Chapter III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Venoms and antivenoms

Pooled and dried crude Russell's Viper (*Daboia russelii*) venom (RVV) of Burdwan [EI RVV (B)] and Nadia districts [EI RVV (N)] of West Bengal, eastern India (EI), and Maharashtra, western India (WI) origins were purchased from licensed venom dealers - Calcutta Snake Park, Kolkata, and Haffkine Institute, Mumbai, respectively. Pooled RVV from southern India (SI) from Irula Snake Catchers' Industrial Cooperative Society (ISCICS), Chennai was a gift from Premium Serum and Vaccines Pvt. Ltd., Pune. Dried venom samples were stored in desiccators at room temperature (~23 ˚C) until further use.

Lyophilized equine polyvalent antivenom (PAV) (raised against *Naja naja*, *D. russelii*, *Bungarus caeruleus* and *Echis carinatus*) samples were obtained from Bharat serum and Vaccines Ltd. (BSVL) (Batch no. A05315029, expiry date: January, 2019), Premium Serum and Vaccines Pvt. Ltd. (PSVPL) (Batch no. 012015, expiry date: December, 2018), Virchow Biotech Pvt. Ltd. (VBPL) (Batch no. 012005, expiry date: May, 2018) and Biological E Limited (Bio-E) (Batch no. A1604216, expiry date: March, 2018). Lyophilized monovalent antivenom (MAV) raised against RVV was obtained from Vins Bioproducts Limited (VINS), India (Batch No. 30AS11001, expiry date: April, 2016).

3.1.2 Chromatographic columns, matrices, and other fine chemicals

Prepacked chromatographic columns such as HiLoad 16/600 Superdex 75 pg gel filtration column (1.6 × 60 cm), HiTrap Q FF anion-exchange column (1.6 × 2.5 cm), NHS-activated Sepharose 4 Fast Flow matrix for antivenomics study, and PD-10 column for desalting were purchased from GE Healthcare, Uppsala, Sweden. Acclaim 300 C_{18} reversed-phase ultra high-performance liquid chromatography (RP-UHPLC) column (2.1 × 150 mm, 3 μm) was purchased from Thermo Fisher Scientific, Bremen, Germany. Sinapinic acid matrix and α-cyano-4-hydroxycinnamic acid matrix for MALDI-TOF-MS analysis were purchased from Sigma Aldrich, USA. C_{18} ZipTip pipette tips were purchased from Merck Millipore, USA. Nylon syringe filters and 0.2 μ membranes were purchased from Genetix Biotech Asia Pvt. Ltd., New Delhi, India, and Riviera, Mumbai, India, respectively. Pre-stained protein molecular markers were

purchased from Thermo Fisher Scientific (#26616; 10-170 kDa or #26619; 10-250 kDa), and BioRad (#16103777; 2–250 kDa). Blood coagulation proteins such as bovine and human fibrinogen, thrombin, factor Xa, and prothrombin were purchased from Calbiochem, Germany or Sigma-Aldrich, USA. Kits for prothrombin time test (PT) and activated partial prothrombin time test (APTT) were purchased from Tulip Diagnostics Pvt. Ltd., Mumbai, India, and r2 Hemostasis Diagnostics India Private Ltd, Ghaziabad, India.

Chemical modifiers such as PMSF, IAA and DTT; metal chelator such as EDTA, reagents for buffer preparation such as HEPES, Tris-buffer, phosphate buffered saline, etc., reagents for SDS-PAGE analysis, solvents such as acetonitrile and methanol, and all other chemicals and reagents were of analytical grade and purchased from Merck, Germany or Sigma-Aldrich, USA.

3.2 Methods

3.2.1 Proteomic analyses of WI, EI and SI RVV

3.2.1.1 Fractionation of WI RVV by gel filtration (GF) followed by anion-exchange (AEX) chromatography

Lyophilized WI RVV (200 mg dry weight) was dissolved in 1.0 ml of 25 mM HEPES buffer containing 50 mM NaCl, pH 7.0 for 4 h at 4 ˚C. The venom solution was then centrifuged at 10,000 rpm for 10 min at 4 ˚C and the clear supernatant was filtered through a 0.2 µ membrane syringe filter. The filtrate was then fractionated on a HiLoad 16/600 Superdex 75 pg column (1.6 × 60 cm) pre-equilibrated with the above buffer. The GF column was coupled to an AKTA Purifier 10 fast protein liquid chromatography (FPLC) system (GE Healthcare, Sweden) operated via Unicorn 5.31 control software. The flow rate was 10 ml/h, and fractions of 2.0 ml were collected in 15 ml centrifuge tubes placed in an automated fraction collector (Frac-950). The elution of protein was monitored at 280 nm. The GF peaks were pooled and assayed for protein content (section 3.2.2.1), enzymatic activities (section 3.2.2.2), and pharmacological properties (section 3.2.2.3).

The GF fractions were pooled according to their protein content, then desalted by passing through a PD10 column, and the desalted fractions were lyophilized (Labconco, Model: 7670061, USA). The dried fractions were re-dissolved in 0.5 ml of

buffer A (20 mM Tris-HCl, pH 7.4) and fractionated on a HiTrap Q FF anion-exchange column (1.6 × 2.5 cm) coupled to the AKTA Purifier 10 FPLC system. The column was washed with two column volumes (CV) of buffer A to elute the unbound proteins; the bound proteins were eluted by a 0 to 50% linear gradient of buffer B (20 mM Tris-HCl, pH 7.4 containing 1.0 M NaCl) for 40 min at a flow rate of 1.0 ml/min. Fractions of 1.0 ml were collected and the protein content of each fraction was estimated (section 3.2.2.1). The pooled anion-exchange peaks were desalted, lyophilized, and then subjected to tandem mass spectrometry analysis for protein identification (section 3.2.1.5 to 3.2.1.7).

3.2.1.2 Fractionation of EI RVV by GF chromatography

Lyophilized EI RVV (B) and EI RVV (N) (45 mg dry weight) were dissolved in 1.0 ml of 25 mM HEPES buffer containing 50 mM NaCl, pH 7.0 for 4 h at 4 ˚C and centrifuged at 10,000 rpm for 10 minutes at 4 ˚C. The clear supernatant was passed through a 0.2 μm membrane syringe filter and the protein content of filtrate was estimated (section 3.2.2.1). The filtrate was fractionated on a HiLoad 16/600 Superdex 75 pg column (1.6 × 60 cm) coupled to an AKTA Purifier 10 FPLC system. The resolved protein peaks were pooled and then assayed for protein content (section 3.2.2.1), enzymatic activity (section 3.2.2.2), and pharmacological properties (section 3.2.2.3). The pooled GF peaks were desalted, lyophilized, and then subjected to tandem mass spectrometry analysis for protein identification (section 3.2.1.5 to 3.2.1.7).

