ ~0.3) was centrifuged at 10,000 rpm for 15 min at 4 ºC and the pellet comprising of bacterial cells were re-suspended in

1X PBS, pH 7.4. The re-suspended bacterial cells were incubated with different concentrations of purified PLA_2 / Nn(N)CM2 / anticoagulant custom peptide / 1X PBS, pH 7.4 (control) at 37 ºC for 24 h. Antibacterial activity demonstrated by lysis of bacterial cells, if any, was measured as an decrease in the optical density of the bacterial suspension at 630 nm by a plate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, USA). The bacterial cells incubated with ampicillin (100 µg/ml) and tetracycline (100 µg/ml) served as positive control for *B. subtilis* and *E. coli* cells, respectively, under identical experimental conditions.

3.2.10.6 *In vitro* cell-cytotoxicity assay

3.2.10.6.1 Effect on platelet viability

Platelets were isolated from goat PRP as described in section 3.2.10.3.1. 100 µl of washed platelets suspension (1×10^6 platelets/ml) was incubated with 10.0 µg/ml of purified PLA_2 or Nn(N)CM2 for 6 h at 37 ºC, 5% CO_2. For control, equivalent volume of 1X PBS, pH 7.4 was used. After incubation, the platelet suspension was treated with trypan blue. Platelets viability or decrease in number of platelets, if any, was determined by counting the trypan blue stained platelets in a hemocytometer using Motic Images plus 3.0 ML software [8].

3.2.10.6.2 Effect on cancerous and normal mammalian cell lines

The mammalian cell lines U87MG, MCF-7, PC-12 and HEK-293 cells (1×10^3 cells/ml) were grown in DMEM containing 10% heat-inactivated FBS and 1% pen-strep antibiotic solution (Gibco™, Thermo Fisher Scientific, USA) in a 96-well plate at 37 ºC in a 5% CO_2 humidified incubator for 24 h. After allowing the cells to grow and adhere to the culture plates, the medium was replaced with a fresh medium containing purified PLA_2 (10.0 µg/ml or 0.70 µM) / Nn(N)CM2 (12.5 and 25.0 µg/ml containing 0.35 µM and 0.70 µM) / custom peptide (10.0 µg/ml or 12.5 µM) / 1X PBS, pH 7.4 (control). After 24 h of incubation, 100 µl of 1.0 mg/ml of MTT was added to the wells and the reaction mixture was incubated for 4 h at 37 ºC, 5% CO_2 [51]. Thereafter, the cells were treated with 100 µl of DMSO and incubated in dark with constant shaking. The background absorbance of multi-well plates was measured at 690 nm and subtracted from the absorbance at 550 nm. The percent cell viability was calculated as the ratio of

treated cells to the control cells and was expressed as % cell death by comparing the values to that of control (100%) [4,50].

3.2.10.6.3 Flow cytometry analysis to determine the cell cycle kinetics

The effect of purified PLA_2 and anticoagulant peptide on the cell cycle of MCF-7 cells was determined by flow cytometry analysis as described previously [45]. The MCF-7 cells (1.5×10^5 cells/ml) were seeded in 96-well plates and allowed to adhere overnight at 37 ºC. Following day, the old medium (DMEM containing 10% FBS) was replaced with fresh media containing 7-mer peptide (10 µg/ml or 12.5 µM) or purified PLA_2 (10 µg/ml or 0.70 µM) or only growth medium (control) and incubated for 48 h at 37 °C in a 5% CO_2 humidified incubator. Thereafter, the cells were collected by trypsinization and fixed by adding chilled 70% ethanol. The fixed cells were centrifuged and washed with chilled 1X PBS, pH 7.4, following incubation with RNase at 37 ºC for 1 h. Cells were then incubated with propidium iodide stain for 2 h before being analyzed by a flow cytometer (FACscan, Becton Dickinson, Bedford, MA) and the data was analyzed by ModFiT LT software.

