CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Venoms, antivenoms and drugs

Lyophilized *Mesobuthus tamulus* (Indian red scorpion) pooled venom from 10 to 12 scorpions of western Indian (Maharashtra state) origin was obtained from Premium Serum and Vaccine Pvt. Ltd. (PSVPL), Pune, India. Lyophilized equine anti-scorpion antivenom (ASA) was obtained from Haffkine Biopharmaceutical Corp. Ltd., Mumbai, India (HBC) (Batch No.: PRMSC-002, expiry date: Feb. 2022) and Premium Serum and Vaccine Pvt. Ltd. (PSVPL), Pune, India (Batch No.: SS170401, expiry date: Sept. 2021). α1-adrenoreceptor antagonists (AAA) such as Prazosin-HCL, Silodosin and Terazosin-HCL were obtained from Aristo Pharmaceuticals Pvt. Ltd., Mumbai, India; Sun Pharma Laboratories Ltd., India; and Abbott India Ltd, India, respectively. Varespladib was obtained from Sigma-Aldrich, USA (Cat-SML 1100-5MG). The *Caenorhabditis* Genetics Center (CGC), University of Minnesota, USA, provided *C. elegans* wild-type strain N_2. The laboratory inbreeds, pathogen-free, Wistar strain albino rats (180-200 g) and Swiss albino mice (18-20 g), were purchased from M/S Chakrabarty Enterprise, Kolkata, and were used for the experiments. Approval for the animal experiment was obtained from the institutional animal ethics committee (IASST/IAEC/2022/09), and experiments were performed following OECD guidelines. The present work has been applied for a patent (Application number: 202331027004). MitoProbeTM JC-1 Assay kit (Cat- M34152) and pure link RNA mini kit (Cat-12183018A) were obtained from Invitrogen, USA. Verso cDNA Synthesis Kit (Cat-AB-1453/A) was purchased from Thermo Scientific, USA. Mouse IL-1β/ IL-1F2, IL-6, and TNFα Quantikine® Mouse Immunoassay kits (Cat-MLB00C, M6000B and DY410, respectively) were purchased from Biotechne R and D systems, Inc. (USA). All other chemicals were from Sigma-Aldrich, USA, and HIMEDIA, India.

3.1.2 Chromatographic columns, matrices,and other fine chemicals

Sephacryl S-200 (16 × 600 mm) gel filtration column was purchased from Shodex, Japan. Desalting PD-10 column waspurchased from GE Healthcare, Sweden. Acclaim 300 C_{18} RP-UHPLC column (2.1 x 150 mm, 3 µm) was obtained from Thermo Fisher Scientific, USA. C_{18} ZipTip pipette tips were procured from Merck Millipore, USA. Membrane filters (0.2 µ) and nylon syringe filters (0.2 µ) were obtained from Genetix

Biotech Asia Pvt. Ltd., New Delhi, India, and Riviera, Mumbai, India, respectively. Pre-stained protein molecular markers were bought from BioRad (#16103777; 2–250 kDa) and Thermo Fisher Scientific (#26616; 10-170 kDa or #26619; 10-250 kDa).

LAL (Limulus amebocyte lysate) Chromogenic Endotoxin Quantitation Kit was obtained from Thermo Fisher Scientific, USA. Affinity-purified $F(ab')_2$ generated from horse immunoglobulin (IgG) was purchased from Jackson ImmunoResearchInc, USA. Purified horse IgG was obtained from BioRad, USA. Anti-horse IgG HRP (horse radish peroxidase) conjugated secondary antibodyand anti-horse IgG Fc-specific antibody conjugated with HRP were purchased from Sigma-Aldrich, USA. Anti-horse IgA or IgE antibodies conjugated with HRP were procured from Bio-Rad, USA.

Blood coagulation factor proteins such as fibrinogen and factor Xa were procured from Sigma-Aldrich, USA or Calbiochem, Germany. Tris-buffer, HEPES buffer, phosphate buffer saline, solvents like acetonitrile, methanol, SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) reagents and all other chemical and reagents were of analytical grade and procured from Sigma-Aldrich, USA or Merck, Germany.

3.2 Methods

3.2.1 Proteomic analysis of Indian red scorpion (*M. tamulus*) venom

3.2.1.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of MTV

A. 12.5% resolving gel (10 ml) -

1. 1.5 M Tris-HCl (pH 8.8) : 2.5 ml
2. 30% acrylamide-bisacrylamide solution : 4.0 ml
3. Type I water : 3.3 ml
4. 10% SDS : 100 µl
5. 10% ammonium persulphate : 100 µl
6. TEMED (Tetramethylethylenediamine) : 10 µl.

B. 15% resolving gel (10 ml) –

1. 1.5 M Tris-HCl (pH 8.8) : 2.5 ml

2. 30% acrylamide-bisacrylamide solution : 5.0 ml

3. Type I water : 2.3 ml

4. 10% SDS : 100 µl

5. 10% ammonium persulphate : 100 µl

6. TEMED (Tetramethylethylenediamine) : 10 µl.

The above mentioned solution mixture was poured into a gel cassette and allowed to polymerize at room temperature.

C. 4% stacking gel (5 ml) –

1. 0.5 M Tris-HCl (pH 6.8) : 2.5 ml

2. 30% acrylamide-bisacrylamide solution : 0.6 ml

3. Type I water : 1.5 ml

4. 10% SDS : 50 µl

5. 10% ammonium persulphate : 50 µl

6. TEMED (Tetramethylethylenediamine) : 10 µl

Crude MTV (MTV) (80 µg) was dissolved in 1× PBS (phosphate buffered saline), pH 7.4 and centrifuged at 10,000 rpm for 15 min at 4 °C. The resulting supernatant was subjected to 15% SDS-PAGE (8.5 cm × 10.0 cm) under reduced conditions (miniVE vertical electrophoresis system, GE Healthcare, Sweden) [1]. PhastGel Blue R staining (GE Healthcare, Sweden) was used for visualizing the protein bands. The destained protein bands were scanned (Epson Expression 11000XL, USA), and band protein intensities were determined by ImageQuant TL 8.1 software (GE Healthcare, Sweden). The cumulative protein band intensity was considered as 100%, and the intensity of individual protein bands was compared to that to determine the relative abundance by the gel-based method [2].