3.2.1.3 Fractionation of SI RVV by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SI RVV (500 μg protein) as well as GF fractions of WI RVV and EI RVV samples - RVV B and RVV N (40 μg) were analyzed by 12.5% SDS-PAGE with or without reduction of proteins by β-mercaptoethanol and dithiothreitol (DTT) according to the method described by Laemmli [1]. Resolving and stacking gels were prepared as follows -

A. 12.5% resolving gel (10 ml) -

1. Type I water : 3.3 ml

2. 30% acrylamide-bisacrylamide solution : 4.0 ml

3. 1.5 M Tris-HCl (pH 8.8) : 2.5 ml

4. 10% SDS : 100 µl

5. 10% ammonium persulphate : 100 µl

6. TEMED (Tetramethylethylenediamine) : 10 µl.

The gel was poured into a gel cassette and allowed to polymerize at room temperature.

B. 4% stacking gel (5 ml) -

1. Type I water : 1.8 ml

2. 30% acrylamide-bisacrylamide solution : 0.6 ml

3. 0.5 M Tris-HCl (pH 6.8) : 2.5 ml

4. 10% SDS : 50 µl

5. 10% ammonium persulphate : 50 µl

6. TEMED (Tetramethylethylenediamine) : 7 µl

Electrophoresis was carried out initially at 80 V to allow the proteins to stack, followed by 120 V (for resolving gel). Protein bands were visualized by staining the gel with PhastGel Blue R stain (GE Healthcare, Sweden) for overnight (~16 h) and destained with methanol: acetic acid: water (40:10:50) until the background of the gel become clear and the bands were distinct. The protein bands were scanned (Epson Expression 11000XL, USA) and band intensities were analyzed using ImageQuant TL 8.1 software (GE Healthcare, Sweden) [2]. The relative band intensities of individual protein bands were calculated by considering the total band intensity of crude venom as 100%. The approximate molecular weights of the RVV 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 RVV proteins [3].

3.2.1.4 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) analyses of RVV samples

The molecular masses of the WI, EI and SI RVV proteins were determined by MALDI-TOF-MS analysis on a 4800 MALDI TOF/TOF™ Analyser (Applied

Biosystems) as described previously [3]. Briefly, 0.5 µl of RVV (~1.0-5.0 µg protein) in 0.1% TFA was mixed with 0.5 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 masses of RVV proteins were determined in the ranges of 5-20, 21-40, 41-100, and >100 kDa.

3.2.1.5 In-solution or in-gel trypsin digestion of crude venom or its chromatographic fractions

Forty microgram protein of desalted anion-exchange peaks of WI RVV and GF peaks of EI RVV was subjected to reduction by 10 mM DTT for 30 min at 56 ˚C followed by alkylation with 55 mM iodoacetamide (IAA) for 30 min at dark. Thereafter, the samples were digested with sequencing grade trypsin (13 ng/µl in 10 mM ammonium bicarbonate containing 10% acetonitrile) at an enzyme: substrate ratio of 1:30 [2,4] for overnight (~16 h) at 37 ˚C. The trypsin-digested peptides were desalted and concentrated using ZipTip C_{18} following the manufacturer's protocol.

The SDS-PAGE (reduced) lane of SI RVV (stained with PhastGel Blue R stain) was divided into 10 gel sections, the protein bands were excised using sterile scalpels, cut into small pieces, and transferred into autoclaved microfuge tubes. The gel pieces were incubated in 100% ACN to remove the stain and then subjected to reduction (10 mM DTT) for 30 min at 56 ˚C and alkylation (55 mM IAA) for 30 min at dark. After carefully removing all the liquids, 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) [5].

3.2.1.6 Electrospray ionization-liquid chromatography-tandem mass spectrometry (ESI-LC-MS/MS) analysis

The tryptic fragments of WI and EI RVV were reconstituted in 0.1% formic acid and separated on a Zorbax 300SB-C_{18} analytical column (75 µm × 150 mm, 3.5 µm, Agilent), coupled to an Agilent 1200 HPLC, at a flow rate of 300 nL/min. The mobile phase gradient for separation of the peptides was set as: 11% B for 5 min, 11 to 25% B for 20 min, 25 to 53% B for 16 min, 53 to 100% B for 5 min, 100% B for 4 min, and then 11% 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 [6].

The tryptic peptides of SI RVV were separated on a Zorbax C_{18} column (Rapid Resolution HT 2.1 × 50 mm, 1.8 μm) coupled to an Agilent 1260 UHPLC system using the following mobile phase gradient: 0-4 min: 11% B; 4-30 min: 11-30% B; 30-40 min: 30-40% B; 40-50 min: 40-60% B; 50-60 min: 60-90% B. Solvent A and B were 0.1% formic acid and 90% ACN containing 0.1% formic acid, respectively. The column compartment temperature was maintained at 40 ˚C and the flow rate was set to 0.15 ml/min. The eluted peptides were then analyzed on an Agilent 6530 QTOF mass spectrometer connected in-line with the HPLC system via an electrospray ion source. MS and MS/MS spectra were acquired at 100 to 2000 m/z, with a scan rate of 6 and 3 spectra/s for MS and MS/MS, respectively.

3.2.1.7 LC-MS/MS data analysis and determination of relative abundance of RVV proteins/toxins

The raw MS/MS data of WI, EI, and SI RVV were searched and analyzed using Proteome Discoverer 1.3 software (Thermo Fisher Scientific, Bremen, Germany), PEAKS 8.5 software (Bioinformatics Solutions Inc., Ontario, Canada) and Morpheus software (Agilent Laboratories, California, USA), respectively. The search was performed against the Viperidae entries of the non-redundant NCBI database (taxid:

8689). Carbamidomethylation of cysteine and oxidation of methionine residues were set as fixed and variable modifications, respectively. Precursor and fragment mass error tolerances were set to 10 ppm and 0.8 Da, respectively, and up to two missed cleavages were allowed. The false discovery rate (FDR) was kept very stringent (<1%). Further, to improve identification and sequence coverage of RVV proteins, non-specific cleavage at one end (semi-tryptic peptides) was also considered. A contaminant database with 115 protein entries (ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta) was also included in the database search to exclude common contaminating proteins from being identified.