3.2.10.6.4 Effect on rat skeletal muscle cells or myoblasts

3.2.10.6.4.1 Culturing and partial differentiation of rat myoblasts

Rat skeletal myoblasts (L6) (1×10^3 cells/ml) were grown in DMEM containing 10% heat-inactivated FBS and 1% pen-strep antibiotic solution (Gibco™, Thermo Fisher Scientific, USA) in a 96-well plate at 37 ºC in a 5% CO_2 humidified incubator for 24 h. The myoblasts were allowed to partially differentiate by replacing the medium with fresh DMEM containing 2% FBS. The process was repeated after every 24 h for 3 – 5 days.

3.2.10.6.4.2 Bright field imaging and MTT-based myoblasts viability assay

The partially differentiated myoblasts were treated with - (i) purified PLA_2 (0.70 µM), (ii) RP-HPLC purified 2.2 µg/ml or 0.35 µM of the cytotoxin (CTx), (iii) 4.8 µg/ml or 0.62 µM of neurotoxin (LNTx), (iv) re-constituted complexes of CTx (0.35 µM)-LNTx (0.62 µM), and (v) CTx (0.35 µM)-LNTx (0.62 µM)-purified PLA_2 (0.35 µM), and (vi) native PLA_2 cognate complex (12.5 µg/ml and 25.0 µg/ml containing

0.35 μM and 0.70 μM PLA_2, respectively). The concentration of the above 3FTxs / PLA_2 was calculated based on their percent relative abundance in the cognate complex as determined by LC-MS/MS analysis of Nn(N)CM2 (section 3.2.4.3.4) The proteins were purified by RP-HPLC of Nn(N)CM2 (section 3.2.4.3.1). After 24 h of incubation, cytotoxicity was determined by observation of the cells under the bright field of a fluorescence microscope followed by counting using Motic Images plus 3.0 ML software, after acridine orange / ethidum bromide (AO/EB) staining (see section 3.2.10.6.4.3 below), and MTT-based cell viability assay as described in section 3.2.10.6.2. Cytotoxicity was expressed as % cell death by comparing the values to that of control (100%) [4,50].

3.2.10.6.4.3 Effect on myoblast morphology

To study the morphological changes induced in L6 myoblasts by purified PLA_2, CTx, LNTx, reconstituted PLA_2-3FTx complexes and native Nn(N)CM2 complex (concentrations described in section 3.2.10.6.4.2), the treated cells were stained with ethidium bromide and acridine orange (100 μg/ml in 1X PBS, 1:1). After 5 min of incubation at 37 °C in a humidified CO_2 incubator, the cells were washed in 1X PBS for three times and then observed under a fluorescence microscope (Leica DMi8, Leica Microsystems, Germany) at 10 X magnification using rhodamine (for red) and FITC (for green) filters [52]. The treated L6 cells were also observed under a bright field microscope (Leica DMi8, Leica Microsystems, Germany) at 10 X magnification.

3.2.10.6.4.4 Assessment of serum CK and LDH

The cytotoxicity on L6 myoblasts was also confirmed by assaying the release of creatine kinase (CK) and lactate dehydronase (LDH) in growth medium 24 h post treatment with the purified PLA_2 and Nn(N)CM2 using commercial diagnostic kits (Tulip Diagnostics Ltd., India).

3.2.10.6.5 Neutralization of PLA_2 cognate complex-induced cytotoxicity by polyvalent antivenom and anti-PLA_2 antibody

Prior to addition to partially differentiated myoblasts, the PLA_2 cognate complex (12.5 μg/ml) was pre-incubated with graded concentrations of PAV (protein: protein,

w/w ratio of 1:6.25, 1:12.5, and 1:25.0) or with anti-PLA_2 antibody (BioBharati Life Sciences Pvt. Ltd.), at 1:25 w/w ratio for abolishing the PLA_2 activity, for 30 min at 37 ºC. In another set of experiments, the PAV was added to culture media at 1:25 ratio after 60, 120, and 240 min post treatment of partially differentiated myoblasts with PLA_2 cognate complex (12.5 µg/ml). After 24 h of incubation the cytotoxicity was assayed by MTT-based method as mentioned in section 3.2.10.6.2. The cytotoxicity induced by PLA_2 cognate complex was considered as 100% activity and other values were compared to that.