3.2.1.2 Tandem mass spectrometry analysis of tryptic peptides

The detailed methodologies for mass spectrometry analysis described here were adopted from our previously described procedures [2; 3; 4; 5]. Briefly, reduced proteins of MTV separated by SDS-PAGE were divided into 10 gel sections. Individual sections/ protein bands were excised and then subjected to in-gel reduction first by incubating with 10 mM dithiothreitol (DTT). The reduced proteins were alkylated by treating with 55 mM iodoacetamide (IAA) under dark conditions at room temperature. The reduced and alkylated proteins were incubated with proteomics grade trypsin for ~16 h at 37 °C at a 1:30 (enzyme: substrate) ratio [2; 3; 4; 5]. After desalting and concentrating the tryptic peptides by ZipTip C18 (Merck, USA) tips, they were separated on a Zorbax C18 column (Rapid Resolution ht2.1X 50MM, 1.8 μm) coupled to an Agilent 1260 UHPLC system, following the method that we described previously [2; 4]. The eluted peptides were analyzed on an Agilent 6530 Q-TOF mass spectrometer via an electrospray ion source. The MS and MS/MS spectra were acquired at 100 to 2000 m/z, and as described previously, the scan rate of 6 and 3 spectra, for MS and MS/MS, respectively, was used for data acquisition [2; 4; 5]. The raw MS/MS data were searched against the Buthidae family (taxid: 6855) protein entries of the non-redundant NCBI databases using Morpheus software [4; 6]. Precursor and fragment mass error tolerances were set to 2.1 and 0.025 Da, respectively, and two missed cleavages were allowed [2]. Carbamidomethylation of cysteine was used as a fixed modification, and the oxidation of methionine residues was used as a variable modification [2; 4; 5]. The false discovery rate was set to The MS1-based (Summed peptide-spectrum Match Precursor Intensity) label-free quantification technique was used to determine the relative abundance of identified MTV proteins [4; 6]. Eq 1 shows the normalization of spectral intensity by the number of identified peptides against a particular protein acquisition [2; 4].

Mean spectral intensity for protein X

$$= \frac{\Sigma Sspectralintensityagainst MS1\ peptidesofX}{numberofidentifiedpeptidesforX} \qquad (1)$$

Equation 2 was then used to determine the relative abundance of a protein(x) in a particular gel section(Y) [7; 2; 4].

Relative abundance of X in gel section Y

$$= \frac{mean spectral intensity of X in Y}{Total mean spectral intensity of all proteins in Y} \times relative band intensity of Y (\%) \quad (2)$$

3.2.2 Determination of enzymatic activities and some pharmacological properties of MTV

3.2.2.1 Assay of enzymatic activities

3.2.2.1.1 MTV metalloproteinase (SVMP) activity

Metalloproteinase activity of crude MTV (10 µg) was assayed by using azocasein (2 mg/ml) as described by Mukherjee et al., (2016) [3]. The reaction mixture for the assay containing 50 µl assay buffer (50 mM HEPES buffer, 100 mM NaCl, pH 8.0) and 250 µl substrate. The venom sample was incubated in the reaction mixture at 37 °C for 30 min. The azocaseinolytic activity of the venom was stopped by adding 0.5 M ice-cold TCA. The reaction mixture was centrifuged at 2000 g for 5 min. The supernatant was collected, and 100 µl was transferred to wells of a microtiter plate followed by 0.5 M NaOH was added and incubated for 5 min at room temperature. The absorbance was recorded at 450 nm, and the specific activity for metalloproteinase activity was expressed as ΔA450 nm/min/mg protein [8; 3].

3.2.2.1.2 L-amino acid oxidase (LAAO) activity

The LAAO activity of crude MTV (10 µg) was assayed using L-kynurenine as a substrate [3; 9]. The reaction was started by incubating the venom sample in the assay mixture containing 100 mM HEPES buffer (50 µl) and 75 µl L- kynurenine as a substrate (1 mg/ml) at 37 °C for 30 min. Ice-cold trichloro-actetic acid (TCA) was used (0.5 M) to stop the reaction. After that, the absorbance of the reaction mixture was measured at 331 nm. The unit of LAAO activity was defined as n-molkynurenic acid produced/ min under the assay conditions.

3.2.2.1.3 Phospholipase A_2 (PLA_2) activity

PLA_2 activity of crude MTV (10 µg) was assayed by the turbidometric method [10]. Egg yolk substrate was prepared, and the absorbance of the substrate was adjusted to 1.0 at 740 nm using 100 mM Tris-HCl (pH 8.0). The venom sample was added to the

2.0 ml of egg yolk substrate in a microfuge tube and incubated for 10 min at room temperature. For negative control, 1X PBS pH 7.4 buffer was used. The absorbance of the reaction mixture was measured at 740 nm. One unit of PLA_2 activity is defined as a decrease in 0.01 absorbance at 740 nm after 10 min of incubation.

3.2.2.1.4 Fibrino(geno)lytic activity

The protease activity of crude MTV (10 µg) or 1X PBS (control) was determined by incubating the venom sample with fibrinogen (2.5 mg/ml)/fibrin for 3 hours at 37 °C [11]. The fibrinogen/fibrin degradation products were separated by 12.5% SDS-PAGE (reduced), and the percent fibrino(geno)lytic activity was calculated by measuring the degradation of the Aα- chain of fibrinogen/fibrin. The band intensity of the Aα chain of fibrinogen/fibrin molecule after treatment of venom samples was considered as 100% fibrino(geno)lytic activity, and other values were compared to that [12]. The protein band intensities were measured by Image Quant TL 8.1 software (GE Healthcare, Sweden).

3.2.2.1.5 Nucleotidase activities

ATPase and ADPase activity of crude MTV (10 µg) was assayed by the method of Williams and Esnouf (1962) [13] with slight modifications as described by Mukherjee et al. (2016) [3]. The 5'-nucleotidase (AMPase) activity was determined according to the protocol of Sinsheimer and Koerner [14] with slight modificationsas described by Mukherjee et al. [3]. The reaction mixture for ATPase and ADPase activity contained 0.08 M Tris-acetate buffer, pH 7.2, 1.5 mM ATP and ADP, respectively. The AMPase activity reaction mixture contained 300 µl of 0.1 M glycine, pH 8.8, 100 µl of 0.1 M $MgCl_2$ and 150 µl of 1.5 mM AMP as substrate. The venom sample was incubated in the reaction mixture at 37 °C for 30 min. The enzymatic activity of venom in the reaction mixture was stopped by adding 10 µl of ice-cold TCA (10%) and incubating for 10 min at 4˚C. The microfuge tubes containing the reaction mixture were centrifuged at 10,000 rpm for 5 min and 20 µl of supernatant was collected and placed in the microtiter plate wells, followed by adding Milli-Q water to adjust the volume up to 50 µl. Solution A (3.5 ml Milli-Q water, 0.3 g ascorbic acid, 5 ml 1 M HCl, 0.5 ml 10% ammonium molybdate and 1.5 ml 20% SDS) was prepared fresh, and 100 µl was added in each well. The plate was incubated for 10 min on ice, followed by adding 150

µl of solution B (0.35 g sodium citrate, 0.35 g bismuth citrate, and 10 ml 1 M HCl) and the plate was incubated in the dark for 10 min [15; 13]. The changes of colour upon enzymatic activity were measured by taking the absorbance at 695 nm in a microplate reader (Multiskan GO, Thermo Fisher Scientific, USA). One unit of ATPase/ ADPase/AMPase activity was defined as µM of Pi released per min at 37 °C.