The initial criteria chosen for valid protein identification were -

A. Protein and peptide -10logP values of ≥ 30 and ≥ 20, respectively.

B. Presence of at least one unique peptide of toxin per identified entry.

Thereafter, all the protein entries were grouped into families on the basis of database nomenclature and the redundant protein and peptide entries were removed from the dataset on the basis of scores to eliminate the peptides being assigned to more than one protein entry. Subsequently, the LC-MS/MS identified peptides were aligned with homologous proteins from the database and manually examined for the presence of overlapping distinct peptides (topologically equivalent in two or more protein entries). Finally, a protein entry having at least one such overlapping distinct peptide was considered to be valid identification.

The relative abundances of the identified RVV proteins were calculated using MS1 (normalized summed peptide-spectrum match precursor intensity) and/or MS2 (spectral count or normalized spectral abundance factor, NSAF)-based label-free quantification techniques [2,7,8]. Normalization of MS1 spectral intensity and MS2 spectral count (mean) was done by number of identified peptides or protein mass using equation 1.

$$Mean\ spectral\ intensity\ or\ count\ for\ protein\ X = \frac{\Sigma\ spectral\ intensity\ or\ count\ against\ MS1/MS2\ peptides\ of\ X}{number\ of\ identified\ peptides\ or\ molecular\ mass\ of\ X} \quad \text{---1}$$

Thereafter, the relative abundance of a protein (X) in a particular chromatographic fraction (Y) was calculated using equation 2:

$$Relative\ abundance\ of\ X\ in\ chromatographic\ fraction\ or\ gel\ section\ Y = \frac{mean\ spectral\ intensity\ or\ count\ of\ X\ in\ Y}{total\ mean\ spectral\ intensity\ or\ count\ of\ all\ proteins\ in\ Y} \times protein\ yield\ (\%)\ or\ relative\ band\ intensity\ of\ Y \quad ---2$$

The relative abundances of RVV proteins were represented as an average of both the methods (MS1 and MS2) in case of SI RVV proteome.

3.2.2 Enzymatic activities and pharmacological properties of crude RVV and their fractions

3.2.2.1 Assay of protein content

The protein content of crude RVV and its chromatographic fractions was determined according to the protocol described by Lowry et al. [9]. A serial dilutions of 0.5, 1.0, 2.5, 5.0, 7.5, and 10 µg per well of bovine serum albumin (BSA, 1 mg/ml stock solution) in 100 µl of Type-II water was prepared in a 96-well microtitre plate to prepare the standard curve of BSA. Thereafter, 200 µl of alkaline copper sulfate solution (0.5% $CuSO_4.5H_20$ in 1% sodium-potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH in 1:49 ratio) was added to the wells and incubated for 10 min at room temperature (~23 ˚C). Then, 20 µl of Folin-Ciocalteau Phenol reagent (diluted with dH_2O to 1:2 ratios) was added and further incubated at room temperature for 30 min. Thereafter, the absorbance was recorded at 660 nm in a microplate reader (Multiskan GO, Thermo Fisher Scientific). The protein content of the unknown samples was estimated from a plot of absorbance values (Y-axis) vs. amount (µg) of BSA (X-axis).

3.2.2.2 Assay of enzymatic activities

3.2.2.2.1 Phospholipase A_2 activity: Phospholipase A_2 (PLA_2) activity of crude RVV (40 µg/ml) and its chromatographic fractions (10 µg) was assayed by the turbidometric method using egg yolk as substrate [10,11]. Egg yolk substrate (absorbance at 740 nm adjusted to 1.0 by 100 mM Tris-HCl, pH 8.0) was added to microfuge tubes containing crude RVV or GF fractions of 1X PBS, pH 7.4 and the final volume was adjusted to 2.0 ml. The reaction mixture was incubated for 10 min at room temperature. Thereafter, the decrease in absorbance of the reaction mixture was measured at 740 nm in a plate reader (Multiskan GO, Thermo scientific, USA). One unit of PLA_2 activity was arbitrarily

defined as a decrease in 0.01 absorbance at 740 nm after 10 min of incubation with RVV or its fraction [10,11].

3.2.2.2.2 L-amino acid oxidase (LAAO) activity: The LAAO activity of crude RVV (40 µg/ml) and GF fractions (10 µg) or 1X PBS, pH 7.4 (control) was assayed against L-kynurenine substrate [2,12,13]. The venom samples were incubated in assay mixture containing 50 µl assay buffer (100 mM HEPES buffer, 100 mM NaCl, pH 8.0) and 75 µl substrate (1 mg/ml) for 37 ˚C for 30 min. The reaction was stopped by adding 750 µl of 0.5 M ice-cold trichloroactetic acid (TCA), allowed to stand at room temperature for 5 min, and the absorbance was measured at 331 nm in a microplate reader. One unit of LAAO activity was defined as nmol of kynurenic acid produced/min under the assay conditions [2,12,13].

3.2.2.2.3 Protease activity: The proteolytic activity of crude RVV (40 µg/ml) and GF fractions (10 µg) was determined using human fibrinogen (fraction I), fibrin (100 µg), or azocasein (for metalloprotease activity) as substrates [2,14,15]. The fibrin was formed by incubating of 40 µl of 2.5 mg/ml fibrinogen with 3 µl of 10 NIH unit thrombin (0.2 mg/ml) for 1 h at 37 ˚C. The test samples or 1X PBS, pH 7.4 (control) were incubated with fibrinogen and fibrin for 6 h at 37 ˚C. The reaction was stopped by adding 10 µl of 10% (w/v) ice-cold TCA and the tubes were kept in ice for 10 min. The reaction mixture was centrifuged at 10000 rpm for 10 min at room temperature (~23 ˚C). Thereafter, 20 µl of the supernatant was transferred to a test tube and the volume of reaction mixture was adjusted to 50 µl by distilled water (dH_2O). Then 2 ml of alkaline copper sulfate solution (0.5% $CuSO_4.5H_20$ in 1% sodium-potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH in 1:49 ratio) was added and incubated for 10 min at room temperature. Subsequently, 200 µl of Folin-Ciocalteu reagent (diluted at 1:2 ratio) was added and incubated at room temperature (~23 ˚C) for 30 min. The absorbance of reaction mixture against control was recorded after 30 min at 660 nm in a microplate reader. The unit of fibrin(ogen)olytic activity was defined as µg of tyrosine liberated per min per mg of enzyme.

