MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection of plant samples

The plant materials were collected from the Tezpur University campus (26.7010° N, 92.8315° E), Napaam, Sonitpur (26.6739° N, 92.8577° E), Sivasagar (27.0031° N, 94.6450° E), Tinsukia (27.5291° N, 95.6458° E) and Dibrugarh (27.4096° N, 95.1015° E) districts of Assam, India. Based on the traditional knowledge of the local ethnic communities of these places, various parts, e.g. leaves, fruits, roots or barks of plants that are used for treating hypertension, and against cardiac ailments as cardiac tonic were collected and washed under tap water until these are clean and then dried in shade. The dried parts were then finely grounded into fine powder using a motor grinder (Panasonic, India) and stored in air-tight bottles at room temperature (23 ± 2 °C) for further use.

3.1.2 Chemicals

All chromatographic matrices such as DEAE- Cellulose, CM- Cellulose and Sephadex G-50 were purchased from Pharmacia Fine Chemicals, Sweden. Prepacked chromatographic column Hi Prep^{TM} anion- exchange 16/10 column (16 mm x 100 mm) was purchased from GE Health Care, Sweden. Pre-stained protein ladder [PageRuler prestained protein ladder, #26616 (10-170 kDa) or #26619 (10-250 kDa)] was obtained from Thermo-Pierce Fermentas. Chromogenic substrates F3301, T1637, V0882, B2133, V3133, and 10898, and group-specific reagents PMSF, TPCK, TLCK, pBPB, IAA, DTT, metal chelator EDTA were purchased from Sigma-Aldrich, USA. Extracellular matrix (ECM) proteins type-IV collagen, laminin and fibronectin were also procured from Sigma Aldrich, USA; reagents for buffer preparation such as Tris-buffer, PBS etc; reagents for SDS-PAGE analysis such as acrylamide, bis- acrylamide; solvents such as acetonitrile, ethanol, acetic acid and methanol; and all other chemicals and reagents were of analytical grade and purchased from Merck, Germany or HiMedia, Mumbai, India. β-sitosterol from natural soybean and ƙ- carrageenan were purchased from Sigma Aldrich, USA.

Coagulation proteins such as human fibrinogen, human thrombin, factor X, factor Xa, prothrombin, plasminogen, and antithrombin-III were purchased from Sigma-Aldrich, USA. Thromboplastin reagent for prothrombin time test (Liquiplastin®) and cephaloplastin reagent for activated partial prothrombin time test (aPTT) (Liquicelin-E) were obtained from Tulip Diagnostics, India. All diagnostic kits used for analysis of biochemical parameters of plasma such as total protein, glucose, uric acid, creatinine, urea, ALP, CPK, SGOT and SGPT were purchased from Diatek Healthcare Pvt. Ltd., Kolkata, India. Cholesterol assay kit was purchased from Sirus Biocare Pvt. Ltd., Kolkata. Fibrinogen assay kit (FIBRO-TEK) was procured from r2 diagnostic, India.

Commercial anticoagulants warfarin, heparin, argatroban were purchased from Sigma-Aldrich, USA. Thombolytic drugs such as nattokinase were purchased from Healthy Origins, Pittsburg, USA, where as streptokinase and plasmin were obtained from Sigma-Aldrich, USA. Antiplatelet drug Aspirin was obtained from Sigma-Aldrich, USA

3.2 Methods

3.2.1 Preparation of extracts of selected ethno-medicinal plants of Assam and screening for anticoagulant activity

Fresh/dried leaves, flowers, whole fruit, seedless fruit, and seeds were washed thoroughly in tap water and several times in double-distilled water, cut into small pieces, and then ground with a commercial grinder (MX-AC400, Panasonic, India). 100 grams of the dried powder was extracted into 250 ml of different solvents, double-distilled water (type-II) (arium® advance EDI water purification system, Sartoriuous); methanol; ethanol; 50 (1:1) water; 50% ethanol in water; 25% ethanol in water; 12.5% ethanol in water. The extracts were first filtered through muslin cloth and then through 0.45 µM pore-sized filters paper (Whatman, USA). The filtrate were centrifuged (Multifuge X1R, Thermo scientific) at 10,000 rpm for 10 min at 4 °C. The supernatant was collected, dried under vacuum in a rotary evaporator (RV-10, IKA, Germany), weighed, and stored at 4 $^{\circ}$C until further use. Plasma re-calcification time and fibrinogenolytic of crude aqueous extract were determined as described in sections 3.2.8.1, and 3.2.7.4.1, respectively. However, the *Leucas indica* was not initially considered for investigation; however, studies from our laboratory have discovered the presence of anticoagulant activity in the aqueous leaf extracts of this plant and it was

considered for the screening. The plants showing best anticoagulant activity were selected for further studies.

Table 3.1. Code used of the different plant extracts.

Code used	Plant name	Vernacular name	Part used
LI	*Leucas indica*	Dorun	Leaf
CA	*Centella asiatica*	Bor manimuni	Leaf
SI	*Solanum indicum*	Bhi-tita	Fruit
MC	*Momordica charantia*	karela	Fruit
HR	*Hibiscus rosasinensis*	Jaba phool	Flower
AS	*Allivum sativum*	Naharu	Clove

3.2.2 Identification of selected plant species

Aqueous shade dried leaves of *L. indica* and whole fruit extract of *M. charantia* showing best activities were selected for our further studies. The identity of *L. indica* and *M. charantia* plants were confirmed by Botanical Survey of India (BSI), Shillong, Meghalaya and a voucher specimen were deposited (Accession number: 37604 and 12309 for *L. indica* and *M. charantia* respectively).

3.2.3 Optimization of extraction conditions of *L. indica* and *M. charantia*

3.2.3.1 Optimization of stirring time of extraction

100 g of *L. indica* shade dried leaves/whole fruit of *M. charantia* was homogenized in a blender during 10 min and the extraction was carried in double distilled water for 1, 2, 4, 6, 8, and 12 h at 4 $^{\circ}$C. The extracts were then prepared as stated in section 3.2.1

3.2.3.2 Optimization of pH of water

The pH of the double distilled water was adjusted to 6.5, 7.0, 7.4 and 8.0 by adding 0.1N HCl or 0.01 N NaOH and the extraction was carried out for 4 and 1 h for shade dried leaves of *L. indica* and whole fruit of *M. charantia* at 4 $^{\circ}$C. The extracts were then prepared as described in section 3.2.1

3.2.3.3 Optimization of temperature for extraction

The extraction was carried out for 4h and 1 h for shade dried leaves of *L. indica* and whole fruit of *M. charantia*, respectively, in double distilled water adjusted to pH 7.4at two different temperatures [4ºC and room temperature (23 ± 2ºC)]. The extracts were then prepared as stated in section 3.2.1.