3.2.2.1.6 Hyaluronidase activity

Hyaluronidase activity of crude MTV (10 µg) was assayed by the turbidometric method described by Pukrittayakamee et al., with slight modification as mentioned by Kalita et al. [8; 16]. The venom sample was incubated in the reaction mixture (Na-acetate buffer-0.2 M Na-acetate, 0.15 M NaCl, pH 6.0) containing 10 µg of hyaluronic acid (substrate). The reaction was carried out at 37 ˚C for 30 min and stopped by adding 200 µl of 2.5% cetyltrimethylammonium bromide (CTAB) prepared in 2% NaOH solution. The absorbance of the reaction mixture was measured at 405 nm, and a decrease in the turbidity was recorded. One unit of enzyme activity was defined as a decrease in turbidity by 1% as compared to the control, and activity was expressed as U/mg protein [8; 16]

3.2.2.2 Assay of pharmacological properties

3.2.2.2.1 Effect on plasma clotting time

The effect of MTV on plasma clotting time was assessed by using platelet poor plasma (PPP) from goat blood. Goat blood obtained from slaughter house, and was collected in 3.8% tri-sodium citrate and the blood was centrifuged at 4300 rpm for 10 min at 4 °C [17]. The pellet was discarded and the yellowish supernatant was termed as platelet-poor plasma (PPP) and it was used within 4 hours after its collection. The plasma clotting activity of crude venom on PPP was determined by Ca-clotting time [18; 3]. For the control, 1X PBS instead of venom was added to PPP. One unit of coagulant or anticoagulant activity was defined as a decrease or an increase of 1 second of clotting time of PPP incubated with crude MTV (10 µg) compared to control PPP [18].

3.2.2.2.2 Effect of haemolytic activity

Hemolytic activity of MTV (10 µg) was assayed against 2.0 mL of 5% (v/v) goat washed erythrocytes as described previously [19]. Hemolytic activity was expressed as

percent hemolytic activity where absorbance of 0.1% Triton X-100- treated erythrocytes was considered as 100% activity.

3.2.2.2.3 Effect on platelet agglutination/aggregation induced by the venom

The reduction in platelet agglutination/ aggregation was monitored by using washed platelets. Platelet rich plasma (PRP) was isolated from whole blood by centrifuging the citrated goat blood at 300 g for 20 min at 4 °C [20; 18; 21]. The supernatant containing PRP was carefully transferred into a fresh tube by pipetting. The platelets were isolated from PRP by centrifuging at 650 g for 10 min, and the platelets were settled down as pellets. The pellet was washed twice and finally re-suspended in Tyrode's buffer (5.0 mM HEPES, 13.0 mM NaCl, 2.7 mM KCl, 12.0 mM NaHCO3, 0.42 mM Na2HPO4, 1.0 mM MgCl2, 0.1% glucose and 0.25% BSA). Platelets were isolated by centrifuging the PRP at 650 g for 10 min. The pellet containing platelets was washed twice in Tyrode buffer, and the absorbance was adjusted to 0.15 at 650 nm. MTV-induced reduction in platelet agglutination/ aggregation was determined by incubation of crude MTV (10 µg) with washed platelet or Tyrode's solution (control) at 37 °C for 6 h in a CO_2 incubator. The platelets were stained with trypan blue and counted in the hemocytometer using Motic Images plus 3.0 mL software [8].

3.2.2.2.4 Effect of amidolytic activity

The inhibitory effect of venom on the amidolytic activity of factor Xa was determined as described by Saikia et al. [19]. Briefly, different concentrations of MTV (1 µg to 10 µg) were preincubated with 0.1 µg of FXa (22 nM) isolated from human plasma (Calbiochem, Merck, USA) for 60 min at 37 °C. After that, 2.0 µL of 10 mM F3301 (CH3COO-D-CHA-Gly-Arg-pNA-AcOH-chromogenic substrate of FXa, 0.2 mM final concentration) was added to the reaction mixture. The release of p-NA (p-nitro aniline) was observed for 20 min at 30 s intervals at 405 nm in a microplate reader (Multiskan GO, Thermo Fisher Scientific, USA). A control was run in parallel where FXa was pre-incubated with 1× PBS, pH 7.4 only (100% activity), and other values were compared to that.

3.2.3 Assessment of immunological cross-reactivity between MTV and commercial anti-scorpion antivenom by ELISA and immunoblot analysis

The immunerecognition of MTV against commercial scorpion antivenom was evaluated by ELISA and immune-blot analysis [2; 3; 5]. For ELISA, 100 ng of venom (protein) was coated in a 96-well microtiter ELISA plate (in triplicate) overnight at 4 °C, followed by washing the wells threetimes with 1× PBS containing 0.05% tween-20. The commercial ASA (200 ng per well) was used as the primary antibody, and antihorse IgG HRP-conjugated antibody (1:2000 dilution) was used as the secondary antibody to detect the primary antibodies. The substrate 3,3′,5,5′-tetramethylbenzidine/ hydrogen peroxide (TMB/H_2O_2) (Sigma-Aldrich, USA) was used to develop the ELISA plates. The commercial ASA-binding capacity of MTV was determined by calculating the EC_{50} value (the median effective concentration for the antivenom binding activity), defined as the concentration of antivenom that produced 50% binding of venom antigens (corresponding to half-maximum absorbance). The EC_{50} value was determined using GraphPad Prism software [7; 4].