In another set of experiments, the reaction mixture of fibrin(ogen)olytic activity (prior to addition of TCA) was separated by 12.5% SDS-PAGE under reduced conditions [3,15,16]. The gel was stained with PhastGel Blue R stain and destained with methanol: acetic acid: water (40:10:50). The densitometric analysis of the gel was done

for quantitative assessment of fibrinogen or fibrin degradation using ImageQuant TL 8.1 software (GE Healthcare, Sweden). The fibrinogen/fibrin incubated with 1X PBS served as control and considered as 0% degradation and other values were compared to that.

Azocaseinolytic activity of crude RVV (40 µg/ml) and GF fractions (10 µg) or 1X PBS, pH 7.4 (control) was assayed against azocasein substrate (2 mg/ml). The venom samples were incubated in an assay mixture containing 50 µl assay buffer (50 mM HEPES buffer, 100 mM NaCl, pH 8.0) and 250 µl substrate (2 mg/ml) for 37 ˚C for 30 min. The reaction was stopped by adding 0.5 M ice-cold TCA followed by centrifugation at 2000 g for 5 min. Thereafter, 100 µl of supernatant was transferred to wells of a microtiter plate and 100 µl of 0.5 M NaOH was added. After incubation for 5 min at room temperature, the absorbance was noted at 450 nm. Metalloprotease specific activity was expressed as ΔA450 nm/min/mg protein [2].

3.2.2.2.4 Esterolytic activity: Crude RVV (40 µg/ml) and GF fractions (10 µg) was assayed for esterase activity against 10 mM Nα-p-tosyl-L-arginine methyl ester hydrochloride (TAME) and Nα-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) substrates as described by Costa et al. [17] and modified by Mukherjee and Mackessy [3]. An increase in absorbance by 0.01 unit at 244 nm during the first 10 min of the reaction at 37 ˚C was defined as one unit of TAME-esterase activity whereas, an increase in 0.01 absorbance unit at 254 nm during the first 5 min of the reaction at 37 ˚C was defined as one unit of BAEE-esterase activity [3].

3.2.2.2.5 Phosphodiesterase (PDE) activity: The PDE activity of crude RVV (40 µg/ml) and GF fractions (10 µg) was assayed by the spectrophotometric method described by Sulkowoski and Laskowoski [18] with slight modifications. Briefly, the assay mixture (100 µl) contained 10 µM $MgCl_2$, 200 µM Tris-HCl, pH 9.0, and 40 µM bis-*p*-nitrophenyl phosphate (bNPP) substrate. The reaction was initiated by adding crude RVV or GF fractions and incubated at 37 ˚C for 10 min. The increase in absorbance was noted at 400 nm and the unit of PDE activity was expressed as micromoles of *p*-nitrophenol released per min (using 17,600 as the molar extinction coefficient of bis-*p*-nitrophenyl phosphate).

3.2.2.2.6 Nucleotidase activities: ATPase, ADPase and AMPase (5′-nucleotidase) activities of crude RVV (40 µg/ml) and GF fractions (10 µg) were determined using

adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) as substrates (1.5 mM), respectively [2,19,20]. AMPase activity was assayed in a reaction mixture containing 300 µl of 0.1 M glycine, pH 8.8, 150 µl of 1.5 mM AMP substrate and 100 µl of 0.1 M $MgCl_2$.

ATPase and ADPase activities were assayed in 0.08 M Tris-acetate buffer, pH 7.2 containing 1.5 mM ATP and ADP as substrate, respectively [2,20]. The reaction mixture containing crude RVV or GF fractions or 1X PBS, pH 7.4 (control) was incubated at 37 ˚C for 30 min. The reaction was terminated by adding 10% ice-cold TCA (10 µl), centrifuged at 10,000 rpm for 5 min and 20 µl of supernatant was transferred to the wells of a microtitre plate and the volume of the wells was adjusted up to 50 µl by Type-II water. Thereafter, 100 µl of solution A (3.5 ml Type-II water, 0.3 g ascorbic acid, 5 ml 1 M HCl, 0.5 ml 10% ammonium molybdate and 1.5 ml 20% SDS) was added to the wells and incubated for 10 min on ice. This was followed by addition of 150 µl of solution B (0.35 g bismuth citrate, 0.35 g sodium citrate, 10 ml 1 M HCl) and incubation in dark for 10 min [21]. Thereafter, the absorbance was measured at 695 nm in a microplate reader. One unit of ATPase/ADPase/AMPase activity was defined as micromoles of inorganic phosphate (Pi) released per min at 37 ˚C [2,19,20].

3.2.2.2.7 Hyaluronidase activity: The hyaluronidase activity of crude RVV (40 µg/ml) and GF fractions (10 µg) was assayed according to the method of Pukrittayakamee et al. [22] with slight modifications. Briefly, the reaction mixture contained Na-acetate buffer (0.2 M Na-acetate, 0.15 M NaCl, pH 6.0) and 10 µg of hyaluronic acid as a substrate. The assay mixture containing crude RVV or GF fractions or 1X PBS, pH 7.4 (control) was incubated at 37 ˚C for 30 min and the reaction was terminated by addition of 200 µl of 2.5% cetyltrimethylammonium bromide (CTAB) in 2% NaOH solution. The decrease in turbidity of the reaction mixture was recorded at 405 nm in a microplate reader. One unit of hyaluronidase activity was defined as 1% decrease in turbidity as compared to control and activity was expressed as U/mg protein [22].

3.2.2.3 Assay of pharmacological properties

3.2.2.3.1 Effect on plasma clotting activity: The plasma clotting activity of crude RVV (40 µg/ml)/GF fractions (10 µg)/1X PBS, pH 7.4 (control) was assayed using platelet poor plasma (PPP). The PPP was prepared by centrifugation of citrated goat blood (collected from slaughterhouse in 3.8% trisodium citrate) at 4300 rpm for 20 min

at 4 ˚C. The yellowish supernatant representing PPP was transferred to a fresh tube, kept in ice, and used within 4 h of its isolation [11,16]. Crude RVV or GF fraction was incubated with 300 µl of PPP for 3 min at 37 ˚C, followed by addition of 40 µl of 250 mM $CaCl_2$ and the clotting time was recorded by a stopwatch. PPP incubated with 1X PBS, pH 7.4 served as a control. One unit of pro-coagulant or anticoagulant activity was defined as 1 s decrease or increase in the clotting time of PPP in presence of test sample as compared to control [11,16,23].