3.2.3.4 Optimization of sonication time for extraction

The extraction was carried out for 4h and 1 h for shade dried leaves of *L. indica* and whole fruit of *M. charantia*, respectively, in double distilled water adjusted to pH 7.4at room temperature (23 ± 2ºC). The extracts were then sonicated for 5, 10 and 15 min and extract preparation is described in detail in section 3.2.1.

3.2.4 Preparation of anticoagulant active fraction from *L. indica*

The dried extract (25.0 mg dry weight) was dissolved in 500 µl of 20 mM potassium phosphate buffer pH 7.4, and centrifuged (Multifuge X1R, Thermo scientific) at 10,000 rpm for 10 min at 4 °C to settle the undissolved particles, if any. The clear supernatant was filtered through 0.2 µM syringe filter (Minigen nylon syringe filter, Genetix Biotech Asia Pvt. Ltd.) and then loaded on a Hi Prep^{TM} anion- exchange column (pre-equilibrated with the above buffer) attached to a Fast Protein Liquid Chromatography (FPLC) system (AKTA purifier 10, Wipro-GE Healthcare Biosciences, Upsala, Sweden). After washing the unbound as well as non-specifically bound fractions with two volumes of equilibration buffer, the bound fractions were eluted using 0.1 -1.0 M NaCl gradient at a flow rate of 1 ml/min at 4 °C. Two ml fraction was collected and elution of fraction was measured at 280 nm. The protein content, fibrinogenolytic activity, and plasma re-calcification time of the fractions were estimated as described in sections 3.2.7.2, 3.2.7.4.1 and 3.2.8.1, respectively.

The fractions showing the significant anticoagulant as well as fibrinogenolytic activity were pooled, concentrated using speed vacuum system (Centrivap concentrator, Labconco) and then fractionated through CM-Cellulose cation-exchange column (20 mm X 60 mm) pre-equilibrated 20 mM potassium phosphate buffer, pH 7.4. Fractions were eluted using 0.1M -1.0 M NaCl gradient at a flow rate of 0.5 ml/min. Fraction elution was monitored at 280 nm and 2.0 ml fraction was collected The protein content,

fibrinogenolytic activity and plasma re-calcification time of all the fractions were determined as described in sections 3.2.7.2, 3.2.7.4.1 and 3.2.8.1, respectively.

The fractions showing the highest anticoagulant as well as fibrinogenolytic activity were pooled, lyophilized and dissolved in 100 µl of 20 mM sodium phosphate buffer, pH 7.4. The solution was filtered through 0.2 µm syringe filter and fractionated on Shodex KW-803 gel filtration column (5 µm, 8 x 300 mm) pre-equilibrated with the same buffer. Fractionation was carried out with equilibration buffer containing 150 mM NaCl at a flow rate of 0.5 ml/min at 4 °C in a UHPLC system (Dionex Ultimate Mate 3000 RSLC, Dreieich, Germany). Fraction elution was monitored at 280 nm and 1.0 ml fraction was collected. The protein content of the peak showing highest anticoagulant and fibrinogenolytic activity was determined and it was selected for further study.

3.2.5 Preparation of active anticoagulant fraction of *M. charantia*

The crude extract showing the highest anticoagulant activity was processed to prepare an active anticoagulant preparation. For this purpose, the crude aqueous unripe whole fruit extract was prepared by stirring 100 g of the dried power from the whole fruit extract in 250 ml of deionized water for 1 h at room temperature (23 ± 2ºC). This was followed by sonication of the mixture (Labsonic® M, Sartorius) for 10 min, with 1 min intervals, at room temperature (23 ± 2ºC). The mixture was then filtered through muslin cloth and by Whatman No. 1 filter paper (0.45 µm). The filtrate was mixed with rice husks (previously winnowed and washed several times with lukewarm water and dried at room temperature) at a 2:5 ratio (100 ml of the supernatant was mixed with 250 g of rice husks) and stirred for 1 h at room temperature. As a control, the rice husks were stirred with water under identical conditions. The mixture was then filtered through a muslin cloth, and the filtrate was collected and named "flow-through". The rice husks were then stirred with 25 ml of deionized water, and then filtered through Whatman No. 1 filter paper. The filtrate was collected and named the "active anticoagulant fraction" (AAF). The AAF was vacuum-dried, weighed, and stored at 4ºC for further use. The protein content, fibrinogenolytic activity, and plasma re-calcification time of the fractions were estimated as described in sections 3.2.7.2, 3.2.7.4.1 and 3.2.8.1, respectively.

3.2.6 Biophysical characterization

3.2.6.1 Purity of preparation and molecular mass determination

3.2.6.1.1 SDS-PAGE analysis

The homogeneity and molecular mass of the isolated fraction/protein (10-50 µg) was determined by SDS-PAGE analysis with or without reduction of protein by β-mercaptoethanol. Preparation and casting of polyacrylamide gels (12% or 15%) were done as described by Laemmli [1]. The composition of 10%, 12% and 15% resolving gels with their 4% stacking gel are shown in Table 3.2 where as the composition of running buffer and 3X loading dye is shown in Table 3.3 and Table 3.4, respectively. The buffers and loading dye were prepared freshly for SDS-PAGE analysis. For analyzing the samples under reducing conditions, 5.0 µl of the loading dye containing β-mercaptoethanol (30.0 µl $ml^{-1}$of loading dye) and DTT (2.0 mM) was added and boiled at 100 °C for 5 min prior to loading in the gel. Electrophoresis was carried out at a constant current of 15 mA until the dye front reached the bottom of the gel. Protein bands were visualized either by staining with Coomassie Brilliant Blue R250 or by silver staining [2]. For staining with Coomassie Brilliant Blue R-250, gels were incubated (overnight) in Coomassie Brilliant Blue R-250stain dissolved in methanol:acetic acid: distilled water (4:1:5, v/v/v). The gels were destained using destaining solution (distilled water containing 40% methanol (v/v) and 10% acetic acid (v/v), until the background of the gels were free from stain.

