Biological and Biochemical Potential of Sea Snake Venom and Characterization of Phospholipase A₂ and Anticoagulation Activity

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Abstract This study is designed to isolate and purify a novel anti-clotting protein component from the venom of *Enhydrina schistosa*, and explore its biochemical and biological activities. The active protein was purified from the venom of *E. schistosa* by ion-exchange chromatography using DEAE-cellulose. The venom protein was tested by various parameters such as, proteolytic, haemolytic, phospholipase and anti-coagulant activities. 80 % purity was obtained in the final stage of purification and the purity level of venom was revealed as a single protein band of about 44 kDa in SDS-polyacrylamide electrophoresis under reducing conditions. The results showed that the Potent hemolytic activity was observed against cow, goat, chicken and human (A, B and O positive) erythrocytes. Furthermore, the clotting assays showed that the venom of *E. schistosa* significantly prolonged the activated partial thromboplastin time, thrombin time and prothrombin time. Venomous enzymes which hydrolyzed casein and gelatin substrate were found in the venom protein. Gelatinolytic activity was optimal at pH 7.5 and ¹H NMR analysis of purified venom was the base line information for the

structural determination. These results suggested that the *E. schistosa* venom holds good promise for the development of novel lead compounds for pharmacological applications in near future.

Keywords Venom · Hemolytic · Proteolytic activity · Activated partial thromboplastin time (APTT) · Thrombin time (TT) · Prothrombin time (PT)

Abbreviations

BSA	Bovine serum albumin
EDTA	Ethylene diamine tetra acetic acid
PBS	Phosphate buffered saline
CaCl ₂	Calcium chloride
HCl	Hydrochloric acid
<i>E. schistosa</i>	<i>Enhydrina schistosa</i>
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	Tris(hydroxymethyl) aminomethane
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
RT	Recalcification time
APTT	Activated partial thromboplastin time
PT	Prothrombin time
TT	Thrombin time

Introduction

Snake venoms are complex mixtures of proteins including phospholipases A₂, myotoxins, hemorrhagic metalloproteases and other proteolytic enzymes, cytotoxins, cardiotoxins and others. PLA₂s (phosphatidylcholine 2-acylhydrolases) is one

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of the major components of snake venom and some venom PLA₂s were reported to be anticoagulants. These enzymes are present in venom in several homologous forms that differ in their ability to affect the coagulation cascade. Thus, they may act either exclusively on the extrinsic pathway (the so-called “weak” anticoagulant PLA₂s) or on both the extrinsic pathway and the prothrombinase complex (“strong” anticoagulant PLA₂s) [1]. They are diverse family of lipolytic enzymes that specifically catalyze the hydrolysis of fatty acid ester bonds at position 2 of 1, 2-diacyl-*sn*-3-phosphoglycerides to produce free fatty acids and lysophospholipids [2]. There is a tremendous molecular diversity of snake venom PLA₂s, with both active and catalytically inactive forms, that results in a wide spectrum of toxin action, such as neurotoxic, cardiotoxic, myotoxic, necrotic, anticoagulant, hypotensive, hemolytic, hemorrhagic and edema-inducing activities [3].

Over the years, a number of toxins that affect blood circulation have been isolated and characterized from various snake venoms [4]. Within each family of snakes, the venom components seem to be comparatively common and similar to one another. Nerve toxins are generally found in the Hydrophidae and Elapidae venoms whereas hemorrhagic and myonecrotic toxins are generally found in the venoms of the Viperidae and Crotalidae families of snakes [5]. Snake venoms contain a potent cocktail of toxins that have evolved to interfere with critical physiological processes including blood coagulation, fibrinolysis, neuronal signaling, blood flow and filtration, and inflammation [6]. Therefore it is mainly considered to be used in the treatment of various diseases which include infection, hematological, inflammatory, cardiovascular, malignant etc. In addition to understand the evolving of venoms; characterization of the proteins and the content of snake venoms also has a number of potential benefits for basic research, clinical diagnosis, development of new research tools and drugs or potential clinical use, and for antivenom production [7].

There are many species of venomous snakes in the world. The venom of sea snakes contains potent neurotoxins which bind to the acetylcholine receptor in the neuromuscular junction [7]. Utilizing this property, many investigators are using snake neurotoxins to elucidate the mechanism of neurotransmission across the synapses or neuromuscular junction. Mostly these types of venom having both the anticoagulant and procoagulant activity that may leads to the concurrent thrombosis, bleeding disorders and disseminated intravascular coagulation. Thus, the aim of this study was to isolate and characterize the venom protein from sea snake (*Enhydrina schistosa*) and to determine the in vitro biological and pharmacological properties of venom.