Prothrombin time (PT) and activated partial thromboplastin time (APTT) of crude RVV (40 µg/ml) and GF fractions (10 µg) against goat PPP were determined using commercial liquiplastin and liquicelin diagnostic kits, respectively following manufacturer's protocol. Briefly, crude RVV or GF fractions or 1X PBS, pH 7.4 (control) was incubated with 100 µl of PPP (pre-warmed at 37 ˚C for 3 min) for 3 min at 37 ˚C. Thereafter, 200 µl of liquiplastin reagent (pre-warmed at 37 °C for 3 min) was added, the contents of the tube were mixed by gentle shaking and the clotting time was recorded by a stopwatch. For determination of APTT, crude RVV or GF fractions or 1X PBS, pH 7.4 (control) was incubated with 100 µl of PPP (pre-warmed at 37 ˚C for 3 min) for 3 min at 37 ˚C. Thereafter, 100 µl of liquicelin reagent (pre-warmed at 37 °C for 3 min) was added and further incubated at 37 ˚C for 3 min. The clot was initiated by adding 100 µl of pre-warmed 25 mM $CaCl_2$ solution and the time of appearance of first clot/thread was recorded by a stopwatch.

3.2.2.3.2 FXa-like activity: The prothrombin activation (FXa-like activity) property of crude RVV was analyzed by 12.5% SDS-PAGE of prothrombin activation products under non-reduced conditions [15]. The reaction mixture (10 µl) containing prothrombin (15 µg), and RVV (40 µg/ml) was incubated at 37 ˚C for 3 h. Prothrombin treated with factor Xa (0.1 µg) and 1X PBS, pH 7.4 served as positive and negative control, respectively. Thereafter, the reaction products were separated in 12.5% SDS-PAGE under non-reducing condition. The prothrombin activation property was expressed as the amount of meizothrombin and/or thrombin formed, as calculated by the densitometry of their bands using ImageQuant TL software 8.1 (GE Healthcare, Sweden).

3.2.2.3.3 Haemolytic activity: Direct and indirect hemolytic activity of crude RVV (40 µg/ml)/GF fractions (10 µg)/1X PBS, pH 7.4 (control) was assayed against 2.0 ml of

5% (v/v) goat washed erythrocytes [24,25]. For the preparation of washed erythrocytes, goat blood was collected in 3.8% tri-sodium citrate and centrifuged at 4300 rpm for 15 min at 4 ˚C. The pellet containing the erythrocytes was washed twice with isotonic 20 mM potassium phosphate buffer, pH 7.4 containing 150 mM NaCl and re-suspended in the same buffer at a final concentration of 5% (v/v). The reaction mixture (2.0 ml) containing 5% (v/v) erythrocytes was incubated for 90 min at 37 ˚C and the reaction was stopped by placing the tubes in ice-cold water. The mixture was centrifuged at 10000 rpm for 10 min at room temperature. The absorbance of the supernatant was recorded at 540 nm against appropriate control. The haemolytic activity was expressed as percent haemolysis, where the absorbance of erythrocyte suspension treated with 0.1% Triton X-100 was considered as 100% haemolysis [24,25]. For the assay of indirect haemolysis, the assay mixture was supplemented with 10 µl of egg-yolk phospholipids before assay of haemolysis as stated above [25].

3.2.2.3.4 Platelet modulation activity: Platelet-rich plasma (PRP) was prepared by centrifugation of citrated goat blood at 300 g for 20 min at 4 ˚C [15,23,26]. Crude RVV (40 µg/ml)/GF fractions (10 µg)/1X PBS, pH 7.4 (control) was added to 100 µl of PRP (pre-warmed at 37 ˚C for 5 min) in a 96-well microplate. Absorbance was measured continuously at 540 nm for 300 s at an interval of 15 s in a form of a kinetic loop. The platelet modulation property was expressed in terms of percent aggregation or deaggregation calculated by equation 3 [15,23]:

$$\%\, aggregation = \frac{A540\ of\ PRP\ before\ addition\ of\ agonist - A540\ of\ PRP\ after\ addition\ of\ agonist}{A540\ of\ PRP\ before\ addition\ of\ agonist - A540\ of\ PPP} \times 100\% \quad \text{---3}$$

Where, A540 denotes absorbance values of PRP (platelet rich plasma) or PPP (platelet poor plasma) at 540 nm.

Reduction in platelet count (thrombocytopenia) was determined by incubating crude RVV (40 µg/ml)/GF fractions (10 µg)/1X PBS, pH 7.4 (control) with 100 µl PRP (2.0 to 5.0 × 10^6 cells/ml) at 37 ˚C for 6 h in a CO_2 incubator. PRP incubated with 1X PBS, pH 7.4 served as control. The platelets were stained with trypan blue and counted in a hemocytometer using Motic Images plus 3.0 ML software [27].

3.2.3 Neutralization of enzyme activities and pharmacological properties of RVV

The neutralization potency of commercial Indian PAVs (BSVL, PSVPL, VBPL and Bio-E) and/or MAV (VINS) towards enzymatic activities and pharmacological properties of crude RVV (40 µg/ml) and/or its GF fractions was determined by pre-incubating venom samples with PAVs and/or MAV at 1:10 (protein: protein) ratio for 30 min at 37 ˚C followed by assay of enzymatic activities and pharmacological properties as stated above (sections 3.2.2.2 and 3.2.2.3). The percent neutralization was calculated by comparing the enzymatic activities/pharmacological properties of RVV and its GF fractions in absence of PAVs or MAV (100% activity) [2,15,23,28].

