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Characterisation and Biological Activity of Stingray Venom (*HimanturaImbricata*)

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ABSTRACT

Stingrays envenomation in humanswere the common accident in the marine and freshwater ecosystem. To determine such effect species Himantura imbricata have been used to elucidate Hemolytic activity, Plasma Coagulation, Fibrin coagulation, Fibrinolytic activity, Activated Partial Thromboplastin Time (APTT),and Prothrombin Time (PT) effects were studied. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Amino acid analysis by RP-HPLC, FTIR spectral analysis and SEM were carried for characterization studies. The results show the of plasma coagulation, fibrin coagulation, of this stingray venom delays the coagulation of citrated plasma. APTT and PT results showed intrinsic and extrinsic coagulation factors that were responsible in time delay when compared with the control. Moreover, these biological experiments and characterization studies aided in understanding the envenoming factors and these results might base the development of treatments for complex diseases.

INTRODUCTION

Stingrays are cartilagenous fishesthat are common in tropical and subtropical marine water throughout the world. These possess one or more barbs in the mid of their tail that act as stingers. These regions are covered with specialized cells, which secreteproteins that cause nociceptive, inflammatory, and necrotic effect (Silva Jr et al., 2015). Accidential contacts of humans with stingray barbs cause epithelial skin destruction and subsequent venom release. Stingrayenvenomation cause severe mechanical injury leading to pain, swelling and muscle cramps due to the venom proteins (Bedore et al., 2014).

Venoms are excellent resources for discovery of enumerous novel bioactive compounds. Currently, more than six molecules derived from different venoms have been approved for treatment of autoimmune diseases to neurological disorders (Chu et al., 2020). The diversed structural features and pharmacological properties of venoms i.e low molecular weight, solubulity, stability and selectivity are affirmed to be an attractive source of noval therapeutics (Fosgerau and Hoffmann 2015). Moreover, venom peptides often have high physico-chemical stability and bioavailability due to their 3D structural features and typically reinforced properties of disulfide bonds (Undheim et al., 2016). These characters of venomous molecules are highly desirable traits for the therapeutic leads. However, most of the venom groups are remains unstudied despite the venom's use for centuries in traditional medicines.

The stingraytoxins have been confirmed to be within the venom are cystatins, peroxiredoxin, and galectin (Bernardes-Oliveiraet al.,

2016).Galectincould cause cell death and cystatinscan inhibit defense enzyme activities. Stingray toxins would also enhance the blood flow in the superficial capillaries which leads to capillary damage(Dos Santos et al., 2017).

In recent years, researchers are targeting marine resources for the noval therapeutic lead molecules. The compounds from the marine fauna and flora are highly stable with diversedphysico-chemical properties. Besides, the research suggest that the marine venoms are unique source of therapeutic leads due to their biochemical compositions. Likewise, venomous fishes have also created fascination towards the biologist for the hunting of bioactive molecules. The venoms are able to provide an insight to the medication improvement for various diseases (de Oliveira Junior., 2016). Current investigations are reported that marine and freshwater stingrays venom have deservedplenty of bioactive compounds with therapeutic properties(rajeshkumar2015), enzymatic activities like phospholipase activity (Abir Ben Bacha2013),antiproliferative activity (rajeshkumar2015), enzyme isolation such as hyalournidase (Marta R. Magalhaes 2008) and other biochemical properties(monteirdossantos 2011).

Based on these background, this study was planned to portray the composition and perform chromatographic evaluation of *Himanturaimbricata* stingray venom. Also the study aim to examine the immunomodulatory and pharmacodynamicproperties of the venomextracted from stings of*Himanturaimbricata*. Thus, the study will give an insight on the stingray venom and their biomedical applications.

MATERIALS AND MEHODS:

2.1.Morphological analysis and Venom extraction:

The stingray, *Himantura imbricate* wascollected from Parangipettai coast, Tamilnadu, India with the aid of local fishermen.The Raw spine was collected and washed with double distillled water and the serration pattern was observed under the Scanning electron microscope.The spines were expelled from the base of the tail and transported to the laboratory under refrigerated condition. The tissue adhering to the stinger spine was scratched and the spines were homogenized in PBS buffer (pH 7.4). The homogenized mixture was centrifuged at 5000g for 10 min and the supernatant (comprising the venom from spine) was recovered. Then, it was stored at –20°C forfurther analysis. (Haddad Jr et al, 2004).