For immunoblot analysis, 100 µg of venom (protein) was subjected to 12.5% SDS-PAGE in reduced conditions, and the proteins were transferred to PVDF (polyvinylidne fluoride) membranes. In the subsequent steps, the membrane was incubated with fat-free skimmed milk (5%) overnight at 4 °C to prevent non-specific binding of proteins. After washing the membrane with 1× TBS (tris buffered saline) containing 0.05% tween-20 (TBS-T), primary antibodies (15 mg/mL of scorpion antivenom) (1:1000 dilution) were added and incubated for 2 h at room temperature. After washing, the membrane was incubated with an antihorse ALP-conjugated secondary antibody (1:15 000 dilutions), and the immunoblot was developed with a 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium (BCIP/NBT) kit (Sigma-Aldrich, USA). The immunoblots were scanned with an EPSON scanner, and the densitometric analyses of the blots were done using ImageQuant TL 8.1 software (GE Healthcare, Sweden) [1].

3.2.4 *In vitro* laboratory analyses of commercial anti-scorpion (*M. tamulus*) antivenoms

3.2.4.1 Physiochemical characterization

The commercial ASAs were visually inspected to determine the consistency, colour, and texture and photographs of the products were taken [22; 23]. The solubility test was done by dissolving the lyophilized ASAs in 10 mL of sterilized de-ionized water (supplied by the manufacturers), followed by centrifugation at 10000 rpm for 10 min to examine any precipitation if present in the solution. The pH of these solutions was also determined (Elutech Instruments, pH 510, USA). The turbidity of the antivenom solution was measured using a turbidimeter (Nephelometer, model-CL52-D, ELICOLtd., India) [22; 23]. For determining the moisture content, the ASAs were heated for 3 h at 105 ◦C in a hot air oven, and the loss of moisture content, if any, was determined by taking the weight of the samples before and after heating [24; 25; 26].

3.2.4.2 Electron microscopic characterization

The lyophilized commercial ASAs were diluted to 10 μg/mL, and the samples were sonicated for 10 min in a water bath sonicator to remove air bubbles. Then the samples were mounted on a copper grid and kept for air drying. The field emission scanning electron microscopy (FESEM) of the ASAs was analysed using a JSM-7200F electron microscope at 15 Kv. Briefly, lyophilized ASAs (1.0 mg) were vertically sectioned with a razor blade and mounted on a sample stub. The samples were then coated with a platinum-palladium layer at 10 mA for 3 min, and a CCD detector was used to capture the images [22]. The commercial ASAs were also analysed by transmission electron microscopy (TEM) using the FEI TECNAI G2 model at magnification of 9900X, 17000X, 38000X, and 71000X at 200 kV. The images were obtained using a GATAN camera [27].

3.2.4.3 Mass spectrometry analyses

The protocol for mass spectrometry analyses of ASAs were adopted from our previous studies [8; 23; 28]. Briefly, 100 μg (protein) of ASAs was reduced with 10 mM dithiothreitol and alkylated (55 mM iodoacetamide) in the dark followed by trypsin digestion (~16 h). After that, LC-MS/MS analysis of desalted and concentrated tryptic

peptides was performed. The Mascot search engine was used to search MS/MS data in the NCBI database against *Equus caballus* protein database (taxid 9796). The searching parameters were set as a precursor and fragment mass error tolerance at 2.1 Da and 0.025 Da, respectively; two missed tryptic cleavage sites were accepted, and oxidation of methionine and carbamidomethylation of cysteine was set as variable and fixed modifications, respectively. The relative abundances of identified proteins in ASAs were calculated using MS1-based label-free proteomics [29]. The mean spectral matches under all the number of significant sequences generated from ASA proteins were calculated [8; 3; 5; 28] by applying the following formula (equation (1)):

$$mean\ spectral\ matches\ of\ proteinX = \frac{Sum\ of\ spectral\ matches\ under\ no.\ of\ significance\ sequence\ of\ proteinX}{Number\ of\ significant\ sequences\ for\ proteinX} - - - -(1)$$

The following formula was used to determine the relative abundance of a protein (X) in ASA (equation 2)

$$Relative\ abundance\ of\ proteinX = \frac{mean\ spectral\ matches\ of\ proteinX}{Net\ mean\ spectral\ matches} \times 100\% - - - -(2)$$

3.2.4.4 FPLC-size exclusion chromatography and SDS-PAGE analyses to determine the purity of the active substance

The procedures described in this section for determining the purity of active substances in commercial antivenom preparation were adopted from previous studies [22; 28; 30]. Before size-exclusion chromatography (SEC) analysis, the protein content of lyophilized ASA was determined [31]. The ASA (60 mg dry weight, which was equivalent to 49 mg protein content of PSVPL ASA and 51.6 mg protein of HBC ASA) dissolved in 1.0 mL equilibration buffer containing 20 Mm Tris-HCl and 150 mM NaCl, pH 7.4, was centrifuged for 15 min at 10,000 rpm, and filtered through 0.2 μm membrane syringe filter. The filtrate was fractionated through an FPLC Sephacryl S-200 (16 × 600 mm) size-exclusion column pre-equilibrated with an equilibration buffer at 4 ◦C [23; 28]. The ASA antibodies were eluted with the equilibration buffer at a flow

rate of 20 mL/h, and a fraction volume of 2.0 mL was collected. Protein elution from the column was measured at 280 nm, and the protein content of SEC peaks was determined [31]. A standard chromatogram of purified $F(ab')_2$ and horse IgG in the same column was used to compare the FPLC SECs of the ASA samples [28]. The crude and SEC peaks of both the ASAs were separated by 12.5% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reduced and reduced conditions [1]. The purified horse IgG and $F(ab')_2$ were also run in similar conditions. The PhastGel Blue R (GE Healthcare, Sweden) staining was done to visualize the gel's protein bands. From a standard curve of protein molecular markers run in the gel, the apparent molecular mass of the proteins in ASA samples was determined. The protein bands were scanned to determine their intensity by using ImageQuant TL 8.1 software (GE Healthcare, Sweden).

3.2.4.5 Determination of Fc content

The Fc content in commercial ASAs, an indicator of pepsin undigested IgG, was determined using ELISA and immunoblot analysis [23]. For ELISA, 100 ng of commercial ASA was coated, and its binding with rabbit anti-horse IgG Fc-specific antibody conjugated with horseradish peroxidase (HRP) (1:1000) was detected post-addition of hydrogen peroxide (H_2O_2)/3, 3′ ,5,5′ -Tetramethylbenzidine (TMB). The Fc content in the purified horse IgG was considered 100%, and the percent Fc content in the ASA samples was determined relative to that [23].