Materials and Methods

Materials and Reagents

Citrated human plasma was kindly provided by Government Hospital, (Portonovo, India) from a pool of healthy donors. Liquicelin E (activated Cephaloplastin reagent) (Sigma Diagnostics, St. Louis, MO, U.S.A), Thromboplastin, Sodium chloride (NaCl), Bovine serum albumin (BSA), Casein and Gelatin, Sodium dodecyl sulphate (SDS), acrylamide, ammonium persulphate, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and Coomassie Brilliant Blue R-250 were purchased from Himedia Chemical Co. (Mumbai, India). The standard protein molecular marker was purchased from Bangalore Genei (Bangalore, India). ‘V’ and ‘Flat’ shaped bottom 96 well plates (Kolkata, India) used in assays in the SpectraMax micro plate ELISA reader (Molecular Devices, USA). All other chemicals and reagents used were of analytical grade.

Collection of Venom

Sea snakes (*E. schistosa*) were collected from various fishing ports [Cuddalore (11°42'23"N & 79°46'55"E) to Nagapattinam (10°45'52"N & 79°51'16"E)] of Southeast Coast of India and were brought alive to the laboratory. Live sea snakes were maintained for several weeks and the venom was collected by milking process using capillary tubes or glass plates in weekly intervals. The collected venom was lyophilized, and then kept at 4 °C until further use.

Purification by Ion Exchange Chromatography

Venom sample of *E. schistosa* was fractionated by ion exchange chromatography using DEAE cellulose (Sigma, India) column. Fresh lyophilized crude venom (100 mg) was dissolved in 5 ml of 0.01 M phosphate buffer at pH 6.4, applied to a DEAE cellulose column (1.5 cm × 16 cm, Sigma). The same buffer with a linear gradient of 0–0.5 M NaCl was used for elution. The column was eluted at a flow rate of 25 ml/h and collected in 4 ml per tube using sterile screw cap tubes. The protein concentration in each fraction was simultaneously monitored by UV-spectrophotometer (UV-160A, Shimadzu, Japan) at 280 nm. Active fractions were pooled, dialyzed against 0.01 M phosphate buffer (pH 6.4) and distilled water and dialyzed venom protein was lyophilized for further analysis.

Protein Quantification

The protein concentration of crude and purified venom protein was determined by the method of Lowry et al. [8] using bovine serum albumin as a standard.

Electrophoresis

The molecular mass of venom protein was determined through Sodium-dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by following the Laemmli [9] method using 10 % resolving gel and 4 % stacking gel. Venom samples (200 µg) were diluted (1:1) with sample buffer (50 mM Tris pH 6.8, 2 % SDS, 20 % glycerol, 2 % 2-mercaptoethanol and 0.04 % bromophenol blue) and were then boiled at 100 °C for 2 min, shaken in vortex for 30 s and loaded onto the gel. After electrophoresis, gel was stained in 0.3 % Coomassie Brilliant Blue R-250 solution followed de-staining with 30 % methanol, 10 % acetic acid and water to reveal protein bands. The molecular size marker, 29–205 kDa (Protein standards, Genei Pvt Ltd, Bangalore, India), was run parallel with venom samples for molecular weight determination.

Proteolytic Activity

The nature of venom protease and molecular weight of *E. schistosa* venom was determined based on the gel observation. Caseinolytic and gelatinolytic activity was determined by dimethylcasein and gelatin as substrates [10]. Briefly, 2 mg/ml of substrates were mixed in 10 % resolving gel (2.3 ml of distilled water, 1.3 ml of 30 % acrylamide-bisacrylamide solution, 1.3 ml of 1.5 M Tris pH 8.8, 50 µl of SDS, 50 µl of APS and finally 3 µl of TEMED) to detect the enzymatic activity. The purified venom protein was (10 µg) mixed in 20 µl non-reducing sample buffer (50 mM Tris pH 6.8, SDS, Glycerol, 10 % bromophenol blue) and loaded into the wells and ran at 20 mA. After electrophoresis, the gel was rinsed in 2.5 % (v/v) of Triton X-100 for 1 h in order to remove SDS and then incubated overnight at 37 °C in buffers of different pH values. The presence of colorless bands were indicated the proteolytic activity on the blue gel. To determine the effect of pH on the proteolytic activity, the gels were incubated in 0.1 M sodium citrate buffer at pH 5.0, 0.1 M sodium phosphate buffer at pH 6.0, 7.0, 8.0 and 9.0. The molecular weight marker (29–205 kDa) was also used in separating gel in the same condition with the substrate.