2.1 Quantification of protein:

Protein concentrationwas determined by the method of Lowry et al. (1951) using Bovine Serum Albumin (BSA) as a standard.5mg/ml of venom (lyophilized powder) stored at -20°C was used for protein estimation.

2.2 Molecular weight determination by SDS- PAGE Electrophoresis:

The protein molecular mass ratio was determined through Sodium Dodecyl Sulfate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using 12% Resolving gel and 5% Stacking gel. Stingray venom sample (150µg) was diluted with sample buffer (50mM Tris P^H- 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.04% bromophenol blue) in the 1:1 ratio. Then, the mixture was boiled at 100°C for 2 min and vortexed for 30s before loaded onto the gel. After running the gel electrophoresis, the gel was stained with 0.3% Coommassive Brilliant Blue R-150 solution with methanol followed by destaining process with destaining solution (30% methanol , 10% Acetic acid and water) for visualizing the protein bands. The venom protein sample was compared with molecular size marker(29-200KDa – Cell signalingTechnology, danvar, US) to determine the molecular weight(Monteiro-dos-Santos, J.,2011).

2.3 Amino Acid Analysis by RP-HPLC method:

The reverse phase High Performance Liquid Chromatography (Merck-Hitachi Lachrome D-7000 HPLC System, Germany) system was used for analysingvenom protein. The crude venom sample was hydrolysed in 6N HCL at 110°C for 6h in sealed vaccum ampoules.The resulting sample was then loaded to RP-high-performance liquid chromatography (HPLC) on a C18 column (250 × 4.6 mm, 5 mm;SHIMADZU) prequilibrated with 0.1% TFA in water and then eluted at a flow rate of 1 ml/min with an acetonitrile linear gradient 0–100% over 60 min.The amino acids were identified and quantified by RP-HPLC by comparing their respective retention time and peak areas with the standard amino acid mixture. The amount of amino acid content was expressed as the number of residues per 1000 residues.

2.4 Infrared spectral analysis:

The sample spectral range was recorded by Infrared Spectra at room temperature using a spectrum two model (Perkin Elmar) in the spectral range 400-4000cm^{-1} via a frequency absorption of the (50µl) sample was taken for determining the functional groups present in venom sample. (matias 2017).

2.5 Biological activities:

1.5.1. Haemolytic activity:

The haemolytic assay of the crude venom was evaluated by the microtitre plate method. Two blood samples (Cow & Chicken) from slaughter house and human blood sample (volunteer) from Tamilnadu government hospital in Parangipettai were collected in EDTA coated tubes. After collection, the blood was centrifuged at 6,000rpm for 5min. The pellet was washed with normal saline (pH-7.4) and supernatant was discarded. The erythrocytes obtained was washed with saline for three times and stored at -20°C. The haemolytic assay was performed in 96 well plate.100 µl of crude venom was mixed with 100 µl of 1% RBC. 1% RBC was taken aspositive control. 100ul of saline was taken asnegative control. The 96 wellplate with samples was maintained for 3hrs at room temperatue and the results were noted. Highest haemolytic dilution taken as Haemolytic Unit(HU) (Chen D et a., 1997).

1.5.2. Plasma Coagulation assay:

The effect of stingray venom on coagulation was assessed through the recalcification time measuredusingthe SpectraMax® Plus 384 microplate reader. This methodology allows to monitor clot formation and kinetic parameters for coagulation through minimalized plate per user. In a final volume of 150µl, 50µl of citrated human plasma was incubated with various concentrations of stingray venom (2, 4, 6 and 8 µg) in 90 µl of 20 mMHEPES, pH 7.4. After incubated for 5 min at 37°C, 10 µlof 150 mM$CaCl_2$ was added and clot formation was observed at 37°C for 20 min, in the SpectraMax system at 650 nm. To get to calcium free coagulation action, EDTA was added rather than $CaCl_2$ to a last grouping of 10 mm. The experiments were rehearsed four times toconfirm every observation (Rajeshkumaret al.,2011).