3.2.10.6.6 Inhibition of PLA_2 cognate complex-induced cytotoxicity by chemical modification of His47 residue

The Nn(N)CM2 (25.0 µg/ml) was pre-incubated with 5.0 mM *p*-BPB at 37 ºC for 30 min. The un-reacted / excess *p*-BPB was separated by filtering the reaction mixture through a 3 kDa cut-off membrane (Pall Corporation, USA). Thereafter, the rat myoblasts were incubated with His-alkylated PLA_2 cognate complex for 24 h at 37 ºC, 5% CO_2, and cytotoxicity, if any, was measured by bright field imaging and MTT assay (described in section 3.2.10.6.4.2).

3.2.11 Binding and internalization of purified PLA_2 to myoblasts

3.2.11.1 Determination of binding of purified EI NnV PLA_2 and Nn(N)CM2 to L6 myoblasts by ELISA

The L6 cells were grown in DMEM as stated above (section 3.2.10.6.4.1). The adherent cells were isolated by trypsinization and were seeded in the wells of a 96-well tissue culture plate at a density of 1×10^3 cells per well. The cells were allowed to adhere to the well surface by incubating them in DMEM under regular growth conditions for 18 h and further allowed to differentiate partially.

The wells coated with L6 myoblasts were blocked using 5% FBS in 1X PBS for 30 min at room temperature and then the wells were washed for three times with PBS-T (10 mM phosphate buffer containing 150 mM NaCl and 0.05% tween-20) for 10 min each with constant shaking. Thereafter, increasing concentrations of purified PLA_2 (0.17 µM, 0.35 µM, 0.70 µM) or Nn(N)CM2 (6.25, 12.5, 25.0 µg/ml) or 1X PBS

(control) were added to the cells and incubated for 30 min at room temperature. The wells were washed with PBS-T for three times followed by addition of 100 µl of 1:2000 diluted rabbit anti-PLA_2 antibodies (100 ng per well) and incubated for 2 h at room temperature. The wells were again washed with PBS-T and 100 µl of 1:5000 diluted anti-rabbit IgG-HRP conjugated secondary antibodies was added and incubated for 2 h at room temperature. After washing the wells with PBS-T, 100 µl of 1X TMB / H_2O_2 was added and the plates were incubated in dark at room temperature for 30 min. The reaction was terminated by adding 50 µl of 2.0 M H_2SO_4 and the absorbance was measured at 492 nm in a microplate reader (Multiskan GO, Thermo Scientific, USA) (section 3.2.10.3.7).

3.2.11.2 Immunofluorescence assay to determine the time-dependent binding followed by internalization of purified PLA_2 in L6 myoblasts

L6 myoblasts grown on cover slips were allowed to differentiate into myoblasts (section 3.2.10.6.4.1) and incubated with 10.0 µg/ml or 0.70 µM of purified PLA_2 in culture medium for 30, 60, 120, and 240 min at 37 ºC, 5% CO_2. The cells were fixed with 4% formaldehyde for 10 min followed by washing three times for 5 min each with ice-cold 1X PBS, pH 7.4. The fixed cells were then permeabilized using 0.1% triton X-100 in 1X PBS, pH 7.4 for 5 min and then washed in ice-cold 1X PBS, pH 7.4 for three times. Thereafter, the cells were blocked with 1% BSA solution in 1X PBS, pH 7.4 containing 22.52 mg/ml glycine and 0.1% tween-20. The cells were then washed with ice-cold 1X PBS, pH7.4 for three times, and incubated with anti-PLA_2 polyclonal antibody (1: 250 in 1X PBS-T) at 4 ºC for overnight. The following day, myoblasts adhered to cover slips were washed with ice cold 1X PBS, pH 7.4 and incubated with 1: 200 diluted (in PBS-T) Alexa Fluor 488 conjugated anti-rabbit IgG (Invitrogen, Thermo Scientific, USA) for 60 min at room temperature (~23 ºC). The cells were then washed, stained with DAPI for 5 min and mounted on glass slides using ProLong® Gold Antifade Mountant (Thermo Fisher Scientific, Germany) and visualized under Leica DMi8 fluorescence microscope at 40 X magnification using FITC (for green) and DAPI (for blue) filters.