For silver staining, the method described by Chevallet et al. [3] was followed. Briefly, the gels were fixed for at least 30 min in fixing solution (40% methanol (v/v), 10% acetic acid (v/v) in ultrapure type-III water (arium® advance EDI water purification system, Sartoriuous). The gels were subjected to silver staining (solution containing 0.2% $AgNO_3$ (w/v) in ultrapure type-III water) after sensitizing the gel using 0.02% $Na_2S_2O_3$ (w/v) in ultrapure type-III water. After 25 min of staining, the gels were developed (till bands were clearly visible) with developing solution (3% Na_2CO_3 (w/v), 0.02% $Na_2S_2O_3$ (w/v), 0.02% 0.5 µl ml^{-1}formaldehyde in ultrapure type-III water). Further development of the gel was stopped by adding 1.4% EDTA (w/v) solution.

For determination of molecular mass by SDS-PAGE analysis, the mobility of the protein under study was compared with the protein molecular standards where a linear

dependency of log molecular weight Vs. migration of protein bands (*Rf* value) was observed.

Table 3.2. Composition of Tris-glycine SDS-PAGE

Composition	**Stacking gel (5 ml)**	**Resolving gel (10 ml)**		
	4%	**10%**	**12%**	**15%**
30% Polyacrylamide(ml)	0.6	3.34	4.0	5.0
0.5 M Tris-HCl, pH 6.8 (ml)	2.5	-	-	-
1.5 M Tris-HCL, pH 8.8(ml)	-	2.5	2.5	2.5
10% APS(ml)	0.1	0.1	0.1	0.1
10% SDS(ml)	0.1	0.1	0.1	0.1
Distill water(ml)	1.7	4.0	3.3	2.3
TEMED(ml)	0.01	0.01	0.01	0.01

Table 3.3. Composition of running buffer

Composition	**(g l^{-1})**
Tris-Cl	15.1
Glycine	72.0
SDS	5.0

Table 3.4. Composition of 3X loading dye

Composition	**10 ml**
Bromophenol blue	10 µl
SDS	0.8 gm
Glycerol	4 ml
0.5 M Tris -Cl	6 ml

3.2.6.1.2 MALDI-TOF-MS analysis

The molecular mass of the purified protein was determined by MALDI-TOF-TOF mass spectrometric analysis (MALDI ToF/ToF Analyzer, 4800 Plus MDS SCIEX, Applied Biosystems) [4,5]. Briefly, 0.5 µl of the purified protein (~ 1.0-5.0 µg) in 0.1% TFA was mixed with 0.5 µl of a-cyano-4 hydroxycinnamic acid matrix (10 mg l^{-1}) or sinapinic acid matrix (10 mg l^{-1}) dissolved in 50% (v/v) acetonitrile containing 0.1 % (v/v) TFA. The mixtures were then spotted onto an Opti-TOF-384 plate (ABSciex), dried, and analyzed in positive linear mode using an acceleration voltage of 25 kV and laser intensity of 3000. Spectra were acquired in low, mid and high molecular rn/z range of 800 - 3,000 Da, 3,000-20,000 Da and 20,000-180,000 Da, respectively [4].

3.2.6.2 LC-MS/MS analysis

For, LC-MS/MS analysis, the protein bands of interest were excised, washed, reduced, alkylated and finally digested by trypsin [6]. Briefly, properly destained reduced protein band was carefully excised from SDS-PAGE with the help of a scalpel, cut to fine pieces under a laminar flow and put in a microfuge tube. The gel pieces were then reduced (10 mM DTT) for 30 min at 56 ˚C and alkylated (55 mM IAA) for 30 min at dark [6]. The proteins in gel were digested with proteomics grade trypsin (50 µl, 13 ng/µl).The samples were then rehydrated for 10 min in ice, overlaid with 25 mM NH_4HCO_3 and incubated at 37 °C for 16 h. The digested peptides were then extracted by adding 5% formic acid (v/v) in 50% acetonitrile (v/v) to the gel pieces. The peptide digest was then vortexed for 15 min at room temperature (~23 °C) and the volume was reduced by subjecting the mixture to SpeedVac [7].

The digested peptides were reconstituted in 15.0 µl of the 0.1% formic acid and were subjected to standard 70 min RP-HPLC-MS/MS analysis with collision induced dissociation as the fragmentation method [8]. The data was searched against Uniprot Swiss-Prot database (non-redundant database with reviewed proteins) from NCBI. The data was searched for identification of protein on the MASCOT 2.4 search engine using Swiss-Prot, TrEMBL, and databases from NCBI and the data was corroborated in Proteome Discoverer 1.3 software (Thermo Fisher Scientific, Bremen, Germany). A minimum of two high confidence peptides were used as a prerequisite to identify the protein. The *de novo* (database independent) sequencing with an average local

confidence (ALC) score of ≥ 50% were derived directly from the MS/MS spectrum using PEAKS 7.0 software and the identified peptides were subjected to a BLAST search in NCBInr, Swissprot protein sequences (Swissprot), and Protein Databank proteins (PDB) against Lamiaceae family protein database and green plant protein database using the blastp algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.2.6.3 Determination of secondary structure of protein

The secondary structure of the purified protein was determined by circular dichroism (CD) analysis (Jasco 1715 Spectropolarimeter, Japan). Briefly, the far UV-CD spectra (190-250 nm) of native protein solution (0.3 mg ml^{-1} in 20 mM potassium phosphate buffer, pH 7.0) in a quartz cuvette with path length of 0.1 cm were recorded at room temperature (~23 °C). The bandwidth was set at 1 run and response time was 2 s. The final spectrum was cumulative of five scans. The CD spectra were corrected by subtraction of buffer blank and expressed in molar ellipticity [ϕ] (degrees cm^2 dmol l^{-1}), using 113 as mean residue molecular mass [9]. Yang's reference was set for the CD analysis. CDPRO CLUSTER software was used to determine the secondary structure of isolated protein as described by us [10].

3.2.6.4 Amino acid compositional analysis

For amino acid composition analysis, protein constituent of active fractions/purified protein (500 µg) was hydrolyzed with 6 N HCl at 110 °C for 24 h under vacuum. The hydrolyzate (25 µl) was mixed with 25 µl of ortho- pthaldialdehyde (Sigma), 3-merceptopropionic acid and 9-fluorenylmethyl chloroformate (Sigma) reagents in borate buffer. This was followed by addition of 8.0 µl of 1M acetic acid and contents were mixed well. The 3 µl of the resulting mixture was injected in Acclaim RSLC 120, C_{18} RP-UPLC column (2.1 X 150 mm, 3 µm, 300 Aº) coupled to ultimate 3000RSLC (Dionex) UHPLC system. After washing the column by eluent A (10 mmol/L Na_2HPO_4, $Na_2B_4O_7$ containing 0.5mmol /L NaN_3), the amino acid derivatives were separated by eluent B (CH_3CN /MeOH/H_20 in 45:45:10) at a flow rate of 0.722 ml/min. The elution was monitored initially at 338 nm for 0-7.2 min and then at 262 nm for the next 7.2 - 10.5 min[11].The concentration of individual amino acid of the purified enzyme was determined against a calibration curve of standard amino acids derivatives run in the same RP-UPLC column under the identical conditions. The amino acids composition of

the purified enzyme was searched in Swiss-prot databases by using the programme AAcompldent of Expert Protein Analysis System (ExPASy) online software (http://www.expasy.org/tool/aacompident)[12,13].