1.5.3. Activated Partial Thromboplastin Time (APTT):

Blood Clotting time (BCT) and Thrombin clotting time (TCT) evaluation of Activated Partial Thromboplastin time(APTT), Prothromin time (PT) and thrombin time (TT) was evaluated in plasma samples of human blood. PT, TT and APTT analysis were identified using commercial kits(AGAPPE, Kerala, India.These tests were monitored for functionally extrinsic and intrinsic pathways respectively. All assays were tested using citrated human plasma from a healthy donor. Result on APTT test was followed by the method of proctor Rapaport using a Liquicelin E (activated Cephaloplastin reagent) (AGAPPE, Kerala, India) as indicated by the manufacturer, using the ELIZA reader kinetic module. Briefly, 50 µl of citrated normal human plasma and 50µlof purified venom protein were mixed inside the plate wells and incubated at 37°C for 3 min. 50µlof APTT assay reagent (Cephaloplastin) was added, and the reaction mixture was incubated for another 3 min. After incubating at 37 °C and shaking for 3 min, 50µlof pre-warmed (37°C) 50 mM $CaCl_2$ was added by using a micropipette. After the addition of the $CaCl_2$, the plate was mixed once and then maintained at 37°C using the apparatus mixer and heating system. Then,absorbance readings at 650 nm wastaken at 10 s intervals. indicated clotting. We chose the time 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.

1.5.4. Effect on Prothrombin Time (PT) and Thrombin Time (TT):

A mixture of tissue thromoplastin from rabbit brain, calcium ions and buffer soutions was used for the evaluation of prothrombin time. Briefly, 50 µl of citrated normal human plasma was incubated in the presence of 100 µl of diagnosis thrombo reagent (rabbit brain thromboplastin reagent) for 2-3min at 37°C. Thrombo reagent was added into the test tube containing 100 µl of plasma and 50 µl of purified E Stingray venom protein. After incubating the mixture for exactly 3 min,100 ml of 25 mM $CaCl_2$ was added to induce coagulation. Buffer solution was used as control in this assay. We chose the time taken for reaching 0.1 absorbance value (onset absorbance) as a measure of time to clot formation. The experiments were carried out twice to confirm the observation. For TT assay, citrated normal human plasma (90 µl) was mixed with a solution of E. Stingray venom (10 µl) was incubated for 2 min. Then, TT assay reagent

(100µl, 5 U/ml) pre-incubated at 37°C for 5 min was added and clotting time was recorded. All samples were prepared in saline solution(Damodharan 2015).

2.6.5.Plasma coagulation activity

Plasma coagulation activity of *H.imbricata* venom and effect of *H.imbricata*venom upon coagulation of human plasma. 50 µl of normal citrated human plasma were incubated with µl of 20 mM HEPES, pH 7.4 at 37°C with different concentration of venom [2(Hi1),4 (Hi2), 6(Hi3),8 (Hi4)] for 5 min. after that coagulation was triggered by adding 10 µl of 10 Mm CaCl2. In the control reaction venom was not added. Reactions (clot formation) were monitored in the SpectraMax Plus system at 650 nm at 37°Cfor 20min.

2.6.6. Fibrinocoagulation assay

Fibrinocoagulation assay was performed based on the method of Shacter et al.(1995). The formation of thrombin catalyzed fibrin clot was monitored by using SpectraMax(Molecular devices) microplate reader. Bovine fibrinogen solution (40µl) at the concentration of 5 mg/ml was incubated in 10 mMTris buffer, pH 7.4,for 5min, with different concentration of venom (4,6 and 8 µg of protein) in a final volume of 90µl in 20 mM HEPES, pH 7.4. After 4 h at 37°C, 10 µl of thrombin solution (0.02 mg/ml) was added and the rate of fibrinogen coagulation was monitored at 650 nm for 20 min. The assay were performed four timed to confirm every observation.

2.6.7. Fibinolytic activity

The fibinolytic activity of the stingray venom was analyzed in a fibrin plate assay by the method followed by Veiga et al (2003). Fibrin plate prepared by a solution containing 25 mg of fibrinogen dissolved in 10 mMTris buffer, pH 7.4 was laid over agarose plates immediately after the addition of 10 µg of thrombin. The plates were incubated at 37°C for 2 h. Then 20 µl of H.imbricata venom were laid over the fibrin plate made already. The same volume of water and plasmin (1 mU) were used as negative and positive controls, respectively. Plates were incubated at 37 °C for 24h and stained with Coomassive Blue. The formation of clear spots in the plate indicated fibrinolytic activity.