3.2.11.3 Confocal microscopy to determine internalization of fluorescein isothiocyanate (FITC)-conjugated purified PLA_2 in myoblasts

In another set of experiment, the purified PLA_2 was conjugated with FITC and thereafter incubated with myoblasts for 30 – 240 min at 37 ºC. Briefly, PLA_2 (10.0 mg/ml) was dissolved in MOPS [3-(N-morpholino)propanesulfonic acid] buffer and incubated with FITC (protein:FITC ratio of 1: 2) for 2 h at room temperature. Then β-mercaptoethanol was added to the reaction mixture to block the free FITC groups. The conjugated PLA_2 (10.0 μg/ml or 0.70 μM) was filtered through a 3 kDa cut-off membrane (Pall Cooperation, USA), and thereafter incubated with myoblasts for 20 – 240 min at 37 ºC. After incubation, the culture media was aspirated out, the cells were washed with ice-cold 1X PBS, pH 7.4, stained with DAPI for 5 min, washed with 1X PBS, pH 7.4, and mounted on glass slides using ProLong® Gold Antifade Mountant. The cells were then visualized under a Zeiss LSM 880 confocal microscope at 63 X magnification using bright field, FITC (488 nm excitation wavelength), and DAPI (358 nm excitation wavelength) filters. To confirm the time-dependent internalization of purified PLA_2, the confocal microscopic images of treated rat myoblasts were subjected to z-stack projection analysis [53] by ZEN System software, ZEISS, Germany. A set of 9 – 15 z-stack images in different planes (separated by 1 μm) was analyzed for time-dependent internalization of FITC-conjugated PLA_2.

3.2.12 Binding of purified PLA_2 and its cognate complex [Nn(N)CM2] to myoblast membrane proteins

3.2.12.1 Isolation of myoblast membrane proteins

Membrane proteins of partially differentiated L6 myoblasts were isolated using the Mem-PER^{TM} Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific, USA). The isolated membrane and cytosolic proteins were quantitated using Bradford reagent (Sigma Aldrich, USA) [21], desalted in PD 10 column (GE Healthcare, Sweden) and freeze-dried (CentriVap Benchtop Vacuum Concentrator, Labconco Corporation, USA) until further use.

3.2.12.2 Immunological assays to determine binding of purified PLA_2 to L6 myoblast membrane proteins (L6MP)

3.2.12.2.1 ELISA to determine binding to L6MP

To determine the binding, 1.0 µg of L6 cytosolic protein (L6CP) or membrane protein (L6MP) / BSA (negative control) was coated in triplicate to a 96-well ELISA plate (Nunc MaxiSorp™, Thermo Fisher Scientific, USA) and incubated for 18 h at 4 ºC. The wells were washed with PBS-T for three times and then blocked with 5% fat-free milk (in 1X PBS, pH 7.4) for 30 min. After washing the wells with 1X PBS-T (three times), 100 µl of 10.0 µg/ml of purified PLA_2 (~1.0 µg per well or 0.70 µM) or 25.0 µg/ml PLA_2-cognate complex [Nn(N)CM2] was added to the wells. An equivalent volume of 1X PBS-T was added to the wells serving as negative controls (BSA). After incubation for 30 min at room temperature, the wells were washed with 1X PBS-T and binding of purified PLA_2 / Nn(N)CM2 to L6MP and L6CP was determined by ELISA as described previously (section 3.2.10.3.7).