Determination of Coagulation Assays

Plasma Preparation and Clotting Assay

The assay was performed according to the protocol described in the United States Pharmacopoeia [11]. Fresh human blood was collected directly into a siliconized test tube containing 3.8 % tri-sodium citrate solution in a proportion of 1:9 (citrate/blood; v/v). It was mixed immediately by gentle agitation. Platelet-rich plasma (PRP) was obtained by centrifugation of blood samples at

5000 rpm for 15 min at room temperature, and the obtained samples were used within 2 h of preparation. The separated plasma was pooled together and kept under refrigeration for the subsequent clotting assay. The effect of venom protein upon coagulation was assessed by using the re-calcification time (RT) assay adopted for the SpectraMax micro plate reader [12]. The procedure allows to monitor the clot formation and to use kinetic parameters for the coagulation process through the micro plate reader. The assay was conducted using a 96 well microplate ELISA reader (SpectraMax, molecular devices co., Sunnyvale, USA) equipped with temperature and shaking controls. The final volume of 50 µl and 100 µl of citrated plasma was incubated with different concentration of purified venom (2, 4, 6 and 8 µg) in 90 µl of 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.4. After 5 min, 10 µl of 50 mM CaCl₂ was added and clot formation was monitored at 37 °C for 20 min, against the SpectraMax system at 650 nm. In the case of calcium independent coagulation activity, EDTA (Ethylenediamine tetraacetic acid) was added instead of CaCl₂ to a final concentration of 10 mM. The experiments were repeated for four times to confirm every observation.

Activated Partial Thromboplastin Time (APTT), Prothrombin Time (PT) and Thrombin Time (TT) Assays

Activated partial thromboplastin time (APTT), Prothrombin time (PT) and Thrombin time (TT) was assayed in plasma samples of human blood. APTT, PT and TT assays were determined using commercial kits (Sigma, Bangalore, India) and screening tests to monitor the functionality of the extrinsic and intrinsic pathways, respectively. All assays were tested using citrated human plasma from a healthy donor.

Effect on Activated Partial Thromboplastin Time (APTT)

The APTT test was performed according to the method of Proctor and Rapaport [13] using a Liquecelin E (activated Cephaloplastin reagent) (Sigma Diagnostics, St. Louis, MO, U.S.A) as indicated by the manufacturer, using the ELIZA reader kinetic module. Briefly, 50 µl of citrated normal human plasma and 50 µl of purified venom protein were mixed inside the plate wells and incubated at 37 °C for 3 min. 50 µl of APTT assay reagent (Cephaloplastin) was added, and the reaction mixture was incubated for another 3 min. After incubating at 37 °C, with shaking for 3 min, 50 µl of pre-warmed (37 °C) 50 mM CaCl₂ was added by using a micropipette. After the addition of the CaCl₂, the plate was mixed once and then maintained at 37 °C using the apparatus mixer and heating system and absorbance readings at 650 nm taken at 10 s intervals. A fast and sharp increase in the absorbance after a lag phase

indicated clotting. We chose the time has taken for reaching 0.1 or 0.05 absorbance value (onset absorbance) as a measure of clotting time, using the 'time to selected absorbance' module included in the instruments software. With this procedure, the entire testing sample results from the whole procedure could be analysed quickly and simultaneously for anti-clotting activity.

Effect on Prothrombin Time (PT) and Thrombin Time (TT) A mixture of tissue thromboplastin from rabbit brain, calcium ions and buffer (Diagnos thrombo reagent) was used for the determination of prothrombin time. Briefly, 50 μ l of citrated normal human plasma was incubated in the presence of 100 μ l of Diagnos thrombo reagent (rabbit brain thromboplastin reagent) for 2–3 min at 37 °C. Thrombo reagent was added into the test tube containing 100 μ l of plasma and 50 μ l of purified *E. schistosa* venom protein. After incubating the mixture for exactly 3 min, 100 μ l of 25 mM CaCl_2 was added to induce coagulation. A buffer solution without venom protein was used as the control. We chose the time has taken for reaching 0.1 absorbance value (onset absorbance) as a measure of time to clot formation was recorded. The experiments were carried out twice to confirm the observation. For TT assay, citrated normal human plasma (90 μ l) was mixed with a solution of *E. schistosa* venom (10 μ l) and incubated for 2 min. Then, TT assay reagent (100 μ l, 5 U/ml) pre-incubated at 37 °C for 5 min was added and clotting time was recorded. All samples were dissolved in saline solution.