3.2.6.5 GC-MS analysis of active fraction

GC-MS analysis of the active fractions was performed using a Agilent GC 7890 system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with a Elite-I, fused silica capillary column (30 mm X 0.35 mm DB-5. For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1ml/min and sample injection volume was2 µl (split ratio of 10:1); Injector temperature 250 °C; Ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min.), with an increase of 10°C/min, to 200 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C[14]. Mass spectra were taken at 70 eV; a scan interval of 0.5seconds and fragments from 45 to 1000 Da. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbomass. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. On the basis of matching, name, molecular weight and structure of the components of the test materials were ascertained.

3.2.6.6 Interaction studies by spectrofluorometric analysis

Interaction of isolated active fraction/components/protein with blood coagulation factors such as thrombin, fibrinogen, and factor Xa was measured by intrinsic fluorescence analysis using a fluorescence spectrometer (LS55, Perkin Elmer) following the protocol described by Saikia et al. [15]. Briefly, the coagulation proteins were mixed with the active fraction/components/protein in a specific ratio and the change in fluorescence intensity at an excitation of 280 nm was measured. The excitation and emission slits were set as 10 nm and emission spectra were recorded from 310-400 nm at room temperature (~23 °C). All the binding experiments were done in triplicates to ensure the reproducibility[15]. The fluorescence intensity of the coagulation protein or isolated fraction/components/protein alone recorded under identical experimental conditions

served as control. On e site binding model was considered for determination of dissociation constant (K_d) using the following equation [5,16]

$$\Delta F = \frac{\Delta Fmax \times C}{Kd + C}$$

Where, **Δ**F: change in the fluorescence intensity;

ΔFmax: Maximum change in the fluorescence intensity;

C: Concentration of the samples

K_d: Dissociation constant

3.2.6.7 ITC (Isothermal Calorimetry) analysis of thrombin and fibrinogen binding

ITC experiments were performed at 37 °C on MicroCal™ iTC-200 system (GE Healthcare) in a high gain mode at a reference power of 10 µcals^{-1}. Human thrombin or fibrinogen (200 µM in 1X PBS buffer, pH 7.4) was titrated against 10 µM of purified protein/component dissolved in the same buffer. A total of 20 injections were made with 300-s time intervals in between [17]. For longer titrations, the syringe was refilled and injections continued into the same cell sample. Control runs were performed in which cell samples and syringe samples were titrated with buffer and the data from these runs were subtracted from the experimental data. Data was fitted by "sequential binding" model and analysis was performed on Origin software[18].

$$q = Vc \sum_{i=1}^{n} \Delta Hi[MXi]$$

where V_C is the volume of the titration cell, n is the number of binding sites, ΔHi is the molar heat of the reaction, and [MXi] is the molar concentration of the receptor (M) bound to i ligands (X). For ITC, in particular, the signal collected at each injection point is proportional to the differential heat (Δq or dq), defined here as the change in the heat content of the cell after a small amount of titrant was added.

3.2.6.8 *In silico* study to determine interaction between active component (BSS) of active fraction of *L. indica* (AFLI) and thrombin

A binding interaction between active component (BSS) and thrombin was determined by AutoDock 4.2 software[19,20]. The initial structure of the thrombin was taken from the protein data bank (PBDCode: 3u69). For the docking study, all water molecules from the protein crystal structure were removed and polar hydrogen atoms were added to the thrombin. On the other hand, the initial structure of the ligand (BSS) was taken from the density functional theory (DFT) optimized structure at a B3LYP level of theory at 6-31+g(d,p) basis set using Gaussin09 software[20]. Prior to the docking calculation, a pre-calculated grid map was acquired by AutoDock to find the binding energy in the region of interest in the receptor molecule. Therefore, a grid box size of 60 Å, 60 Å, and 60 Å (coordinate at X, Y, and Z axis) with a grid spacing of 0.972 Å was used. During the BSS-thrombin interaction, 10 runs were conducted and one docking pose was retained for the ligand.

3.2.7 Biochemical characterization

3.2.7.1 Qualitative analysis of active fractions:

The active fractions of the selected plants were subjected to qualitative chemical tests to determine the presence and/or absence of alkaloids, carbohydrates, flavanoids, proteins, saponins, tannins, phenolic compounds and glycosides.

3.2.7.1.1 Test for Alkaloids

a) Dragendroff's Test: About 0.2 g of the active fraction was warned with 2% H_2S0_4 for two minutes. It was filtered and few drops of Dragendroff's reagent was added. Orange red precipitate indicates the presence of alkaloids [21].

b) Mayer's test: To a few ml of active fraction, a few drops of Mayer's reagent was added by the side of the tube. A creamy white precipitate indicates the presence of alkaloids [22].

3.2.7.1.2 Test for Flavonoids

a) Alkaline reagent test: 0.5 g active fractions dissolved in 2 ml distilled water was treated with 10% NaOH solution; formation of intense yellow color indicates presence of flavonoids [22,23].

b) NH_4OH test: 3.0 ml of active fraction was treated with 10% NH_4OH solution, development of yellow fluorescence indicates a positive test [22,23].

c) Mg turning test: 1.0 ml of active fraction (2.0 mg/ml) was treated with Mg turning and add conc. HCl to this solution add 5 ml of 95% ethanol, formation of crimson red color indicated presence of flavonoids [22].

d) Zn test: 2 ml active fraction (2.0 mg/ml) was treated with Zn dust and conc. HCl development of red color indicated presence of flavonoids [23].

3.2.7.1.3 Test for phenolic compounds

The active fraction (100 mg) was dissolved in 2.0 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green color indicated the presence of phenolic compounds [24].

3.2.7.1.4 Test for gum and mucilage

About 10 ml of the active fraction (2.0 mg/ml) were slowly added to 25.0 ml of absolute alcohol under constant stirring. Precipitation showed the presence of gum and mucilage [22].