For immunoblot analysis, 30 µg protein of ASAs/ horse IgG (positive control) were separated under non-reduced and reduced conditions by 12.5% SDS-PAGE, and proteins were transferred to a PVDF membrane using a semi-dry blotter (Amersham Bioscience, Sweden), the non-specific binding sites were blocked with 5% fat-free skimmed milk. Then membranes were washed three times with wash buffer (1X PBS, pH 7.4 containing 0.1% tween-20), incubated with HRP-conjugated rabbit antihorse IgG Fc specific antibodies (Sigma-Aldrich, USA) at 1:2000 (1.5 mg/mL stock solutions) at room temperature for 1 h, and TMB/H_2O_2 substrate kit (Sigma-Aldrich, USA) was used to develop the blots [8]. The densitometry analysis of the scanned image was done with ImageQuant TL 8.1 software (GE Healthcare, Sweden). The Fc content in the purified horse IgG was considered 100%, and the Fc content in commercial ASAs was measured relative to that [23].

3.2.4.6 Determination of particle size distribution (protein aggregation) by dynamic light scattering (DLS) analysis

The horse IgG and commercial ASAs aggregates for DLS analysis were generated following the standard procedures described previously [32; 27], and aggregate formation was confirmed by SDS-PAGE analysis. The DSL analysis was performed in a NanoPlus, Zeta Potential, and NanoParticleAnalyzer (Particulate Systems, USA) to determine the hydrodynamic size of particles present in ASA at 37 ºC using a diode laser (660 nm) with an output power of 70 mW. The measurement angles were fixed at 160º and 15º at 37 ºC for determining the sizes of the particles. For each run, 70 acquisitions were maintained. The translational diffusion coefficient (DT) measured the hydrodynamic radius (RH) of each particle by using the Stokes-Einstein relation (equation (3)): $DT = kBT / 6\pi\eta RH$ (3)

In the above equation, kB denotes the Boltzmann constant, T defines the temperature in Kelvin, and the solvent's viscosity is represented by η. The particle size and size distribution of ASAs were obtained using the regularized CONTIN method [33] and compared with horse IgG aggregate.

3.2.4.7 Determination of IgA and IgE Contamination

The ELISA and immunoblot analysis were performed to determine the possibility of contamination of IgA and IgE in commercial ASAs [23; 28]. For determining the IgA/ IgE content by ELISA, 100 ng of commercial ASA was coated onto the wells of a 96-well plate. Non-specific binding sites were blocked with skimmed milk and incubated with the HRP conjugated to mouse anti-horse IgA antibodies (1:1000, 1:2000, 1:5000, 1:10000 dilutions) or mouse anti-horse IgE antibodies (1:100, 1:250, 1:500, 1:1000 dilutions). The IgA and IgE in ASAs were detected by colourimetric blot development using hydrogen peroxide (H_2O_2) and 3,3′,5,5′ -tetramethylbenzidine (TMB). For immunoblot analysis, 30 µg (protein content) of ASAs and horse IgG (positive control) were separated by 12.5% SDS-PAGE under non-reduced conditions. The protein transfers to the PVDF membrane, and its blocking and subsequent washing steps are described above. The washed membranes were incubated with HRP-conjugated anti-horse IgA/ anti-horse IgE antibodies (1:2000) (Sigma-Aldrich, USA) at room

temperature, and TMB/ H_2O_2 substrate kit was used to develop the blots [8]. The densitometry analysis of the blots was done as described above.

3.2.4.8 Determination of complement activation and endotoxin contamination

The detailed procedures of blood collection from healthy volunteers (TU ethical permission TU/TUEC/59/08/4017) and isolation of serum and erythrocytes preparation have been described elsewhere [28]. The previously described procedure determined the commercial ASAs-induced complement activation via classical and alternative pathways [23; 34]. The CH_{50} (total hemolytic complement) or AP_{50} value (alternative pathway complement measurement) measured the complement activation property of ASA [34]. The classical (CH_{50} value) and alternative pathways (AP_{50} value) were calculated by comparing the activity of NHS incubated with normal saline (100% activity) [23; 34]. The possibility of bacterial endotoxin contamination in the tested ASAs was assessed using Pierce ™ LAL Chromogenic Endotoxin Quantification Kit (Thermo Scientific, USA), following the instruction of the manufacturer [35].

3.2.4.9 Determination of preservative content

According to WHO guidelines [26], phenol and m-cresol can be used as a preservative for antivenom production for long-term storage. The preservative content in commercial ASAs (100 µL of 1.0 mg/mL dry weight of ASA) was determined by reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) analysis as initially described by Hansen and Døssing (1982) and modified by Patra et al. (2018, 2021a) [23; 28]. The elution of m-cresol was detected at 254 nm, and the percentage of m-cresol in ASA was determined from a standard curve of m-cresol run in the same column under identical conditions [23; 28; 36].

3.2.4.10 Determination of kd value for scorpion venom-ASA interaction

The affinity of antibodies in commercial ASA with scorpion venom can be measured by determining the kd value of interaction. The higher the affinity; the petite will be the kd value. The present study used two biophysical techniques- (i) spectrofluorometric titration and (ii) atomic force microscopic (AFM) analysis to determine the kd value of interaction.

3.2.4.10.1 Spectrofluorometric analysis

The procedure to determine the venom-antivenom interaction by spectrofluorometric analysis was adopted from our previous study [23]. Spectrofluorometric analysis exploits the phenomenon of electron excitation upon collision between venom and antivenom with high energy partiles like photons and other excited electrons. Three amino acid residues with aromatic side chains—phenylalanine, tyrosine, and tryptophan—are responsible for the intrinsic fluorescence of proteins. The latter of these three is particularly significant because of its largest wavelength (close to the UV range) and longest lifespan in terms of both excitation and emission spectra. These characteristics enable its selective detection and make fluorescence measurements easier. Despite being a universal protein, tryptophan fluorescence has mostly been utilised in studies examining protein–protein interactions or structural changes in proteins [37]. Briefly, a fixed concentration of MTV (10 μg/mL) was incubated with different protein concentrations (10–1280 μg/mL) of ASA at room temperature. The reaction mixture was exited at 280 nm. The emission slits were set at 5 nm, the temperature was at 25 $^{\circ}$C, and the emission spectra were monitored from 300 to 500 nm using a Varioskan LUX Multimode Microplate reader (Thermo Fisher Scientific, Denmark). As a control, the fluorescence spectra of MTV /ASA were determined and compared with the relative fluorescence spectra intensity (λmax) achieved from venom-ASA interaction. The change in intensity before and after the interaction (Δλmax) was plotted against the concentration (μg/mL) of ASA using GraphPad Prism 5.0 software [38; 3], and the kd value was determined from the graph.