Hemolytic Activity

Hemolytic activity of venom protein was estimated on various cow, goat, chicken and human (A and O positive) erythrocytes as described earlier by Paniprasad and Venkateshwaran [14]. The micro-hemolytic test was performed in 96 well 'V' bottom microtitre plates and different rows were selected for different blood samples and serial dilution of the purified venom was made into 100 μ l of PBS solution. 1 μ g/ml of purified venom protein sample at various concentrations was incubated with 100 μ l of suspended erythrocytes (1 % (v/v) in Phosphate buffer saline (PBS) (pH 7.4) for 3 h at room temperature. A negative control (1 % erythrocyte suspension in PBS solution) and a positive control (erythrocyte suspension in distilled water) were prepared, to enable calculation of percentage hemolytic unit (HU). Uniform red color suspension in the wells considered as positive hemolysis and button formation in the bottom of the wells was considered as a lack of hemolysis. All assays were carried out in duplicate. The reciprocal of the highest dilution of the purified venom sample showing the hemolytic pattern was taken as one hemolytic unit.

Phospholipase A₂ Activity Assay

The evaluation of phospholipase activity was measured by the method of Marinetti [15], with suitable modifications. Briefly, egg yolk was suspended in Tris-HCl buffer pH 8.0 to an initial absorbance of 1.0 at 740 nm and prepared purified venom sample (2, 4, 6 and 8 μ g) was added to 30 ml of this suspension. Phospholipase activity was assessed as the rate of a linear decrease in optical density over an incubation period of 5–15 min. One unit of phospholipase activity corresponds to the decrease of 0.001 absorbance per min. Activity was expressed as U/mg of venom of six independent experiments.

Amino Acid Analysis

Amino acid analyses of venom protein were performed on a reverse phase HPLC system (Merck Hitachi LaChrome D-7000 HPLC system, Darmstadt, Germany). The purified venom sample was hydrolysed in 6 N HCl at 110 °C for 24 h in vacuum-sealed ampoules. The hydrolysates were analyzed on a Hitachi LaChrome liquid-chromatography system for required times at room temperature. The amino acids were identified and quantified by RP-HPLC through the comparison of their retention times and peak areas with those from a standard amino acid mixture. The amount of amino acid content was expressed as the number of residues per 1000 residues.

¹H NMR Analysis

The purified venom protein sample was analysed by ¹H NMR spectroscopy. ¹H NMR was obtained by using a Bruker Avance 400 nuclear magnetic spectrometer (Bruker DRX 500 Rheinstetten, Germany) operated at 400 MHz. Two milligram of purified venom sample were dissolved in D₂O (denatured water), and denatured potassium hydroxide (KOH), using dimethylsilapentane sulfonic acid (DSS) as a reference and a 20 ppm spectral window was considered. All chemical shifts were given in parts per million (ppm). The ¹H spectra were used to assess the composition of non-volatiles in the of venom sample.

Statistical Analysis

All the experiments were repeated for five independent observations. The values were expressed as the Mean $\pm$ SD of results obtained with the indicated number of animals. The statistical significance of differences between groups was evaluated using the Turkey–Kramer test. *P* value < 0.05 was considered to indicate significance.

Results and Discussion

Protein Concentration and Purification of Venom Protein

One milligram of dried venom was collected from approximately 200–600 g of *E. schistosa* sea snake per week. The freshly milked venom was lyophilized prior to further treatment and the lyophilized venom look-like pale yellowish white in color. The amount of protein in crude and purified samples was estimated as 457 and 786 $\mu\text{g}/\text{mg}$, respectively. The chromatographic techniques of ion exchange column chromatography have been used widely to purify proteins, including those from snake venoms [16]. As a result, fractionation of *E. schistosa* venom using a DEAE cellulose column eluted the venom proteins into four major and six minor peaks (Fig. 1). In the present results of purifying steps along with an overview of the entire fractionation process were summarized in Fig. 1. The yield of purified venom protein was obtained at 80 percent/gm on the dry weight basis. Peak no 8, which was revealed that the highest amount of venom protein and also displayed the maximum PLA2 and anticoagulation activity.