3.2.4 Assessment of immunological cross-reactivity of RVV samples against commercial polyvalent (PAV) and/or monovalent (MAV) antivenom

3.2.4.1 Enzyme-linked immunosorbent assay (ELISA)

For assessment of immuno cross-reactivity by ELISA, 100 ng of RVV or GF fractions were coated onto wells of a 96-well ELISA plate in 100 µl of coating buffer (10 mM phosphate buffer, 150 mM NaCl and 0.1% Na-azide) for overnight at 4 ˚C [2]. Next day, the wells were washed three times with washing buffer (10 mM phosphate buffer, 150 mM NaCl and 0.5% Tween-20), and non-specific bindings were blocked using 5% skimmed milk in washing buffer for 30 min at room temperature (~23 ˚C). Thereafter, the wells were washed three times with washing buffer and 200 ng of PAV or MAV was added and further incubated for 2 h at room temperature. After washing the wells, 100 µl of anti-horse IgG horseradish peroxidase conjugated secondary antibody (1:2000 dilutions) was added to the wells and incubated for another 2 h at room temperature. Thereafter, the excess of secondary antibodies were washed with washing buffer and then 100 µl of 1X 3,3,5,5'-tetramethylbenzidine/hydrogen peroxide (TMB/H_2O_2) substrate was added and the reaction was incubated in dark for 30 min. The reaction was stopped by adding 50 µl of 2 M H_2SO_4 and the absorbance was recorded at 492 nm against blanks in a microplate reader [2].

3.2.4.2 Immunoblot analysis

For immunoblot analysis, crude RVV or GF fractions (100 µg) were separated in 12.5% SDS-PAGE under reduced conditions (section 3.2.1.3) and transferred to Immobilon-P PVDF membrane (Merck, Germany) on a semi-dry gel transfer system (Amersham Bioscience, UK) at 20 mA (1.2 mA/cm^2) for 2 h. The membranes were

blocked by 5% skimmed milk (w/v) prepared in Tris-buffered saline with 0.1% Tween-20 (TBS-T) at 4 ˚C overnight. Thereafter, the membranes was washed with TBS-T, and primary antibody (15 mg/ml PAV or MAV) at a dilution of 1:1000 ratio was added and incubated at room temperature for 1 h. The excess unbound primary antibodies were washed off by TBS-T and the membranes were incubated with alkaline phosphatase conjugated rabbit anti-horse secondary antibody (1:15000 dilutions) at room temperature for 1 h. Thereafter, the blots were developed using 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) kit (Sigma-Aldrich, USA), scanned (Epson Expression 11000XL, USA), and then densitometry analysis of the blots was done using ImageQuant TL software 8.1 (GE Healthcare, Sweden).

3.2.5 Second generation antivenomics of RVV samples against commercial PAVs

The immunological profiling of RVV against commercial PAVs (BSVL and PSVPL) was investigated with a venom-antivenom immuno-chromatographic approach [29,30] with certain modifications described by Patra et al. [31]. The immuno-affinity columns were packed in-house with 2 ml of N-hydroxysuccinimide (NHS) activated sepharose fast flow matrix (GE Healthcare, Sweden), previously stored in isopropanol. The matrices were activated by passing 15 CV of ice-cold 1 mM HCl followed by 2 CV of coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Fifteen mg (protein content) of PAVs (BSVL and PSVPL) were dissolved in 1 ml of coupling buffer and coupled to the immuno-affinity columns for 4 h at room temperature with mild shaking. The excess uncoupled antivenom was washed off with the same buffer and its protein content was determined (section 3.2.2.1) to quantitate the antivenom bound to the affinity column. The non-reactive groups of the immuno-affinity columns were blocked by incubating with 0.1 M Tris-HCl, pH 8.0 at 4 ˚C for overnight. Thereafter, the columns were washed alternatively with 3 CV of high pH (0.1 M Tris-HCl, pH 8.5) and low pH (0.1 M acetate, 0.5 M NaCl, pH 4.0) buffers which was repeated 6 times followed by equilibration with 5 CV of 1X PBS, pH 7.4. Then 500 µg of RVV, dissolved in 1 ml of 1X PBS, pH 7.4 was loaded to the columns and incubated for 2 h at 37 ˚C on a shaker orbiter.

Unbound RVV proteins were eluted from the immuno-affinity columns with 5 CV of 1X PBS, pH 7.4, while the bound proteins were eluted with 5 CV of 0.1 M glycine, pH 2.0 [30] and immediately neutralized by 1 M Tris-HCl, pH 9.0. Both the

fractions were then desalted by passing through a PD10 column and vacuum dried (Labconco, Model: 7670061, USA).

The bound and unbound RVV proteins and 500 µg of crude RVV were then separated by 12.5% SDS-PAGE under reduced conditions. The poorly immunogenic protein bands (present in the unbound fractions) were excised from the gel and then subjected to LC-MS/MS analysis for protein identification, as described in sections 3.2.1.6 and 3.2.1.7. The percentage of unbound RVV proteins was quantified by MS1 (mean spectral intensity) and/or MS2 (NSAF)-based methods [2,7] using the equation 4:

$$\%\ of\ \text{poorly immunogenic protein (X)} = \frac{Mean\ spectral\ intensity\ or\ NSAF\ of\ protein\ X\ in\ PAV\ unbound\ fractions}{Mean\ spectral\ intensity\ or\ NSAF\ of\ protein\ X\ in\ crude\ RVV} \times 100\% \quad ---4$$

3.2.6 Purification and characterization of a novel apyrase enzyme from WI RVV

3.2.6.1 Isolation and purification of RVV apyrase

3.2.6.1.1 Fractionation of WI RVV through GF followed by anion-exchange chromatography

The RVV was fractionated on a HiLoad 16/600 Superdex 75 pg column (1.6 × 60 cm) as described in section 3.2.1.1. Each fraction was screened for apyrase activity (section 3.2.2.2.6).

Gel filtration fractions of WI RVV exhibiting superior apyrase activity were pooled, desalted by passing thorough PD10 column and re-fractionated on a HiTrap Q FF anion-exchange column (1.6 × 2.5 cm) as described in section 3.2.1.1. The resulting protein peaks were then assayed for apyrase (ATPase and ADPase) activity (section 3.2.2.2.6) and protein content (section 3.2.2.1). The anion-exchange peak demonstrating the highest apyrase activity was assayed for purity and molecular mass determination.