3.2.12.2.2 Immunoblot analysis of L6MP binding to purified PLA_2 and Nn(N)CM2

Eighty microgram of L6MP was separated by 12.5% non-reduced SDS-PAGE (two sets of gel run under identical condition). The proteins from one set of gel were transferred to an Immobilon-P PVDF membrane (Merck, Germany) by semi-dry gel transfer system (Amersham Bioscience, UK) at 38 mA (1.2 mA/cm2) for 2 h whereas another set of gel was kept aside. The protein transfer efficiency was verified by 0.5% Ponceau-S red staining of the PVDF membrane as well as PhastGel Blue R staining of the post transfer gel. Thereafter, the membrane was blocked in 5% skimmed milk (w/v) dissolved in TBS-T for overnight at 4 °C. Following day, the membrane was washed three times with TBS-T (10 min for each wash), cut into two pieces and immersed in a small tray containing 5.0 ml of 10 µg/ml (0.70 µM) of purified PLA_2 or 25.0 µg/ml of Nn(N)CM2 (containing 0.35 µM of PLA_2). After incubation for 30 min at room temperature, the membrane was washed with TBS-T and then 1:2000 diluted rabbit anti-PLA_2 antibodies were added and incubated at room temperature for 1 h. After washing the membrane with TBS-T (three times) it was incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (1:5000 dilutions) at room temperature for 1

h. Thereafter, the membrane was washed with TBS-T and the blot was developed using TMB Liquid Substrate System for Membranes (Sigma Aldrich, USA). The gels were scanned on an EPSON scanner using Silver Fast software. The experiment was repeated three times to assure the reproducibility. As a positive control, 80 µg NnV was separated by 12.5% SDS-PAGE under reduced condition, transferred to PVDF membrane and the membrane was developed by using anti-PLA_2 antibody as mentioned above.

3.2.12.2.3 LC-MS/MS to identify the $NnPLA_2$-I binding L6MP

From the second set of gel the L6MP bands showing binding with purified PLA_2 or Nn(N)CM2 were excised and subjected to in-gel trypsin digestion (section 3.2.3.1.1). The tryptic peptides were then separated on an Agilent 1260 UHPLC system and subsequently analyzed on an Agilent 6530 QTOF mass spectrometer (section 3.2.4.3.3). The raw MS/MS data was searched against the *Rattus norvegicus* (taxid 10116) protein entries of the non-redundant NCBI database using Morpheus software (section 3.2.4.3.3).

3.2.12.3 Isolation and purification of PLA_2-binding L6MP

3.2.12.3.1 Affinity purification of PLA_2 binding L6MP

The PLA_2 binding L6MP was isolated by affinity chromatography using immobilized purified PLA_2 as the ligand. Briefly, 200 µl of NHS-activated Sepharose 4 Fast Flow matrix was packed in a 1.0 ml column and incubated with 200 µg of EI NnV PLA_2 for overnight (~18 h) at 4 ºC. The column was washed with 1.0 ml of 1X PBS, pH 7.4 to wash out the unbound PLA_2, if any, which was determined by estimating the protein content [21,22] and assay of PLA_2 activity of post wash buffer. Thereafter, 400 µg of membrane proteins were loaded onto the column and incubated at room temperature (~25 °C) for 2 h with constant shaking. The unbound L6MPs were eluted by washing the column with 5 column volumes (CV) of 1X PBS, pH 7.4; while the bound proteins were eluted with a step gradient of 2 CV of 0.02 mM glycine, pH 2.0, followed by 5 CV of 0.1 M glycine, pH 2.0 [54]. The pH of the eluted bound L6MP was immediately neutralized by adding equal volume of 1 M Tris-HCl, pH 9.0 [13].

3.2.12.3.2 RP-HPLC and identification of PLA_2 binding L6MP

The PLA_2 binding L6MP was quantitated, desalted, vacuum dried, and then subjected to RP-HPLC fractionation (Dionex Ultimate 3000 U-HPLC system, Thermo Fisher Scientific, Bremen, Germany) in an Acclaim C_{18} column (3 µm, 2.1 × 150 mm) pre-equilibrated with 95% of solvent A (0.1% trifluoroacetic acid) and 5% solvent B (90% acetonitrile containing 0.1% TFA). After eluting the hydrophilic proteins with solvent A, the bound L6MPs were eluted with a multi-step gradient of solvent B. The proteins fractions were collected and lyophilized.

L6MP protein (100 ng) from each RP-HPLC peak was coated in a 96 well plate and it's binding with EI NnV PLA_2 was determined by ELISA using anti-PLA_2 antibodies (section 3.2.11.1). The RP-HPLC fraction of L6MP that bind to purified PLA_2 was subjected to in-solution trypsin digestion (section 3.2.3.1.1) followed by LC-MS/MS analysis for protein identification (section 3.2.12.2.3) and quantification (section 3.2.4.3.4).