Determination of Molecular Weight

The electrophoretic pattern revealed that both the crude and purified *E. schistosa* venom showed as separated bands of

Fig. 1 Purification profile of DEAE cellulose-Ion exchange chromatographic separation of venom protein from *E. schistosa*. The crude venom was applied on the column (1.5×16 cm) equilibrated with 0.01 M phosphate buffer (pH 6.4 and a linear gradient of 0.0–0.5 M NaCl in the same buffer was applied (total volume of 300 ml). Four milliliters-aliquots were collected. Full and broken lines indicate the absorbance at 280 nm and protein concentration, respectively

56 & 44 kDa in crude venom (Lane 2) and single band of 44 kDa in purified venom (Lane 3) under reducing conditions (Fig. 2). Which was higher than that of other snakes venom, such as *Porthidium nasutum* snake venom has molecular mass of 15,802.6 Da migrates a single band under both non-reducing and reducing conditions, *Piper umbellatum* and *Piper peltatum* untreated toxin migrated as ~ 15 kDa under reducing conditions and were ponding to the mass of its monomeric subunits in SDS-PAGE. In non-reducing condition, the spanning was reported as 28 kDa (homodimer) to 15 kDa (monomer) [17, 18].

Proteolytic Activity

The proteolytic activity was identified by a zymography experiment using gelatin and casein as the substrates (Figs. 3, 4). The result of the present study revealed that the proteolytic activity was observed in crude and purified venom which was involved degrade distinct proteins are such as casein and gelatin and a clear zone in the gels indicated the enzymatic nature. Gelatinolytic proteolysis was detected as colorless bands in blue gel which ranged from 53 to 238 kDa and the optimal pH for the venom enzyme was involving 5, 6, 7, 8 and 9 (Fig. 3). In addition, mild caseinolytic activity was also observed at molecular weight ranged from 76 to 234 kDa which demonstrated in the venom of the sea snake when casein was used as a substrate (Fig. 4). The hydrolysis of the proteolytic activity of sea snake venom on casein and gelatin were assayed due to the probable involvement of proteases in the instability of biological activities. The profile of enzymatic degradation was similar using distinct substrates, indicated the presence of proteases with broad substrate specificity. The result of the present study suggested that the proteases could contribute to the degradation of proteins and components present in the extracellular matrix for diffusion factors, or they can be directly involved in the degradation

Fig. 2 SDS-polyacrylamide gel electrophoresis of the venom protein (Lane 1—molecular weight markers; Lane 2—crude venom; Lane 3—purified venom) from *E. schistosa*

Fig. 3 Substrate-SDS-PAGE pattern of purified venom from *E. schistosa*, tested by using gelatin and incubated with different pH in 0.1 M sodium phosphate buffer solution. Lane 1 protein marker, Lane 2 pH 6.0, Lane 3 pH 7.0, Lane 4 pH 8.0, Lane 5 pH 9.0

Fig. 4 Substrate-SDS-PAGE pattern of purified venom from *E. schistosa*, tested by using casein and incubated with different pH in 0.1 M sodium phosphate buffer solution. Lane 1 pH 6.0, Lane 2 pH 7.0, Lane 3 pH 8.0, Lane 4 pH 9.0, Lane 5 protein marker

of proteins. Previously, Marsh [19] reported that the venom extracted from anterior regions of the venom ducts of *C. arenatus*, *C. lividus*, and *C. quercinus* displayed significant

proteolytic activity towards casein with optimal activities between pH 5, 6, 7, 8 and 9. The functions of the proteolytic enzyme in *E. schistosa* venom and its mechanism of action are still under investigation. The present study revealed that a new field of investigation may possibly open to understand the anticoagulant, cytotoxicity and proteolytic activity from sea snake's venom. Thus, sea snake's venom possible proves to be vital agents in the biomedical field.

Anticoagulant Activity

The effect of sea snake venom protein on blood coagulation assay was analyzed by incubating different concentration of venom protein (4, 8 and 10 μ g) with human citrated plasma prior to the induction of clotting with calcium chloride. These results suggested that the control reaction was taken 4.5 min to initiate coagulation after the addition of calcium chloride; but the venom sample has been prolonged the clotting time to 7.5, 9.0, 13.5 and 15.0 upon increasing concentration. These results were almost dissimilar with the control and significantly increased Recalcification time (RT).