3.2.6.1.2 Assessment of purity and molecular mass of RVV apyrase

3.2.6.1.2.1 Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

The purity of enzyme preparation was assessed by RP-HPLC analysis on an Acclaim 300 C_{18} column (2.1 × 150 mm, 3 µm) pre-equilibrated with 0.1% (v/v)

trifluoroacetic acid (TFA) (solvent A). The column was washed with solvent A for 2 min, followed by elution of bound proteins with a linear gradient over 12 min from 0 to 100% of solvent B (90% ACN in H_2O containing 0.1% TFA). The flow rate was maintained at 0.5 ml/min and the elution of protein was monitored at 280 nm.

3.2.6.1.2.2 SDS-PAGE analysis

The homogeneity of preparation and molecular mass of apyrase was determined by 12.5% SDS-PAGE under both reduced and non-reduced conditions as described in section 3.2.1.3. Approximate molecular mass of the purified enzyme was determined from a plot of log MW of protein standards vs. *Rf* values [3].

3.2.6.1.2.3 MALDI-TOF-MS analysis

The molecular mass of 2 µg of purified enzyme was determined by MALDI-TOF-MS analysis on a Bruker Daltonics UltrafleXtreme mass spectrometer at 40,000 resolutions (section 3.2.1.4).

3.2.6.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and *de novo* sequencing for identification of apyrase enzyme

Eighty micrograms of purified apyrase were subjected to in-solution trypsin digestion and subsequent ESI-LC-MS/MS analysis on an LTQ Orbitrap Discovery hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described in sections 3.2.1.5 to 3.2.1.7.

For *de novo* sequencing the MS/MS data was analyzed by PEAKS 7.0 software with a threshold average local confidence (ALC) score of ≥50. The *de novo* peptides were then subjected to basic local alignment search tool (BLASTp) search against apyrase sequences of the NCBInr (http://blast.ncbi.nlm.nih.gov/Blast.cgi) database with a restriction set to taxid Animalia (33208).

3.2.6.3 Biochemical characterization

3.2.6.3.1 Catalytic activity and stability of apyrase

ATPase, ADPase, AMPase and PDE activities of the anion-exchange peaks (1 µg) were assayed as described in section 3.2.2.2.6. Since ATPase and ADPase activities

of apyrase enzymes are susceptible to photo-oxidation [32], all the enzyme assays were performed in dark conditions.

ATP, ADP, and AMP hydrolytic activities were also assessed by RP-HPLC analysis as described previously [33] with slight modifications. Briefly, 25 nM of purified apyrase was incubated with ATP, ADP or AMP (1.5 mM) at 37 ˚C for 30 min. The reaction was stopped by adding 10 µl of 10% ice-cold TCA and centrifuged at 10,000 rpm for 10 min. Thereafter, 50 µl of supernatant was separated on an Acclaim 300 C_{18} column (4.6 × 150 mm, 3 µm) pre-equilibrated with 50 mM sodium phosphate buffer, pH 6.0 (solvent A). The bound nucleotides were eluted with the following multi-step gradient of 100% methanol (solvent B): 0% B for 1.4 min, 0-12.5% B in 3.6 min, 12.5% B for 1.5 min, and 12.5-0% B in 2.5 min. The elution of nucleotides at a flow rate of 1 ml/min was monitored at 254 nm. Hydrolysis of ATP and ADP by apyrase (expressed in micromoles) was calculated from the area under standard curves of ATP and ADP eluted from the RP-HPLC C_{18} column under identical conditions.

The stability of enzymatic activity (ATPase and ADPase) of apyrase (25 nM) and the same activity contained in crude RVV (10 µg) was assessed by incubating the purified enzyme or crude RVV at physiological conditions (pH 7.4 and 37 ˚C) for different time intervals (0 to 8 h) followed by assay of their enzymatic activities (ATPase and ADPase) as described in section 3.2.2.2.6.

3.2.6.3.2 Substrate specificity of the apyrase

To determine the substrate specificity, apyrase (25 nM) was incubated with ATP, ADP or AMP or bis-*p*-nitrophenyl phosphate (1.5 mM) at 37 ˚C for 30 min. Thereafter, micromoles of Pi released was estimated as described in section 3.2.2.2.6.

3.2.6.3.3 Effect of metal ions and chemical inhibitors on the enzymatic activity of apyrase

The effect of divalent cations (Mg^{2+}, Ca^{2+}, and Zn^{2+}) on enzyme activity was assessed by incubating apyrase (25 nM) with different metal ions (2 mM final concentration) at 37 ˚C for 30 min, followed by assay of its enzymatic activities (ATPase and ADPase) as described in section 3.2.2.2.6.

Similarly, the effect of different chemical inhibitors such as ethylenediaminetetraacetic acid (EDTA) (5 mM), phenylmethylsulfonyl fluoride (PMSF) (2 mM), DTT (2 mM), and IAA (5 mM) was assayed by incubating apyrase (25 nM) with the chemical inhibitors for 30 min at room temperature followed by assay of its enzymatic activities (ATPase and ADPase) as described in section 3.2.2.2.6.

3.2.6.3.4 Determination of kinetic parameters of apyrase

Michaelis-Menten constant (*Km*) and maximum reaction rate (*Vmax*) values of apyrase catalyzed reaction were determined by incubating different concentrations of ATP or ADP (0.1-2.5mM) with 25 nM of RVV apyrase for 30 min at 37 ˚C, pH 7.4. Thereafter, the enzyme activities (ATPase and ADPase) were determined as described in section 3.2.2.2.6. The kinetic parameters were determined by the Lineweaver-Burk plot (equation 5) using GraphPad Prism 5.0 software [15].

$$\frac{1}{V} = \frac{Km}{Vmax}\frac{1}{[S]} \times \frac{1}{Vmax} \qquad \text{---5}$$

Where, where V is the reaction velocity (reaction rate), *Km* is the Michaelis-Menten constant, *Vmax* is the maximum reaction velocity, and [S] is the substrate concentration.