3.2.4.10.2 Atomic force microscopic (AFM) analysis

Scorpion venom-ASA binding affinity (kd value) was also determined by AFM analysis. The characteristics of the products (venom:antivenom complex) can also be determined by this technique. More is the venom-antivenom interaction, more will be the height of the complex formed. A fixed concentration (50 μg/mL, protein content) of MTV was incubated with graded concentrations of commercial ASA (50–3000 μg/mL, protein content) at room temperature on glass slides, and the reaction mixtures were subjected to AFM analysis (NTEGRA PRIMA, NT-MDT technology). AFM images were analysed using Gwyddion software, and the height of the venom-antivenom complexes was calculated using Origin software with the Gaussian function. Then

percent frequency distribution of the complex particles was plotted against the concentration (µg/mL) of commercial ASAs (PSVPL and HBC) using GraphPad Prism 5.0 software.

3.2.4.11 The spectrofluorometric titration to determine the presence of venom toxins-specific antibodies in commercial ASAs

The procedure to determine the MTV-specific antibody in commercial ASAs was adopted from our previous study [23]. From the spectrofluorometric titration curve, the total venom-specific antibody available in each vial of commercial ASAs was calculated by the following formula (equation (4)).

$$Total\ venom\ -\ specfic\ antibodies\ in\ a\ vial\ (mg)\ = \frac{Amount\ of\ venom\ used\ in\ spectrofluorometric\ titration\ (mg)}{Amount\ of\ ASA\ in\ which\ shows\ saturation\ with\ the\ venom\ (mg)} \times total\ amount\ of\ immunoglobulin\ content\ [IgG\ and\ /\ or\ F(ab')2]\ in\ a\ vial\ (mg) \quad (4)$$

The following equation did Determination of venom-specific antibody (%) present in each vial

$$Percentage\ of\ venom\ -\ specfic\ antibodies\ in\ a\ vial = \frac{Percentage\ of\ venom\ -\ specfic\ antibodies\ in\ a\ vial}{Total\ immunoglobulin\ content\ [IgG\ and/or\ F(ab')2]\ in\ a\ vial\ (mg)} \times 100$$

(5)

3.2.5 Computational (*in silico*) analysis to compare the binding efficiency of AAAs between α1- adrenergic receptor (α1A, α1B, and α1D) in human and mouse and homologous receptor (SER6) in *Caenorhabditis elegans*

As the 3D protein structures for the selected proteins were not available in RCSB (Research Collaborator for Structural Bioinformatics) Protein Data Bank, the protein structures were taken from the predicted structure database of AlphaFold [39; 40], where DeepMind has applied artificial intelligence (AI) driven methods to solve the

protein structures accurately. The AlphaFold predicted structures were docked against the ligands (drug molecules), *viz.*, Prazosin-HCL, Terazosin-HCL, and Silodosin using AutoDock Vina [41].

3.2.5.1 Preparation of the ligand 3D structures for docking

The 2D structures of the ligands to be docked, *viz.* Prazosin-HCL [PMCID: 68546], Silodosin [PMCID:5312125], and Terazosin-HCL [PMCID: 44383] were downloaded from the PubChem Compound Database of NCBI. 3D models of the ligands were generated using CORINA classic 3D software [42; 43] and subjected to energy minimisation using UCSF Chimera [44].

3.2.5.2 Protein-ligand docking

The AutoDockVina, a widely used and fast Open source program for molecular docking [41], was used for this study through the Python-based PyRx virtual screening platform [45].

3.2.6 Determination of *in vivo* neutralisation potency of commercial ASAs, AAAs, and Ascorbic acid in *C. elegans* model

3.2.6.1 Cultivation and synchronization *C. elegans* worms (N_2 generation)

Wild-type N_2 nematodes were kept in Petri dishes containing nematode growth medium (NGM, composed of 3.0 g NaCl, 2.5 g peptone, 17 g agar, autoclaved, and supplemented with 24 mL phosphate buffer, 1 mL 1 M $CaCl_2$, 1 mL 1M $MgSO_4$, and 1 mL of 5 mg/mL cholesterol dissolved in ethanol) [46] and fed with OP_{50} *Escherichia coli*. The synchronised L1 larvae of N_2 nematodes were transferred to the NGM containing *E. coli* OP_{50} at 20°C for 48 h. Then late L4-young adult stage worms were transferred to a 50 mL sterile conical centrifuge tube and spun for 2 min at 1150 x g to pellet the worms. After aspirating the remaining liquid, worms were transferred to 250 mL of S Basal inoculated with *E. coli* OP_{50}. The worms were monitored by visualizing a drop of the culture under the stereo-microscope (Labomed CZM-4). Mid-L1, mid-L2, mid-L3, and mid-L4 larvae were harvested after approximately 8, 18, 25, and 37 h, respectively, at 20°C [47].

3.2.6.2 Determination of lethal concentration 50 (LC_{50}) of MTV in *C. elegans*

For determining the LC_{50} value (the concentration of MTV at which 50% of the larvae died), L4 stage N_2 worms (50 worms/well) were grown in NGM containing graded concentrations of MTV (33.3 to 500 µg/ mL) for 24 h at 20°C. The control larvae were raised in NGM only. The worms were fed with *E. coli* OP_{50} *ad libitum* and observed for survival and paralysis/ death under a stereo-zoom microscope. Those worms that showed no movement after exposure to light and gentle tapping were considered dead or paralysed. Notably, the paralysis induced by MTV was irreversible. The LC_{50} value was calculated from regression analysis of the dose-response curve by comparing dead or paralysed worms with controls. The dead *C. elegans* are completely inactive but the paralysed *C. elegans* shows negligible movement upon tapping.

3.2.7 Determination of dose- and time-dependent neutralisation of MTV-induced toxicity in *C. elegans* by commercial ASAs, AAAs, and Ascorbic acid

The toxicity of a fixed concentration of commercial ASA (2 mg), AAAs (100 µM), and Ascorbic acid (2 µg) in *C. elegans* (50 worms per well) was determined as described in section 3.2.6.2.