RESULTS AND DISCUSSION:

3.1. Morphological structure of stingray spine:

Scanning electron microscope revealed the spine structure of the stingray H. imbricate (Fig 1). It consisited of two spines and it confirmed that the spine does not have any injectory system. The fish possess serrated structure like spine. Totally 30 to 35 small arrow shaped and serrated spines were arranged literally in both the left and right sides.The serrated spine distance for one sting to another sting is 0.6mm.SEM observation revealed that the breadth of the spine tip ranged from 50 to 200um. The barbs of the spine were enveloped by epithelial tissues and the breadth varied between 50um to 180um. The middle portion of the spine was hard and thick under SEM analysis. Similar phenomenon in their study was observed with the spines having ventral grooves with glandular tissue, enveloped by the sheath(Kalidasan et al., 2014). During the stinging act, this sheath breaks and the venom is mechanically expressed in the wound. Further, the serrated margins of the spiny barb breaks the sheath widening the wound and breaks apart from the spine.

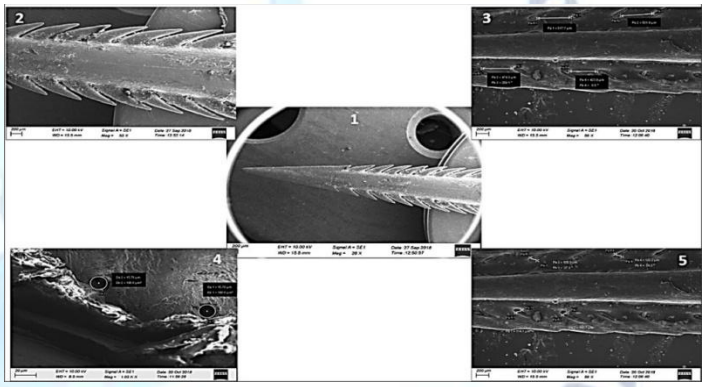

Figure 1: SEM images of Stingray – 1)Serrated spine with barb; 2)venom producing Spine covered with mineral content 3)single barb sheath width (200 µm) view of one barb to another barb distance; 4)inner view of single barb depth ; 5)Cross view of spine barb

3.2. Protein Estimation:

The amount of crude protein was estimated by lowry method with Bovine serum albumin as standard. The concentration of venom protein was found to be 658 µg/ml. Further the chromatographic technique of High performance liquid chromatographic was used for purifying the proteins and stored for further analysis.

3.3. SDS-PAGE Electrophoresis:

The electrophoretic profile of *H. imbricata* venom protein showed four prominent bands at the molecular range of 19 to 66kDa (Fig 3). In a similar study, SDS-PAGE profiling of Brazilian stingray, *Potamotrygon cf. henlei*venom protein showed

intense bands with approximately 70 kDa, around 40 and 50 kDa, and one last band presented approximately 15 kDa and also at 30-35 kDa (Monteiro-dos-Santos et al., 2011). In addition, 12% SDS-PAGE gel analysis presented a single band with a molecular weight of approximately 16 kDa in *P. cf. henlei*venom extract (Conceicao et al., 2012).Similary, 14-25kDa proteins were observed from the venom of *A. membranaceus*which exhibited good anti-cancer activity respectively (Sivaramakrishnan et al, 2019).

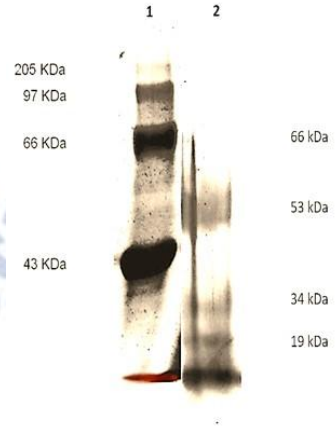

Fig 2: SDS-PAGE electrophoretic profile of Stingray Venom protein

3.4. FTIR analysis:

The functional groups of venom protein were determined through the FTIR analysis (fig 3). Table 1 depicts the characteristic functional groups present in the venom. The amide I band stretching at 1661.84 cm^{-1} attributed to different secondary structures as mentioned in previous literatures [Laustsen, A et al.,2015]. The β-sheet vibrations at the range of 1640 and 1620 cm^{-1} was not observed, whereas peaks between 1650 and 1660 cm^{-1} were basically assigned to α-helical absorption. Similar observations were made in the venom extract of *H. gerrardi*, *H. imbricata* and *Pastinachussephen* stingray spine (Uthaya Siva et al., 2013).