The turnover number and specificity constant (kinetic efficiency) of apyrase against ATP and ADP were calculated by equation 6 and 7, respectively.

$$Kcat = \frac{Vmax}{[Et]} \qquad \text{---6}$$

Where, *Kcat*, *Vmax* and [Et] represent turnover number, maximum reaction rate, and enzyme concentration, respectively.

$$Specificity\ constant = \frac{Kcat}{Km} \qquad \text{---7}$$

3.2.6.3.5 Determination of carbohydrate content and glycosylation of apyrase

The carbohydrate (neutral sugar) content of the RVV apyrase was estimated by the phenol-sulfuric acid method described by Dubois et al. [34] using D-glucose as a standard. Briefly, serial dilutions of 0.01, 0.025, 0.05, 0.1, 0.15, and 0.2 µg per well of D-glucose (0.01 mg/ml stock solution) in 20 µl of Type-II water was prepared in a 96-well microtitre plate to prepare the standard curve of D-glucose. Thereafter, 50 µl of 5%

phenol (w/v) was added to the wells and incubated for 10 min at room temperature (~23 ˚C). Then, 250 µl of 96% sulfuric acid (v/v) was added and further incubated at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was measured at 490 nm against a reagent blank in a microplate reader (Multiskan GO, Thermo Fisher Scientific). The carbohydrate content of the unknown sample (apyrase) was calculated from the standard curve of absorbance (490 nm) vs. amount of D-glucose (µg).

The extent of N-linked oligosaccharides in apyrase was determined by incubating 40 µg of the denatured enzyme (by heating at 100 ˚C for 10 min) with PNGase F (500 units) for 4 h at 37 ˚C. Denatured (without PNGase treatment) and native (unheated) apyrase incubated with 1X PBS, pH 7.4 under identical experimental conditions served as controls [3]. Thereafter, the reaction products were subjected to 12.5% SDS-PAGE analysis under reduced conditions (section 3.2.1.3). Percentage of glycosylation was calculated by comparing the molecular masses of native and PNGase-treated apyrase (equation 8).

$$\%\ of\ N-linked\ oligosaccharides\ = \frac{MW\ of\ native\ Ruviapyrase - MW\ of\ PNGase\ treated\ Ruviapyrase}{MW\ of\ native\ Ruviapyrase} \times 100\% \qquad ---8$$

The glycoprotein nature of apyrase was further assessed by incubating 50 µg of enzyme with Concanavalin A matrix (0.5 ml) for 30 min at room temperature. The matrix was then packed in a 2.0 ml column. The column was washed with 10 CV of equilibration buffer (20 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl) to elute the Concanavalin A unbound protein, if any. The bound protein was then eluted with 20 CV of equilibration buffer containing 0.5 M and 1.0 M glucose. The elution of protein was monitored at 280 nm. The unbound and bound fractions were assayed for protein content (section 3.2.2.1), and ATPase and ADPase activities (section 3.2.2.2.6).

3.2.6.3.6 Determination of interaction between ATP/ADP and apyrase by spectrofluorometry analysis

The interaction between ATP or ADP with the apyrase was assessed by spectrofluorometry analysis as described earlier [23,35,36]. The fluorescence spectra of a fixed concentration (50 µM) of ATP or ADP in presence of different concentrations of apyrase (0.25-2.5 nM) dissolved in 20 mM Tris-HCl, pH 7.4 were measured in a final

volume of 600 µl. The reaction mixture was excited at 260 nm and its emission spectra were recorded at a range of 300 to 500 nm against blank (buffer). The slit length was maintained at 10 nm. The change in the λmax for each curve (ΔF) was fitted to one site-specific binding curve (equation 9) using GraphPad Prism 5.0 software and the *Kd* values for the interactions were determined in the same software.

$$\Delta F = \frac{\Delta Fmax \times C}{Kd+C} \qquad \text{---9}$$

Where, ΔF is the change in fluorescence intensity of ATP or ADP in the presence of apyrase, ΔFmax is the maximum change in fluorescence intensity of ATP or ADP when saturated with apyrase, and C is the concentration of apyrase [37].

3.2.6.4 Pharmacological characterization

3.2.6.4.1 Assessment of haemolytic activity and cytotoxicity of apyrase against MCF-7 cells

The *in vitro* cell cytotoxicity, if any, of RVV apyrase was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based method on human breast adenocarcinoma MCF7 cells (1×10^4 cells/ml) cultured in 96-well culture plates in Dulbecco's modified eagle medium (DMEM) containing 10% heat inactivated foetal bovine serum [3,13]. The cells were allowed to grow and adhere to culture plates at 37 ˚C in a 5% CO_2 humidified incubator for 24 h. Thereafter, the medium was replaced with a fresh medium containing 125 nM of RVV apyrase and incubated for 48 h at 37 ˚C in the CO_2 humidified. The cytotoxicity of apyrase, if any, was determined by MTT-based assay following manufacturer's protocol (Sigma-Aldrich, USA) and expressed as percent cell death by comparing with a standard curve of control cells [3,13].

Haemolytic activity of the apyrase (25 nM) was assessed against 2.0 ml of 5% (v/v) goat washed erythrocytes as described in section 3.2.2.3.3.

3.2.6.4.2 Plasma clotting activity of RVV apyrase

Plasma clotting activity of the apyrase (25 nM) was assessed against goat PPP as described in section 3.2.2.3.1.

3.2.6.4.3 Platelet modulation and effect on ADP-induced platelet aggregation by apyrase

The effect of apyrase (60 nM) on goat PRP was studied as described in section 3.2.2.3.4. In another set of experiments, 100 µl of PRP was pre-incubated with apyrase (60 and 120 nM) or 1X PBS, pH 7.4 (control), for 5 min at 37 ˚C prior to the addition of ADP (30 µM). The platelet aggregation induced by ADP (30 µM) was considered as 100% activity and other values were compared with that [23]. The inhibition of ADP-induced platelet aggregation by apyrase was determined by considering the platelet aggregation induced by ADP as 100% activity.

3.2.6.5 Immunological characterization

3.2.6.5.1 Neutralization of enzyme activity of RVV apyrase by commercial PAV and MAV

Neutralization potency of commercial MAV and PAV against ATPase, ADPase, and platelet deaggregation activities of apyrase (1 µg) was assessed as described in section 3.2.3.

3.2.6.5.2 Immuno cross-reactivity of apyrase towards commercial PAV and MAV

The immuno cross-reactivity of apyrase (100 ng) with commercial MAV and PAV (200 ng) was studied by ELISA as described in section 3.2.4.1.

3.2.7 Statistical analysis

Student's *t*-test and ANOVA were done using Sigma Plot 11.0 for Windows (version 10.0) and MS Excel, respectively, to test the significance of difference in activities with respect to control and between crude RVVs or GF fractions. The same was also employed to test the significance of difference in immuno cross-reactivity and neutralization potency of commercial PAVs against enzymatic activities and pharmacological properties of RVV. A value of $p \leq 0.05$ was considered statistically significant.

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