3.2.13 *In silico* analysis to determine interaction of PLA_2 and its cognate complex [Nn(N)CM2 fraction] with coagulation factors and vimentin

3.2.13.1 Structure prediction of interacting proteins/peptides

The structure prediction of EI NnV PLA_2 is described in section 3.2.6.1. The complete FASTA sequence of the different proteins of the PLA_2 cognate complex – P15445 (purified PLA_2), P25671 (LNTx), and P86538 (CTx) were obtained from NCBI database and submitted to I-TASSER software for structure prediction [27]. The PDB structures for four regions of the rod region of vimentin were available at the Protein Data Bank data (https://www.rcsb.org/) with accession numbers 3s4r (chain B: 99 – 189), 3uf1 (chain A: 146 – 249), 3trt (chain A: 261 – 335) and 1gk4 (chain D: 330 – 407). Molecular dynamics for each of these structures were run using MDWeb server (http://mmb.irbbarcelona.org/MDWeb/) [55] and for docking analysis chains B, A, A and D of 3s4r, 3uf1, 3trt and 1gk4, respectively, were used [56].

3.2.13.2 Docking analysis with vimentin and predicting free-energy of interaction

The binding of PLA_2 with each of the structures of vimentin was studied using ClusPro 2.0 [28] and FireDock servers [57]. The docking servers calculate the global energy of interaction and the model with the lowest energy was considered as the best model [57]. The vimentin structures (mentioned above) showing the best interaction with PLA_2 was further considered for studying their binding with Nn(N)CM2 and its individual components. The Nn(N)CM2 was obtained by docking its individual components using ClusPro 2.0 server (https://cluspro.bu.edu/). Further, PDBSum server tool (http://www.ebi.ac.uk/pdbsum) was used to determine the residue-to-residue interactions between PLA_2 and its cognate complex [Nn(N)CM2] to vimentin [30,31].

In order to calculate the predicted values of ΔG (free binding energy) and K_D (dissociation constant) of PLA_2-vimentin interaction, the full sequences of PLA_2 and vimentin were submitted to PPA-Pred2 (Protein-Protein Affinity Predictor) online server (https://www.iitm.ac.in/bioinfo/PPA_Pred/) [58].

3.2.14 Validation of *in silico* docking results

3.2.14.1 ELISA to determine binding of purified PLA_2 to vimentin

To confirm the results of *in silico* studies by wet lab experiment, 1.0 µg of RP-HPLC purified vimentin was coated on a 96 well plate for overnight. Prior to addition of 10.0 µg/ml of PLA_2 or 25.0 µg/ml of Nn(N)CM2, the vimentin was pre-incubated with anti-vimentin antibody (ab92547, Abcam, USA) raised against the tail region of vimentin or 1X PBS, pH 7.4 (control) for 30 min at room temperature. Thereafter, binding of PLA_2 or Nn(N)CM2 to treated vimentin was assessed by ELISA as described in section 3.2.11.1. The binding of PLA_2 or Nn(N)CM2 to vimentin was considered as 100% binding and other values were compared to that.

3.2.14.2 Spectrofluorometry analysis to determine dose- and time-dependent binding of purified PLA_2 and its cognate complex [Nn(N)CM2] to vimentin

The interaction between purified PLA_2 and its cognate complex with vimentin was determined by our previously described method [10] with slight modifications. Briefly, to determine the dose-dependent binding, graded concentrations of PLA_2 (0.17

– 0.70 µM) or Nn(N)CM2 (6.25 – 25.0 µg/ml containing 0.17 – 0.70 µM of PLA_2) were added to the wells of a Nunc™ F96 MicroWell™ Black Polystyrene Plate (Thermo Fisher Scientific, Denmark) containing vimentin (10.0 µg/ml) in a reaction mixture of 100 µl. For time-dependent binding study, 0.17 µM of PLA_2 or 2.5 µg/ml of Nn(N)CM2 was incubated with 10.0 µg/ml of vimentin for different time intervals (7.5-60 min) in a 100 µl reaction mixture. The reaction mixtures were excited at 280 nm with a monochromator light source. The slit length was maintained at 12 nm and emission was monitored from 300 to 500 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, USA). As a control, the fluorescence spectrum of individual protein was also determined and compared with the relative intensity of the fluorescence spectra (λmax) of PLA_2 and its cognate complex [Nn(N)CM2] with vimentin. The change in λmax (Δλmax) was plotted against the concentrations (µg/ml) or pre-incubation time (min) of purified PLA_2 / Nn(N)CM2 with vimentin using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, USA).