3.2.7.1.5 Test for fixed oils and fats

A drop of concentrated active fractions (10 mg/ml) were pressed in between two filter papers and kept undisturbed for 4 h. Oil stain on the paper indicates the presence of oils and fats [22].

3.2.7.1.6 Test for glycoside

Active fractions (5.0 mg) dissolved in glacial acetic acid and few drops of ferric chloride and concentrated sulphuric acid was added, and formation of reddish brown

coloration at the junction of two layers and the bluish green color in the upper layer indicated the presence of glycoside [22].

3.2.7.1.7 Test for steroid

20 mg of the active fraction was treated with 2.5 ml of acetic anhydride and 2.5 ml of chloroform. Then concentrated solution of sulfuric acid was added slowly and red violet color was observed for terpenoids and green bluish color for steroids [25].

3.2.7.1.8 Test for tannins

To 0.5 ml of active fraction (20 mg /ml), 1ml of water and 1- 2 drops of ferric chloride solution was added. Blue color was observed for gallic tannins and green black for catecholic tannins [26].

3.2.7.1.9 Reducing sugar test

To 0.5 ml of active fraction (20 mg/ml), 1.0 ml of water and 5- 8 drops of Fehling's solution was added at hot and observed for brick red precipitate for reducing sugar [25].

3.2.7.2 Estimation of protein content

The protein content of crude aqueous leave extracts/active fractions/protein was determined according to the protocol described by Lowry et al. [27]. A serial dilution of 0.5, 1.0, 2.5, 5.0, 7.5, and 10 µg per well of bovine serum albumin (BSA, 1 mg/ml stock solution) in 100 µl of dH_2O was prepared in a microtitre plate. Thereafter, 200 µl of alkaline copper sulfate solution (0.5% $CuSO_4.5H_20$ in 1% sodium-potassium tartrate and 2% Na_2CO_3 in 0.1 N NaOH in 1:49 ratio) was added to the wells and incubated for 10 min at room temperature (~23 °C). Then, 20 µl of Folin-Ciocalteau Phenol reagent diluted to 1:2 ratio (Folin: dH_2O) was added and further incubated at room temperature for 30 min. The protein content of the unknown samples was calculated from a standard protein calibration curve established by plotting the absorbance at 660 nm vs. concentrations of BSA.

3.2.7.3 Casein/plasma protein hydrolysis activity

Digestion of casein/albumin/globulin was evaluated calorimetrically[28]with slight modifications [29]. Briefly, 1.0 ml of 1% (w/v) casein/albumin/globulin dissolved in 20 mM potassium phosphate buffer, pH 7.4 was incubated with specific amount of fractions (1.0 µg/ml)/component/protein (0.2 µM) for 90 min at 37 °C. After the stipulated time interval, the reaction was stopped by addition of 0.5 ml of 10% (w/v) ice-cold trichloroacetic acid (TCA). The reaction mixture was centrifuged (5000 rpm, 15 min) and 2.0 ml of 2% (w/v) Na_2C0_3 in 0.1 N NaOH was added to the mixture and incubated for 10 min at room temperature (~23 °C). This was followed by the addition of 0.5 ml of Folin- Ciocalteu's reagent (1:2 dilutions). After 30 min, absorbance of the mixture was measured at 660 nm. One unit (U) of proteolytic activity has been defined as n mole equivalent of tyrosine formed min^{-1} which is calculated from a standard tyrosine curve [13,15].

3.2.7.4 Fibrinolytic/fibrinogenolytic activity assay

3.2.7.4.1 Colorimetric assay: The quantitative fibrinolytic assay was done following the protocol described by Mukherjee et al. [30]. Briefly, 40.0 µl of fibrinogen solution (2.5% w/v) was clotted with human thrombin (3 µl, 10 NIH U/ml) at room temperature (~23 °C) for 60 min. To the fibrin clot, specific amount of the fraction (5.0 µg/ml)/component/protein (0.2 µM)was added and incubated at 37 °C for 30 min in a final reaction mixture of 90.0 µl adjusted with 1X PBS, pH 7.4.

For quantitative fibrinogenolytic assay, instead of fibrin clot, 40.0 µl of fibrinogen solution (final concentration 2.5 mg/ml) was taken in a microfuge tube. The reaction was stopped by adding 10.0 µl of 10% ice-cold TCA and the supernatant obtained after centrifuging the reaction mixture at 3000 rpm for 10 min was used to determine the release of free amino acids (tyrosine) at 660 nm using Folin-Ciocalteus reagent. One unit (U) of fibrino(geno)lytic activity has been defined as n mole equivalent of tyrosine formed min^{-1}. Human plasmin (1.0 µg/ml) assayed for fibrinolytic/fibrinogenolytic activity under similar conditions served as a positive control.

3.2.7.4.2 SDS-PAGE analysis

To study the time course of fibrin/fibrinogen degradation, if any, a specific amount of the fractions (5.0 µg/ml)/protein (0.2 µM) or human plasmin/ nattokinase/streptokinase (0.2 µM) was incubated with fibrin/fibrinogen (see section 3.2.7.4.1) for different time intervals (30 min to 180 min) at 37 °C and the fibrin/fibrinogen degradation products were separated by 12% SDS-PAGE. The protein bands were visualized by staining with Coomassie Brilliant Blue R-250 as described in section 3.2.6.1.1. For control, 0.1 ml of PBS (1X, pH 7.4) instead of fraction/components/protein was added to the fibrin/fibrinogen solution and incubated under identical experimental conditions. The gel was scanned and band intensities were analyzed using ImageJ software.

3.2.7.5 RP-HPLC analysis of fibrin(ogen) degradation products

The active fractions (5.0 µg/ml)/protein (0.2 µM)or human plasmin/nattokinase catalyzed fibrin or fibrinogen degradation products were separated on a RP-C_{18} (300 A, 2.1 mm x 150 mm) column coupled to a UHPLC system (Ultimate 3000, Dionex, Germany). After washing the column with solvent A (5% acetonitrile with 0.1 % TFA) for 5 min, the bound proteins were eluted with a linear gradient of solvent B (95% acetonitrile with 0.1 % TFA) from 0% to 60% of B over 20 min at a flow rate of 1.0 ml min^{-1} at room temperature (~23 ºC).RP-HPLC elution profile of undigested fibrin/fibrinogen served as a control [13,19].