Different amounts of commercial ASA (375 to 1500 µg) were pre-incubated with 125 µg/mL (LC_{50} value) of MTV (venom: antivenom; protein: protein) for 30 min at 37ºC, and the mixtures were added to synchronised late L4-young adult stage nematodes into the 48-well plates (each well contains 50 worms/ 200 µl reaction mixture); the wells containing *C. elegans* with NGM were considered as controls. The worms were kept at 20°C, and their survival and paralysis/ death were determined at 24 h post-treatment by eye quantification using a stereo-zoom microscope.

In another set of experiments, different concentrations (6.25 to 50 µM) of AAAs viz. Prazosin-HCL, Silodosin, and Terazosin-HCL, and Ascorbic acid (vitamin C) (0.2 to 2.0 µg) were mixed with LC_{50} concentration of MTV, and the mixtures were transferred to 48-well plates containing 50 worms /well. Veraspladib (400µM), a known inhibitor of the PLA_2 enzyme of venom [48], was used as a negative control because MTV lacks the PLA_2 enzyme [49]. The survival and paralysis/ death were determined at 24 h post-treatment as described above. The result was expressed as the per cent viability of worms in control and treated groups at each time interval. The neutralisation potency of commercial ASA, AAAs, and Ascorbic acid was determined.

For determining the time-dependent neutralisation of MTV-induced toxicity in *C. elegans*, the optimum concentration of each inhibitor (as determined from the results of the above experiments), viz. commercial ASAs, AAAs, and Ascorbic acid was added to the culture of *C. elegans* (50 worms/ 200µL reaction mixture) at 0 min (immediately after venom treatment) to 120 min post-treatment with MTV (LC_{50} value). The survival and paralysis/ death of the worms were determined 24 h post-treatment, as described above.

3.2.8 *In vivo* neutralisation of MTV-induced generation of reactive oxygen species and alteration of mitochondrial transmembrane potential in *C. elegans* by ASA, AAAs, and Ascorbic acid

For determining the neutralisation of reactive oxygen species (ROS) generation, the late L4-young adult stage nematodes were transferred into the 48-well plates (containing 50 worms in each group) and then treated with (i) LC_{50} value of MTV, (ii) reaction mixture of MTV (LC_{50} value) and optimum dose of AAAs/ commercial ASAs/ Ascorbic acid, and (iii) NGM (control). The worms were fed with *E. coli* OP_{50} and incubated for 6 h at 20 °C. Afterwards, worms were washed twice with 1X M9 buffer, set with a fluorogenic probe 2',7'-dichlorofluorescein-diacetate H_2DCFDA stain at 37 °C, for 5 h in the dark, followed by washing twice with 1x M9 buffer. Then worms were placed on agarose bed slides and covered with a cover clip. Then fluorescence intensities of 2',7'-dichlorodihydrofluorescein (DCF) produced by intracellular ROS were analysed on a confocal laser microscope (TCS SPE, Leica, Wetzlar, Germany) with excitation and emission wavelength at 480 nm and 530 nm, respectively [50].

The MTV-induced mitochondrial transmembrane potential was determined using 5,5',6,6' -tetrachloro-1,1',3,3'–tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye. Late L4-young adult stage *C. elegans* (50 worms) were treated with MTV (LC_{50})/ NGM (control)/ carbonyl cyanide m-chlorophenylhydrazone (CCCP; positive control)/ MTV (LC_{50}) mixed with an optimum dose of AAAs/ commercial ASAs/ Ascorbic acid. After 12 h at 20°C, the worms were washed twice with 1X M9 buffer, incubated with JC-1 with stain at 37ºC for 5 h in the dark, and then washed twice 1X M9 buffer. Then worms were placed on agarose bed slides and covered with a cover clip. The fluorescence intensities were determined using a confocal laser microscope (Leica DMi8) with an excitation wavelength of 488 nm and emission wavelength of 533 ± 30 nm (FL-1 green channel) and 585 ± 40 nm (FL-2 red channel). The alteration of

transmembrane potential by crude venom was considered 100% activity, and other values were compared to that.

3.2.9 The *in vivo* neutralisation of MTV-induced lethality in *C. elegans* with individual components of the formulation and their combinations

Synchronised nematodes were treated with individual components of formulation such as commercial ASA (PSVPL), AAA (Prazosin-HCL), Ascorbic acid (vitamin C), their combinations, and different concentrations of the formulation against LC_{50} (125 µg/ml) value of MTV. The compositions of the components of the formulations are described in Table 3.1. The percent survivability of *C. elegans* was determined as described above (section 3.2.6.2).

Table 3.1 Composition of the components used for formulation.

S. No.	Component of the formulation	Solubility
1	ASA (%venom specific antibodies: 6.29%)	Water/ 1xPBS
2	AAA (Prazosin-HCL)	Water/ 1XPBS and then heating at 50-60 °C for 3-5 min
3	Ascorbic acid	Water/ 1XPBS

3.2.10 *In vitro* DPPH free radical-scavenging activity of different concentrations of the formulated drug, individual components of the formulation, and their combinations

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity was measured according to the method of Chen et al. (2020) [51]. Briefly, a 100 µL sample of the formulated drug, individual components of the formulation, and their combinations were mixed with a DPPH radical solution of 100 µL (2×10^{-4} mol/L, dissolved in 95% ethanol) in a 96-well plate. Then the solution was incubated for 30 min at room temperature. The absorbance of the solution at 517 nm was immediately measured by the Enspire microplate reader (Perkin Elmer, Inc., Baesweiler, Germany). The

percentage of DPPH free radical-scavenging activity of formulated drug and components of the formulation was calculated as:

DPPH radical scavenging activity (%): $[1-(A1-A2)/A_0]X100$----------[1]

Where A_0 represents the absorbance of 100 µL of 95% ethanol (v/v) with a DPPH radical solution of 100 µL at 517 nm, A_1 the absorbance of the sample (100 µL) with DPPH solution (100 µL), A_2 represents the absorbance of the sample (100 µL) solution with 95% ethanol (100 µL).

3.2.11 *In vivo* neutralisation of MTV-induced generation of reactive oxygen species (ROS) and alteration of mitochondrial transmembrane potential in *C. elegans* by different concentrations of the formulated drug and individual components of the formulation, and combination of thereof

For determining the neutralisation of reactive oxygen species (ROS) generation, the late L4-young adult stage nematodes were transferred into the 48-well plates (containing 50 worms in each groupwith 200 µL reaction mixture) and then treated with (i) LC_{50} value MTV, (ii) reaction mixture of MTV (LC_{50} value) and the individual component of the formulation and different concentrations of formulation and, (iii) NGM (control)/CCCP1 (positive control). MTV-induced ROS production was determined as described above in section 3.2.6.2.