When, the present venom protein sample was pre-incubated with 50 and 150 μ l of citrated plasma for 20 min, RT increased by almost three times, as compared with control (Fig. 5). The normal plasma was unable to clot in the absence of calcium chloride and also in the presence of EDTA along with venom protein. In humans, normal hemostasis depends on the complex interaction of clotting of blood plasma and aggregation of platelets through the release of clotting factors [20]. On contacting directly with foreign material, blood could activate the immune complement system and trigger the blood plasma coagulation cascade, resulting in harmful effects such as acute inflammatory reaction and thrombus formation. Present investigation, in invitro anticoagulant potential and the levels of coagulation factors were assayed in normal human plasma with APTT, PT and TT reagents. In humans, normal hemostasis depends on the complex interaction of clotting of blood plasma and aggregation of platelets through the release of clotting factors [21]. The APTT assay measures the activity of all coagulation factors in the intrinsic pathway. The TT assay screens fibrin polymerization process measuring the formation time of fibrin from fibrinogen after the addition of known amounts of thrombin to the plasma sample, and the PT assay measures the activity of the extrinsic pathway [21].

As seen in Table 1, *E. schistosa* venom has negligibly higher coagulation times from the control and the effects of the concentration of venom on the APTT, PT and TT values. These results suggested that the APTT, PT and TT of venom protein was effectively prolonged compared to

Fig. 5 Effect of coagulation activity of the venom protein from *E. schistosa* tested by human plasma. Fifty micro liters of normal citrated human plasma was incubated with 90 μ l of 20 mM HEPES, pH 7.4, at 37 $^{\circ}$ C with different concentration of venom, 2, 4, 6 and 8 μ g of protein for 5 min. After that Coagulation was triggered by adding 10 μ l of 150 mM CaCl_2 . In the control reaction no venom was added. Reactions (clot formation) were monitored in the SpectraMax system at 650 nm at 37 $^{\circ}$ C for 20 min. Each point represents mean $\pm$ SD of four independent experiments

control (saline solution) in a dose dependent manner. APTT, PT and TT of venom protein increased rapidly with the increase of concentration, and reached coagulation about 174.59 ± 0.60 , 334.57 ± 0.73 and 124.87 ± 0.29 s at 50 and 100 μ l of plasma, respectively. At the same time in APTT, PT and TT of control were only reached about 44.11 ± 0.48 , 33.66 ± 0.28 and 27.9 ± 0.35 s at 50 and 100 μ l of plasma. It is suggested that the highly sulfated chitosan mainly affected the intrinsic pathway, and has little effect on the assays reflecting extrinsic pathway. The results of the present study were similar on *Scanthophagus argus* venom also no shown any disturbance in the plasma clotting time [22]. But, similar to protease from *Vipera labetina* [23], *Naja naja* [24], *Naja melanolenca*, *Naja pallid*, *Crotalus argus* venom prolonged the coagulation of citrated plasma and shown the anticoagulant activity [25].

The results of the present study suggested that the anticoagulant activity significantly increases with increasing concentrations of *E. schistosa* venom protein and this could also be the reason for hemorrhagic activity at the site of envenomation. However, in other species, these activities appear to be represented by separate but closely related

proteolytic enzymes [26]. Indian venomous snakes *Echis carinatus* and *N. naja* take about 13 times less time for the clot formation, where as *Vipera russelli* crude venom takes about 2.23 times less time for clot formation, as their APTT and PT times was far less than the normal platelet poor human plasma. Therefore, one could make use of this venom protein for the treatment of coagulation disorders, thus proving their superior procoagulant efficacy over the existing commercial pharmaceutical preparations.