3.2.7.6 Determination of kinetic parameters of fibrinogenolytic activity

A specific amount of purified protein/nattokinase (0.2 µM) was incubated with graded concentrations of fibrinogen (1.0 – 6.0 µM) for 30 min at 37 °C, pH 7.4. Fibrinogenolytic activity of purified protein/ nattokinase was assayed as described in section 3.2.5.3. The kinetic parameters (*Km* and *Vmax*) of fibrinogenolytic activity were determined by non-linear regression analysis using the GraphPad Prism 6.03 software using the following equation [4,16]:

$$V = \frac{Vmax\,[S]}{Km + [S]}$$

Where, V is the reaction rate (velocity) at a substrate concentration [S]

Vmax: maximum rate of the reaction that can be observed in the reaction

Km is the Michaelis constant

3.2.7.7 Effect on extracellular matrix (ECM) proteins

ECM proteins such as laminin, type-IV collagen, and fibronectin were incubated separately with the protein in a substrate:enzyme ratio of 15: 1 (w/w), in a total volume of 20.0 µl of 20 mM potassium phosphate buffer, pH 7.4 at 37 °C for 4 h[16]. The reaction was stopped immediately after the stipulated time interval by chilling in ice and adding 5.0 µl denaturing buffer containing SDS and β-mercaptoethanol (see section 3.2.4.2). The degradation products were analysed on 10% SDS-PAGE after staining with 0.25% Coomassie Brilliant Blue R-250 as described in section 3.2.6.1.1. The gel was scanned and band intensities were analyzed using ImageJ software.

3.2.7.8 Thrombin inhibition studies

3.2.7.8.1 Inhibition of amidolytic activity of thrombin

Human thrombin (3.0 µl, 10 NIH U ml^{-1}) was incubated with active fractions (1.0 µg/ml) /component (7.5 µM) /purified protein (200 nM) for 30 min at 37 °C [13]. Thereafter, chromogenic substrate for thrombin (T1637, *N*-(p-Tosyl)-Gly-Pro-Arg-p-nitroanilide acetate) was added (0.2 mM) and the volume of reaction mixture was adjusted to100 µl with 1X PBS, pH 7.4. Substrate hydrolysis was measured by monitoring the change in absorbance at 405 nm for 20 min at 20 s intervals in a microplate reader (MultiSkan GO, Thermo Scientific, USA). Residual activity of thrombin due to the fraction/peptide was calculated by considering the activity of thrombin (absorbance after 20 min of substrate hydrolysis) in the absence of inhibitor as 100%. Dose response curve for thrombin inhibition was fitted using GraphPad Prism 6.03 to calculate the Hill coefficient and IC_{50} value [5,13,16].

3.2.7.8.2 Determination of inhibitory constant (*Ki*) for thrombin inhibition

Thrombin (3.0 µl, 10 NIH U ml^{-1}) was pre-incubated with two different concentrations of the isolated protein (0.2 and 0.4 µM) at 37°C for 30 min. Various concentrations (0.1-0.8 mM) of chromogenic substrate for thrombin (T1637) was added to the mixture in a final reaction volume of 100 µl adjusted with phosphate buffer saline (1X, pH 7.4). The release of para- nitroanilide (pNA) at 405 nM was determined after 10 min

incubation at 37°C in a microplate reader (MultiSkan GO, Thermo Scientific, USA). For kinetic analysis, the reaction rate (V) was plotted against substrate concentration (S) at each inhibitor concentration and the data was fit to a hyperbolic Michaelis-Menten model using GraphPad Prism 6.03 software [16].

The inhibitory constant (*Ki*) was determined using the mixed model for enzyme inhibition using the same software. The inhibitory constant was determined by the software using the model given below,

$$V_{maxApp}=\frac{V_{max}}{1+\frac{I}{\alpha Ki}}$$

$$K_{mApp}=\frac{K_m}{1+\frac{I}{\alpha Ki}}$$

$$V=\frac{V_{maxapp}\times S}{K_{mapp}+S}$$

Where, αI is the inhibition constant, expressed in the same units as I, *Vmax*App and *Vmax* denotes the maximum velocity in the presence and absence of the inhibitor, respectively whereas *Km*App and *Km* represents the Michaelis constants in the presence and absence of inhibitor, respectively.

3.2.7.8.3 Effect on fibrinogen clotting activity of thrombin

The effect of active fractions /component /purified protein on the fibrinogen clotting activity of thrombin was assayed as described by Ciprandi et al. [31] and modified by Mukherjee and Mackessy[4] and Thakur et al.[16]. Briefly, Human thrombin (3.0 µl, 10 NIH U/ml) was incubated with different concentrations of the active fractions (0.25-3.0 µg/ml) /component (1.25-12.5 µM) /protein (25 – 600 nM) for 30 min at 37 °C. This mixture was added to fibrinogen solution (in 1X PBS, pH7.4) and final reaction volume was adjusted to 100 µl adjusted with PBS (1X, pH 7.4). The time of fibrin clot formation, if any, was monitored by visual inspection [4,16].

3.2.7.8.4 Determination of effect of active component on thrombin inhibition by AT-III in the presence or absence of heparin

The effect of active fraction/component/purified protein on thrombin inhibition produced by antithrombin-III (AT-III) in the presence or absence of heparin was determined as per the protocol described by Arocas et al. [32]. Briefly, thrombin (3 µl, 10 NIH/ml) was pre-incubated with active component (BSS) (7.5 µM, dissolved in 1X PBS, pH 7.4) for 30 min at 37 ºC in a final reaction mixture of 100 µl adjusted with 1X PBS, pH 7.4. The heparin-free AT-III (100 nM) in the presence of 0.5 U/ml heparin was added to the samples and the residual activity of thrombin was measured by hydrolysis of its chromogenic substrate T1637 at 10 min at 405 nm in a microplate reader [4].

3.2.7.9 Factor Xa inhibition studies

3.2.7.9.1 Effect on the prothrombin activation property of factor Xa

The effect of the active fractions/component/purified protein on the physiological function (i.e. prothrombin activation) of factor Xa was studied by pre-incubating a fixed concentration of active fractions/component/purified protein or 1X PBS (control) with FXa (0.13 µM) in 20 mM sodium phosphate buffer, pH 7.4 at 37 °C for 30 min [13,16]. Thereafter, 1.4 µM prothrombin (physiological substrate for FXa) was added and the reaction mixture was incubated at 37 °C for 4 h. The prothrombin degradation products were analyzed by 12.5% SDS-PAGE under reducing conditions [16]. Prothrombin incubated with PBS (1X, pH 7.4) and factor Xa (0.13 µM) under identical assay conditions served as negative and positive controls, respectively[16].The gel was scanned and analyzed by ImageJ software (version 1.47) (Wayne Rasband, NIH, USA) to calculate the percent degradation of prothrombin by active fractions/component/purified protein considering the band intensity of prothrombin treated with FXa as 100% .