The MTV-induced mitochondrial transmembrane potential was determined using 5,5',6,6' -tetrachloro-1,1',3,3'–tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye. For the assessment of neutralisation MTV-induced alteration of mitochondrial transmembrane potential, late L4-young adult stage *C. elegans* (50 worms) were treated with MTV (LC_{50})/ NGM (control)/ CCCP (positive control)/ MTV (LC_{50}) mixed with components of the formulation and different concentrations of the formulation. After 12 h, fluorescence intensities were determined, as mentioned in section 3.2.6.2.

3.2.12 Restoration of MTV-induced expression level of genes involved in apoptosis, detoxification, and stress response by the formulated drug was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Synchronised L4-young adult stage nematodes (approximately 500 worms) were treated with LC_{50} dose (125 µg/mL) of MTV/ NGM (control group) and incubated at 20 ºC for 24 h [50]. After 24 h of treatment, worms were washed, followed by total RNA extraction using a pure link RNA mini kit. The purity and concentration (A260/ A280) of the RNA were measured using a nanodrop spectrophotometer. Then c-DNA was synthesised with 1 µg of extracted RNA using a Verso c-DNA Synthesis Kit. To determine gene expression, q-RT-PCR was performed with the help of SYBR Green (Applied Biosystems, USA) in a real-time PCR machine (Applied Biosystem, USA). The amplified genes were pro-apoptotic (ced-3 and ced-4), anti-apoptotic (ced-9), detoxification GSTs (gst-6, gst-7), catalases (ctl-1), Thioredoxin-1 (trx-1) and SOD (sod-3), and heat-shock proteins HSP (hsp-60). Each gene's relative expression was examined using the $2^{-\Delta\Delta Ct}$ method [52; 50]. The house keeping gene act-1 was used to normalise each gene expression.

3.2.13 Validation of *in vivo* neutralisation of MTV-induced toxicity by formulated drug and combinations of commercial ASA and AAA in Wistar strain albino rats

In our study, we used Wistar strain albino rats because the lethal effect of MTV in rats is higher than that of mice reported by Tiwari and Deshpande in 1993 [53]. Further, it is very difficult to get the sufficient volume of blood require for the experiments from the mice which can easily be available from rats. The rats were acclimatised at 22 ± 3° C with a relative humidity of 30–70% and fed with a standard diet of "Amrut" procured from Krishna Valley Agrotech LLP, Pune, Maharashtra, India, and water *ad libitum*. They were maintained for a 12:12h light-dark cycle and divided into six groups of n=6 as per approval from the institutional animal ethics committee (IASST/IAEC/2022/09).

3.2.13.1 Neutralisation of hyperglycemia and prolonged tail bleeding time

The venom yield for an adult MTV is 1.5 mg [54]. Therefore, an average 60 kg adult can receive a maximum of 1.5 mg MTV in a sting equivalent to 25 µg/kg (or 5 µg/ 200 g of rat) 0.5 ug/20g for an adult human of 60 kg weight. Individual groups of Wistar strain albino rats (200 ± 10 g, n=6) were injected intravenously with 25 µg of MTV

(approximately fivetimes higher than the amount of MTV injected in one sting to human). The venom was dissolved in 0.20 ml of 1XPBS and injected (i.v.) into venom-injected groups of rats (n=6); the group of rats injected with 1XPBS served as a control.

Wistar strain albino rats (n=6) were injected (intravenously or i.v) with formulated drug (formulation 2), combinations of ASA and AAA [ASA (1500 µg): AAA (50 µM] and ASA (187.5 µg): AAA (3 µM)) as well as the individual component of the formulation simultaneously with MTV (25 µg/200 g or 125 µg/kg of rats). Blood glucose content (mg/dL) of each group was measured by an accu-chek active glucometer at 0 to 240 min (at an interval of 30 min) by tail prick method [55]. All the groups were observed for 24 h for any physical or behavioural change, viz., body weight, food and water intake, defecation and urination, grip strength, and death.

After 24 h of observation, the tail bleeding time of each Wistar strain albino group treated with MTV and formulated drug/ MTV (venom-treated group)/ PBS (control group) was determined by transverse amputation of the rat tail tip followed by immersing the tail in saline at 37 ºC and monitoring the bleeding time [55].

3.2.13.2 Neutralisation of changes in serum biochemical parameters

After 24 h post-injection, rats were sacrificed using chloroform (1%), and blood was collected immediately by cardiac puncture. The serum was isolated by centrifugation of the blood samples at 2200 rpm at 4 ºC for 15 min. Biochemical parameters of blood serum viz. alkaline phosphatase (ALKP), serum glutamic pyruvic transaminase (SGPT), and creatinine were analysed by BeneSphera C61 semi-automatic biochemistry analyser using commercial diagnostic kits following the manufacturer's instructions.

3.2.13.3 Neutralisation of morphological alterations in vital organs

The kidney, heart, liver, lung, testis, and ovary of treated and control groups were dissected 24 h post-observation to determine the possible morphological alterations. Tissues were cut and washed extensively in 1XPBS to remove the adhered blood clots and fixed in 10% buffered formaldehyde. The fixed tissues were dehydrated in grade concentrations of alcohol and embedded in paraffin wax. Sections 4–5 mm thick were

processed routinely for light microscopic observation after hematoxylin-eosin staining (H&E) for pathological studies described by [56].

3.2.14 Determination of MTV-induced inflammatory cytokines levels in Swiss albino mice

Swiss albino mice (22-24 g) were divided into two groups (control and treated). Group of mice (n=6) were injected with 200 µL 1X PBS were considered a control. A group of mice injected (intravenously or i.v) with 25 µg of MTV dissolved in 1X PBS in the tail vein were considered as treated. Mice were maintained and used according to animal welfare international recommendations.

At 24 h of venom injection, blood was collected by cardiac puncture, and the level of blood plasma pro-inflammatory cytokines was determined. According to the manufacturer's guide, the plasma levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNFα) were assayed by ELISA using themice-specific immunoassay kits **(**R&D Systems**).**

3.2.15 Statistical analysis

The significance of the difference for more than one data set was analysed by a one-way ANOVA in Graph Pad Prism 5. The significant difference between the two data sets was determined by the Student's t-test analysis using Sigma Plot 11.0 for Windows (version 10.0). When the p-value between the two data sets was ≤ 0.05, it was considered to be a significant difference.

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