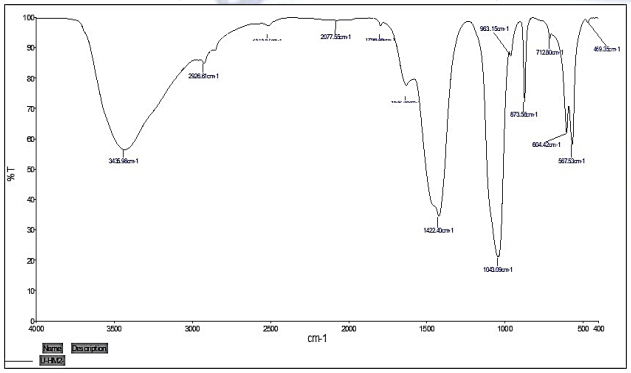

Figure3: FTIR analysis for stingray venom protein

Table:1FTIR spectral data for stingray venom protein

Sr. No.	Wavenumber (cm^{-1})	Functional group	Vibration mode
1.	3400.97	NH	Asymmetric stretching secondary
2.	2949.98	CH_3	Asymmetric stretching
3.	2888.41	CH_2	symmetric stretching
4.	2527.49	CH_3	Asymmetric stretching
5.	1661.84	C=N	Amide-I stretching
6.	1453.82	O-CH_2	Scissors
7.	1412.82	O-CH_2	Twist out of the plane
8.	1113.48	CH	bending in the plane
9.	1052.40	PO_4^{-2}	symmetric stretching
10.	1030.58	CH	bending in the plane
11.	667.28	NH	out of plane deformation of Protein

3.5. Amino acid analysis by RP-HPLC:

In the present study, 21 amino acid standard residues and venom sample residues were investigated.In the amino acid composition of *H. imbricata* venom, lysine and phenyl alanine were observed as the major residues. However, among the 21 amino acids analyzed, all of them were present in the venom sample. Low molecular weight 'Lysine' has been found to increase bleeding time as it highly interacts with certain anticoagulants (Muralidharan-Chari et al., 2017). It has been reported that glycine and glutamine are important neurotransmitters where they have the ability to be both excitatory (stimulate brain and nervous system activity) and inhibitory (silence the activity) in our system (Hernandes and Troncone, 2009).

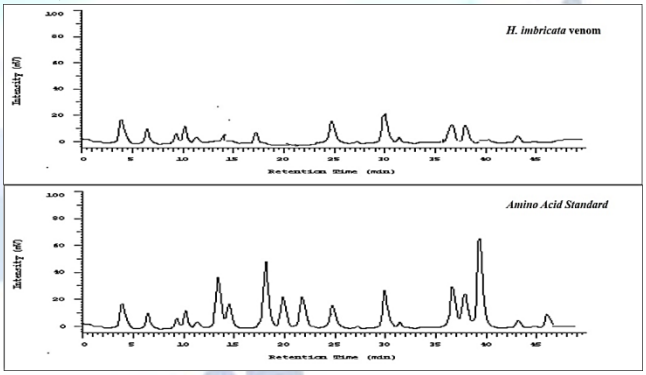

Figure 4: Acmino acid content analysis for stingray venom protein

Table:2 Amino acid analysis data for stingray venom protein

Sr. No.	Amino Acid	1000/Residues
1.	Aspartic acid	13.27
2.	Glutamic acid	4.44
3.	Asparagine	3.52
4.	Glutamine	9.75
5.	Serine	378.82
6.	Glycine	22.30

7.	Threonine	17.82
8.	Arginine	24.00
9.	Alanine	18.58
10.	Cysteine	3.52
11.	Tyrosine	38.46
12.	Histidine	2.22
13.	Valine	3.82
14.	Methionine	34.99
15.	Iso-leucine	40.72
16.	Phenyl alanine	38.12
17.	Leucine	337.76
18.	Lysine	44.14
19.	Proline	4.21
20.	Tryptophan	5.00
21.	Taurine	2.22