3.2.7.9.2 Determination of inhibitory constant (*Ki*) for factor Xa inhibition

Factor Xa (0.02 µM) was pre-incubated with two different concentrations of purified protein(0.2 and 0.4 µM) at 37°C for 30 min. Various concentrations (0.1-0.8 mM) of chromogenic substrate for thrombin (F3301, *N*-Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide acetate) was added to the mixture in a final reaction volume of 100 µl adjusted with

phosphate buffer saline (1X, pH 7.4). The release of pNA at 405 nM was determined after 10 min incubation at 37°C in a microplate reader (MultiSkan GO, Thermo Scientific, USA). For kinetic analysis, the reaction rate (V) was plotted against substrate concentration (S) at each inhibitor concentration and the data was fit to a hyperbolic Michaelis-Menten model using GraphPad Prism 6.03 software [13,16]. The inhibitory constant (*Ki*) was determined using the mixed-model for enzyme inhibition using the above software. The inhibitory constant was determined by the software as described in section 3.2.7.8.2.

3.2.7.10 Determination of plasminogen activating assay

1 µM of active component (β-sitosterol, BSS) in 20 mM phosphate buffer pH 7.4 / 1X PBS, pH 7.4 containing 0.1% DMSO (control) was incubated with 20 µl of plasminogen (10 U/ml in 1X PBS buffer, pH 7.4) at 37°C for 20 min [13,30]. The formation of plasmin from plasminogen was determined by adding 0.2 mM of V0882 (D-Val-Leu-Lys-p-nitroanilidedihydrochloride) and the absorbance was monitored at 405 nm in a plate reader (Multiskan Go, Thermo Scientific, Waltham, USA) continuously for 15 min against the reagent blank. As control, plasminogen was incubated with V0882 only. The activity of streptokinase (1 µM) under identical experimental condition was used as a positive control [30]. To determine the plasmin-like activity of component, if any, the component (1 µM) was directly added to the 0.2 mM of V0882 (D-Val-Leu-Lys-p-nitroanilidedihydrochloride) and the absorbance was monitored at 405 nm in a plate reader (Multiscan Go, Thermo Scientific, Waltham, USA) continuously for 15 min against the reagent blank.

In an another set of experiment, to determine the role of soybean protease contamination, if any, in the component preparation, the active component (BSS) (1.0 µM) was pre-incubated with 2 mM phenylmethylsulfonylfluoride (PMSF) (serine protease inhibitor) for 30 min at 37 ºC and then the plasminogen activation property was performed as described above.

3.2.7.11 Biochemical characterizations of active fractions and purified protein

3.2.7.11.1 Chemical modification of active site of purified protein

The active site of the purified proteinwas chemically modified with group specific reagents [13,30]. Different inhibitors viz. phenylmethylsulfonyl fluoride (PMSF, serine protease inhibitor), Ethylenediaminetetraacetic acid (EDTA, metal chelator), Tosyl phenylalanyl chloromethyl ketone (TPCK, chymotrypsin inhibitor), Tosyl-L-lysyl-chloromethane hydrochloride (TLCK, trypsin inhibitor), Dithiothreitol (DTT, reducing agent for disulphide bonds), Indole-3-acetic acid (IAA, cysteine modifier) and p-bromophenylacyl bromide (Pbpb, histidine modifier) were tested for chemical modification of the active site of the purified protein. Briefly, specific amount of the purified protein (02 µM) was incubated with different inhibitors (at 2.0 mM final concentration except EDTA which was tested at 5.0 mM final concentration) at room temperature (~23 °C) for 30 min. Thereafter, the respective enzymatic properties viz. fibrinolytic/fibrinogenolytic activity and plasma re-calcification time of the protein was analyzed in their respective assay systems as described in sections 3.2.7.4.1, and 3.2.8.1, respectively. The enzymatic or anticoagulant activity of protein in the absence of inhibitor was considered as 100% activity and all other values were compared with that.

3.2.7.11.2 Determination of optimum temperature and pH for enzymatic activity

To study the effect of temperature on the catalytic properties of the active purified protein (0.2 µM) was incubated with 2.6 µM fibrinogen/fibrin for 30 min at different temperatures (10 – 80 °C). Thereafter, fibrinogenolytic/fibrinolytic activity of the active fraction/protein enzyme was assayed as described in section 3.2.7.4.1

To determine the optimum pH for the functioning of the purified protein, specific amount of the purified protein (0.2 µM) was incubated with fibrinogen/fibrin for 30 min at a 37 °C and different pH ranging from pH 2.0-12.0. The different buffers used for this study were 20 mM sodium acetate buffer, pH 5.0-6.5; 20 mM potassium phosphate buffer, pH 7.0-7.5 and 20 mM Tris-HCl buffer, pH 8.0-9.5. The fibrinogenolytic/fibrinolytic activity was analyzed as described in section 3.2.7.4.1.

3.2.7.11.3 Stability study

Stability of aqueous solution of active fractions/purified protein (2 mg/ml) at 4 °C, -20 °C as well as room temperature was determined by withdrawing 1.0 µg of enzyme at an interval of 7 days for 60 days and then assaying anticoagulant activity as described in section 3.2.8.1.

An anticoagulant unit at 0 day was considered as 100% activity, and activity at various time intervals was compared with that. The effect of freeze-thawing on anticoagulant activity was determined by freezing the active fractions/purified protein at −20 °C followed by thawing at room temperature, and this process was repeated 5 times [3]. The residual activity after each cycle of freeze-thawing was determined and compared with the control (anticoagulant activity before freeze-thawing) [4].

3.2.8 Pharmacological characterization

3.2.8.1 Plasma re-calcification time

The plasma clotting activity of the isolated fraction/purified component/purified protein was determined by the method of Angulo et al. [33] with some modifications as described by Mukherjee & Maity [34]. Briefly, blood from goat/ human volunteers who were not under any medications was collected in 3.8% trisodium citrate (9: 1 ratio) and platelet-poor plasma (PPP) was prepared by centrifuging the citrated blood twice at 4300 rpm for 15 min at 4 °C. The PPP was used within 4 h of collection. For the assay of plasma re-calcification time, specific amount of the isolated fraction/ component/purified protein(in a final volume of 20.0 µl) was added to 300.0 µl of pre-warmed PPP at 37 °C and the mixture was incubated for 3.0 min at 37 °C. To the mixture, 40.0 µl of 250 mM $CaCl_2$ was added and the time taken for the appearance of first visible fibrin thread/clot formation was recorded with the help of a stop watch. As a control, plasma aliquots were incubated with 20.0 µl of 20 mM potassium phosphate buffer, pH 7.4, and the coagulation time was determined identically. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP as compared to the clotting time of normal PPP (control) under identical assay conditions [5,30,35].