3.2.15 Assessment of *in vivo* toxicity and therapeutic potential of the purified PLA_2 and 7-mer anticoagulant custom peptide

3.2.15.1 Toxicity assessment

Acute *in vivo* toxicity of the purified PLA_2 and the anticoagulant peptide was validated in Wistar strain rats by following the OECD / OCDE guidelines 425 (2001). Briefly, Wistar strain rats (n=3, either sexes; 150 – 180 g) were intravenously (*i.v.*) injected with 4.0 mg/kg of PLA_2 / peptide dissolved in 0.2 ml 1X PBS, pH 7.4. The control group (placebo) of rats (n=3) received equal volume of 1X PBS, pH 7.4. The animals were observed at regular intervals for up to 72 h for any behavioral change [40]. After 72 h of treatment, the rats were sacrificed and blood was collected immediately by cardiac puncture [40,45]. Hematological parameters of the blood and biochemical parameters of serum of treated and control group of rats were analyzed by Hematology Auto Analyzer MS4-S (Melet Schloesing Laboratories, Osny, France) and auto analyzer (Biochemical Systems International SRL Model 3000 evolution, Florence, Italy), respectively.

3.2.15.2 Histological study of major organs

Histopathological study of the heart, liver, and kidney tissues of the treated and control groups of rats was done as described previously [4,45]. Briefly, the animals were treated with 4.0 mg/kg of purified PLA_2 / 7-mer custom peptide by *i.v.* administration. After 72 h of injection, the animals were euthanized by diethyl ether and major organs were dissected. Heart, kidney and liver tissues were fixed cut into smaller pieces, washed in 1X PBS, and fixed in 10% formaldehyde solution until further use. Thereafter, the tissues were dehydrated by passing through increasing concentrations of ethanol (30 – 100%) and embedded in paraffin (Paraplast TM resin). The blocks of tissues were sliced into 5 µm sections using a microtome, dehydrated by gradient concentrations of ethanol, dual stained by hematoxylin and eosin, and mounted on a coverslip for microscopy. The tissues were observed under a light microscope (Leica DM 3000) for alterations due to PLA_2 / peptide-induced pathology, if any.

3.2.15.3 Assessment of *in vivo* anticoagulant activity

To determine the *in vivo* anticoagulant activity, Wistar strain rats (n=3, either sexes), weighing 150 – 180 g, were intravenously (*i.v.*) injected with 0.4 mg/kg of body weight 7-mer peptide / purified PLA_2 / argatroban (positive control) / heparin (positive control). Blood was withdrawn post 60 min of injection from the control as well as 7-mer peptide/ PLA_2 / argatroban / heparin-treated groups of rats by retro-orbital bleeding [59]. The whole blood clotting time (WBCT), re-calcification time of PPP, PT, APTT (described in section 3.2.10.2) and tail bleeding time were determined as previously described [40,59].

3.2.15.4 Assessment of *in vivo* antithrombotic potential

Either sexes of Wistar strain albino rats (n=4), weighing 150 – 180 g, were intravenously injected with two doses (0.2 mg/kg and 0.4 mg/kg body weight) of 7-mer peptide / argatroban. The control (placebo) groups received an equivalent volume of 1X PBS, pH 7.4. After 30 min of treatment, κ-carrageenan (0.9 mg/kg in 0.2 ml of 1X PBS, pH 7.4) was injected in the tail vein of the rats and wine coloured thrombus formation in the tail was observed for 48 h [59-61]. The thrombus formation was quantitated by densitometry scanning of the photographs of thrombus formed in the tails of control and

treated rats using ImageJ software (NIH, USA). The thrombus formed in the tails of control rats was considered as 100% and other values were compared with that.

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