3.6. Coagulation Assays:

3.6.1. Haemolytic activity assay:

This Himanturaimricata venom assay was performed on two different blood (chicken, and human) and followed by serial dilutuion.The venom showsmaximum activity in human erythrocytes(10^{-7})and minimum activity in chicken erythrocytes(10^{6})as shown in fig4.The haemolytic activity of these venom are comparatively significant with earlier findings (Veeruraj, A et al.,2008). The specific haemolytic activity of stingray venom on , Chicken and Human RBC's was found to be 12.23 & 31.63 HT/mg respectively.As per report of Damotharan et al., 2016, a hemolytic activity of 51 HU was determined for sea snake venom. It is evident that all sea water venomous fishes and snakes venom possesses haemolytic activity. Hence, such venomous protein could be utilized in pharmaceutical industries in term of cytotoxicity.

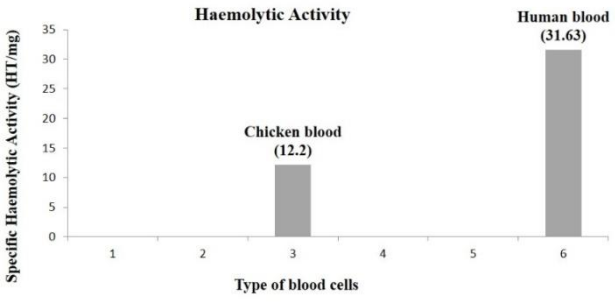

Figure 5: Graphical representation of Hemolytic activity of Stingray

3.6.2. APTT and PT determination:

The anticoagulant property of the venom was determined through the APTT and PT assays (Fig or Table 5). The APTT assay measures the activity of all coagulation factors in the intrinsic pathway and the PT assay measures the activity of extrinsic pathway. In the APTT assay, the control showed a clotting time of 2 min 40 s but the *H. imbricata*venom delayed the coagulation time for 3 min 25 s. In the PT determination assay, the control coagulated the plasma within 16 sec, whereas the venom sample reaction took 22 sec to clot. In the APTT and PT assay of sea snake venom, the clotting time was extended to 44.11 ± 0.48 and 33.66 ± 0.28 s (Damotharan et al., 2016). 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 preparation.

Sample	Concentration(µg)	APTT(s)	PT(s)
Control(PBS)	-	104	-
Stingray Venom	2	158	16
	4	205	19
	6	>300	128
Heparin	2	135	11
	4	>300	39

Table:3 APTT and PT determination for stingray venom protein

3.6.3. Plasma coagulation assay:

The blood coagulation activity of venom samples was assessed by the plasma coagulation assay. To investigate the effect of venom samples upon clotting, citrated chicken plasma was incubated with venom samples prior to the induction of coagulation by calcium. From fig 6, H.imbricata venom sample showing the clotting time of human plasma is clear. At the same time, control plasma and venom samples take more than 5min to initiate coagulation.After the addition of calcium, plasma incubated with venom sample procured around 190sec to start coagulation. The venom protein sample showed increase in Recalcification Time (RT) by almost three times, as compared with control (Fig. 6). The normal plasma was unable to clot in the absence of calcium chloride and also in the presence of EDTA along with venom protein. Thus, we may infer that stingray venom have good interference with the blood coagulation cascade. This result was in conglomeration with the RT study of *Enhydrinaschistosa* (sea snake) venom, where the sample increased the RT by three times in comparison with the control (Damotharan et al., 2016). Normal blood coagulation cascade is a tightly regulated pathway operated by the combined action of both extrinsic and intrinsic pathways. These pathways converge at factor X activation and become a common cascade in generating fibrin clots. A similar concept was also explained in the catfish venom studies conducted by Abirami et al., 2014.