Hemolytic Activity

In the present study, the purified venom protein has showed the hemolytic activity was expressed as μ g/ml. Among the erythrocytes of goat, cow, chicken and human blood (A, B and O), the maximum haemolytic activity was found in human blood and the minimum activity was found in cow blood. These results suggested that the haemolytic activity displayed is most vulnerable to complete lysis provoked by the sea snake venom protein. This observation is in agreement with the findings reported for potent hemolytic and cytotoxic activity of purified crude venom from *Actina equine* and various sea anemones [27–29]. In the present study, haemolytic activity of purified venom protein showed maximum and minimum activity at 512 and 64 HU for human & cow blood, respectively. At the out set, the sea snake venom has indicated the most ability to damage the membrane of erythrocytes. Which was similar to those of venom from other snakes *S. verrucosa*, *S. horrida* and *S. trachynis*, which shows positive haemolytic activity [27]. Almost all sea water venomous fish and snakes venom possesses haemolytic activity. Therefore, this type of venom protein could play a vital role in pharmaceutical application in term of cytotoxicity.

Phospholipase A₂ Activity

Phospholipase A₂ activity was observed in different concentration of venom such as, 4300.00 & 1090 U/mg for 8 μ g, 3680.00 & 930 U/mg for 6 μ g, 3240.00 & 860 U/mg for 4 μ g and 2860.00 & 620 U/mg for (2 μ g), respectively (Fig. 6). The result suggested that the maximum amount of venom protein in phospholipase A₂ activity was recorded 4300.00 U/mg, which was similar to those found in other venoms, like the venom extract of *P. falkneri* and *D. guttata* [30] and the myotoxicity provoked by *Bothrops* spp. Since PLA₂ enzymes affect almost every vital organ or tissue, one could get ‘addresses’ to many types of cells and tissues. All myotoxic variants with phospholipase activity are anticoagulants in vitro, prolonging the time of recalcification of platelets poor plasma [31], thus suggesting that phospholipid hydrolysis is required for this effect, but there are controversies about anticoagulant action mechanisms

Table 1 Effect of sea snake venom on prolongation of APTT, PT and TT in human plasma in vitro

Compounds	APTT (s)	PT (s)	TT (s)
Control	44.11 ± 0.48	33.66 ± 0.28	27.9 ± 0.35
Venom sample	174.59 ± 0.60	334.57 ± 0.73	124.87 ± 0.29

induced by PLA₂ from snake venoms, as evaluated by Kini [1]. In this work we tested the possibility of monoclonal antibodies to be able to inhibit phospholipase activity interfering in anticoagulant activity. Because many of these venoms lack the complexity of viperid and elapid venoms and the single step isolation method presented here will allow rapid isolation of elapidae PLA₂s. It is clear that elapidae PLA₂s are homologous with those found in other venoms. Such a possibility should be provided a strong impetus for studying structure function relationships of PLA₂ enzymes and identification of more pharmacological sites.

Amino Acids Composition

The amino acid composition of venom protein from *E. schistosa* is shown in Table 2. In the results, the purified venom consist of nine different major amino acids, such as leucine, histidine, isoleucine, methionine, asparatic acid, valine, threonine, cysteine and phenylalanine were found and contributed 74.96 % of total amino acids (determined after hydrolysis with 6 N hydrochloric acid) residues. Among these, Leucine was major (13.59 %) amino acids and Serine is a lowest concentration (0.5 %). The individual concentration of glutamic acid, asparagine, serine, glutamine, glycine, arginine, alanine, tyrosine, lysine, proline and tryptophan were found to be less than 5 % to the total amino acids composition, which was similar to the other sources of snakes venom peptides were observed in the sea-snake and cobra toxin [32]. More than ten amino

acids are functionally important for neurotoxins such as Gln7, Ser8, Gln10, Lys27, Trp29, Asp31, Arg33, Ile36, Glu38 and Lys47. Among these, Lys27, Trp29, Asp31, Arg33, Glu38 and Lys47 are containing both in short chain and long chain a neurotoxins [33]. The basic amino acid residues, such as lysine and arginine may play important roles in blocking the acetylcholine receptor sites on the postsynaptic membrane. Among them glutamine and glycine are important neurotransmitters and the presence of these amino acids might play a role in the usage of the sea snakes envenomation.