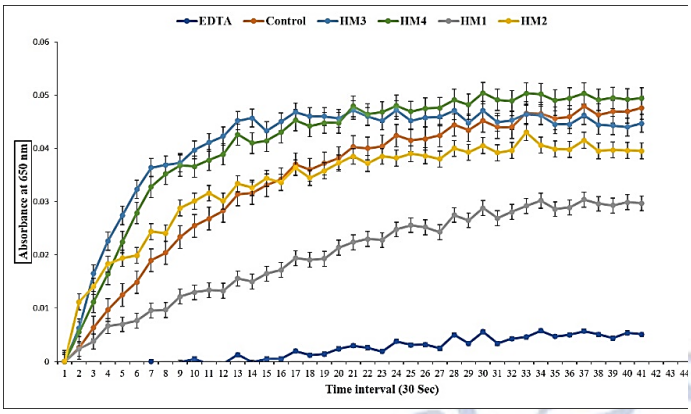

Figure 6: Plasma coagulation assay for stingray venom protein

3.6.4 FIBRINOCOAGULATION:

In order to analyze whether the delaying in coagulation was due to the effect of venom on fibrinogen present in the plasma, the thrombin catalyzed fibrinocoagulation assay was performed. In this assay, fibrinogen was incubated with venom prior to the addition of thrombin and the formation of the fibrin clot was monitored at 650 nm. In the reactions fibrinogen incubated with H.imbricata venom, led to the formation of less amount of fibrin clot formed was decreasing with increasing concentration of venom.

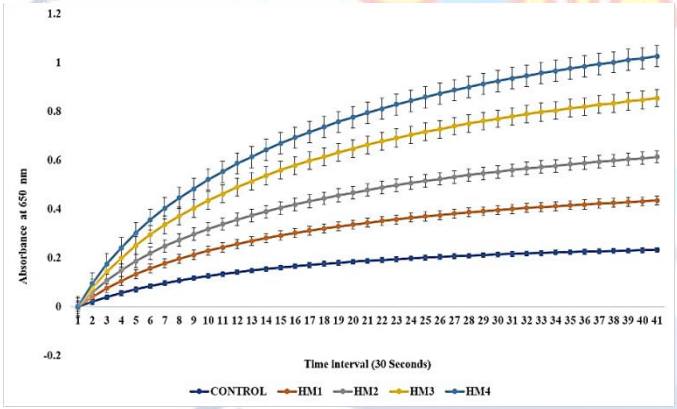

Figure 7:Fibrinocoagulation assay for stingray venom protein

3.6.5 Fibrinolytic Activity:

Fibrinolytic activity of *H.imbricata*venom. Fibrinogen (2 mg/ml, Tris Buffer, pH 7.4) was mixed with 10 µl of thrombin, prior to the clot formation the mixture was laid over the agarose plate and allowed to incubate for 2 h at 37° C. same volume of water and plasmin solution (1 mU) used as negative and positive controls, respectively the plate was stained with Coomassie Blue.

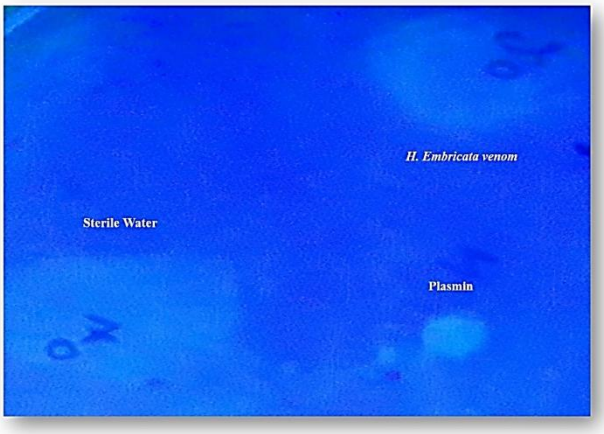

Figure 8: Fibrinollytic assay for stingray venom protein

Conclusion:

Venomous animals have evolved as a vast array of toxins for prey capture and defense. These toxins are directed towards the research of pharmacological targets such as neuromuscular, cardiovascular, hematological and cytotoxic properties. the present study contributes to the biological study of venom in the coagulation pathways and extracellular matrix components through various enzyme activities. Anticoagulation property of the venom was determined through the APTT and PT assay. Our results indicate that the intrinsic and extrinsic pathway observed could be triggered by venom. It could be inferred from the present study that *H.imbricata* venom possesses a diverse mixture of peptides, enzymes and pharmacological active components. These properties could be targeted for the treatment of wide variety of life threatening human diseases. However, further studies are required to isolate and find the mechanism of action of these molecules in the venom.

Acknowledgments

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