¹H NMR Spectra

The characterization of purified venom protein of *E. schistosa* usually give ¹H-NMR spectra with sharp lines and very good signal dispersion that is readily amenable to resonance assignment and structural determination (Fig. 7). The ¹H NMR (400 MHz) results suggested that the compound contained nine protons and the relative responsible of chemical shifts (δ) were represented in Table 3. Even though, the H₂O signal continuously observed at 4.8 ppm. However, the 90 % of the isolated venom consisted of small molecules. The results of the present study suggested that the presence of various peaks were responsible for methylene and methyl groups of neurotoxin protein

Fig. 6 Phospholipase A2 activity (U/mg) of *E. schistosa* venom was used different micrograms (2, 4, 6 and 8 µg). The absorbance was determined at 740 nm using a spectrophotometer

Table 2 Amino acid composition of *E. schistosa* venom (residues per 1000 total amino acid residues)

S. no	Amino acid	1000/Residue
1	Asparatic acid (Asp)	87.44
2	Glutamic acid (Glu)	45.32
3	Asparagine (Asn)	27.32
4	Serine (Ser)	4.83
5	Glutamine (Glu)	19.34
6	Glycine (Gly)	49.47
7	Threonine (Thr)	66.65
8	Arginine(Arg)	10.5
9	Alanine (Ala)	18.57
10	Cysteine (Cys)	51.48
11	Tyrosine (Tyr)	21.69
12	Histidine (His)	101.96
13	Valine (Val)	67.89
14	Methionine (Met)	89.54
15	Iso-leucine (Ile)	97.96
16	Phenyl alanine (Phe)	50.85
17	Leucine (Leu)	135.87
18	Lysine (Lys)	15.67
19	Proline (Pro)	12.88
20	Tryptophan (Try)	24.77

(around 3.183–1.143 ppm). The ^1H NMR spectral signals at 3.183 (HC–OH and –OR), 2.350, 2.060 (HC–SR), and 1.147 (HC–C–(C=O)Ar) were represented for methyl group and the signals at 2.527 (HC–O–O) was assigned as a protons of methane. The signals at 2.484 (HC–C $\equiv$ N), 2.389 (HC–(C=O)R) and 1.164 (HC–C–CH₂) were confirmed as protons of methylene group and the final signal at 0.773 (NH₂) which responsible for the amino group. Previously it is reported that, the absence of five quaternary methyl signals at 0.82, 1.17, 1.19, 1.24 and 1.31 in the ^1H -NMR spectrum, fraction-1 is presumably non-terpenoidal in nature [34]. Furthermore, in ^1H -NMR spectral widths are quite narrow, so that this condition is easily fulfilled. Quantitative ^1H - as well as ^{13}C -NMR spectroscopy has been employed for the analysis of commercial antipyretic preparations containing aspirin, caffeine and phenacetin or ethenzamide [35], and complex mixture of the drug [36]. Núñez et al. [17] investigated that the anionic group in the molecule is probably not the only requirement for inhibition, since compound 2 presents a hydroxyl group, but did not inhibit PLA₂ activity. Thus, the present results of venom peptides well recognized PLA₂ activity and increase the anticoagulants activity. The NMR results showed that the direct NMR-spectroscopic analysis can be used to screen complex biological samples such as sea snake venom for the presence of small molecules.

Fig. 7 ^1H NMR (400 MHz), spectrum of *E. schistosa* venom

Table 3 ^1H NMR spectrum of venom peptides with proton chemical shifts

S. no	Chemical shift (ppm)	Structure	Types of proton
1	3.183	HG–OH and –OR	Methyl
2	2.527	HC–O–O	Methine
3	2.484	HC–C $\equiv$ N	Methylene
4	2.389	HC–(C=O)R	Methylene
5	2.350	HC–SR	Methyl
6	2.060	HC–SR	Methyl
7	1.164	HC–C–CH ₂	Methylene
8	1.147	HC–C–(C=O)Ar	Methyl
9	0.773	NH ₂	Amine

Conclusion

In conclusion, sea snake (*E. schistosa*) venom contains components that possess biological and biochemical potential of proteolytic, haemolytic, phospholipase activities and also pro-coagulant and functional relationships of anti-coagulant activities. Venom of sea snakes has an obvious place in the coagulation laboratories both for routine assay of coagulation factors and as reagents. Some of these routine applications have been adopted as the preferred option in clinical applications of snake venom materials.

which have been less successful although there has been a lack of well-designed clinical trials. Present study insists that furthering the research in identifying the target proteins (receptors) for venom phospholipase A₂ will help to unravel the molecular mechanism underlying the various pharmacological activities of these enzymes, which will be useful for the development of novel anti-coagulating lead compounds and drug delivery system and targeting a particular tissue or organs. Therefore, this novel venom protein molecule deserves further studies to elucidate their mechanisms of action and potential pharmacological applications impending use for therapeutic field.

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Conflict of interest The authors declare no conflict of interest.

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