3.2.8.2 Prothrombin time test

The prothrombin time (PT) of goat PPP was tested with commercial Liquiplastin kit [5,16]. Briefly, 0.1 ml of PPP was taken in a test-tube and placed in a water bath for 3 to 5 min at 37°C. Then specific amount of active fractions/component/purified protein was added to the PPP and the mixture was incubated in a water bath for 3 min at 37°C. To the tube, 0.2 ml of liquiplastin reagent (pre-warmed at 37 °C for 3 min) was added and the tube was gently shaken to mix the contents. The first appearance of visible fibrin thread/clot formation was recorded with the help of a stop watch. As a control, plasma aliquots were incubated with 20 µl of 20 mM potassium phosphate buffer, pH 7.4, and the coagulation time was determined identically. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP as compared to clotting time of normal PPP (control) under identical assay conditions.

3.2.8.3 Activated partial thromboplastin time test

The activated partial thromboplastin time (aPTT) of goat PPP was tested by using commercial Liquicelin-E kit. Briefly, 0.1 ml of PPP was taken in a test-tube and placed in a water bath for 3 to 5 min at 37°C. Then specific amount of active fractions/components/protein was added to the PPP and the mixture was incubated in a water bath for 3 min at 37°C. To the tube, 0.1 ml of liquicelin reagent (pre-warmed at 37°C for 3 min) was added and the mixture was again incubated in a water bath for 3 min at 37 °C. Following incubation period, 100 µl of pre-warmed $CaCI_2$ (25 mM) added to the plasma-Liquicelin mixture. The first appearance of visible fibrin thread/clot formation was recorded with the help of a stop watch. As a control, plasma aliquots were incubated with 20 µl of 20 mM potassium phosphate buffer, pH 7.4, and the coagulation time was determined identically[16]. One unit of anticoagulant activity has been defined as 1.0 s increase in the clotting time of PPP as compared to clotting time of normal PPP (control) under identical assay conditions

3.2.8.4 Platelet modulating activity

The collection of blood from healthy volunteers (who were not under medication) was approved by the Tezpur University Ethical Committee. Platelet rich plasma (PRP) was prepared from citrated human blood by following the procedure described by Bednar et al.[36] and modified by Dutta et al. [5]. First, platelet rich plasma (PRP) was isolated

from goat blood by centrifuging fresh blood at 250 x g for 10 min at 4 ºC. The supernatant containing the PRP was carefully extracted and platelets were isolated. Briefly, PRP was centrifuged at 650 x g for 10.0 min and the pellet was washed twice in a tyrode buffer (5.0 mM Hepes, 13.0mM NaCl, 2.7 mM KCl, 12.0mM $NaHCO_3$, 0.42 mM Na_2HP0_4, 1.0 mM $MgCl_2$, 0.1 % glucose and 0.25% BSA). The pellet was resuspended in the same buffer and the volume was adjusted with tyrode buffer to give an absorbance of 0.15 at 650 nm.

The agonist (6.2 nM collagen type IV or 30 µM ADP or 15 µM arachidonic acid)-induced aggregation of PRP/washed platelets was measured continuously at an interval of 15 s for 5 min at 30 °C in a 96-well microplate reader (Multiscan GO, Thermo Scientific, USA) at 540 nm [5]. In another set of experiments, isolated fraction (1-5 µg/ml)/ component (1- 15 µM)/ purified protein (0-600 nM) / was added to 100 µl of the PRP and the absorbance was measured continuously at 540 nm for 5 min as stated above. Aspirin served as a positive control. The percent platelet aggregation after 300 s of incubation of platelets with agonists was calculated as below [5].

$$\text{Percent aggregation} = \frac{\text{A540 of the PRP before the addition agonist} - \text{A540 of the PRP after the addition of agonist}}{\text{A540 of the PRP before the addition of agonist}} X100$$

In another set of experiments, PRP was pre-incubated with different concentrations of active fraction (1-5 µg/ml)/ component (0-15 µM)or purified protein (0-600 nM) for 5 min prior to addition of collagen (6.2 nM) / ADP (30 µM) / arachidonic acid (15 µM). The aggregation induced by the identical concentration of collagen/ADP/arachidonic acid was considered as 100% activity and decrease in active fractions/components/ protein-induced platelet aggregation (antiplatelet activity) was compared with that [5].

3.2.8.5 *In vitro* hemolytic activity

The hemolytic activity of was determined using the method as described by of Mukherjee et al. [37]. Briefly, 9 ml of blood was collected from healthy goat in sterile tubes containing 1.0 ml of 3.8 % tri-sodium citrate (anticoagulant), centrifuged at 4300 rpm for 15 min. The platelet poor plasma (PPP) was discarded, and the pellet was re-suspended in PBS and washed thrice with 1X PBS (pH 7.4), and then diluted to 0.5% in PBS (pH 7.4). The active fractions/components/protein to be tested was placed in

different tubes and after that 2 ml erythrocyte suspension was added to each tubes, gently inverted and incubated at 37 °C for 90 min. Total hemolysis (100%) was achieved by adding deionized water/ 1 % Triton X-100 to the erythrocyte suspension and 1X PBS was used as negative control under identical experimental condition. After incubation, all the tubes were centrifuged at 10,000 rpm for 10 min to pellet out RBCs. The supernatant was carefully separated out and the absorbance was measured at 540 nm for released hemoglobin using UV-Vis spectrophotometer. The percentage of hemolysis was calculated by using the following formulae:

$$\% \text{ hemolysis} = \frac{(\text{OD samples} - \text{OD negative control})}{\text{OD positive control} - \text{OD negative control}} \times 100$$

3.2.8.6 *In vitro* cytotoxicity assay

3.2.8.6.1 MTT-based assay

The *in vitro* cytotoxicity of the isolated fractions/protein was tested against human embryonic kidney cells (HEK 293 cell line), cultured and maintained in Dulbecco's modified eagle medium (DMEM)[38]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). Briefly, 2 x 10^4 cells were seeded in 96-well tissue culture plate and allowed to adhere by growing at 37°C and 5% CO_2. After 24 h of seeding, the culture medium (DMEM) was replaced with fresh medium containing different concentrations of the active fractions/components/protein. For control, cells were treated with 20 mM potassium phosphate buffer, pH 7.4. After 48 h of treatment, 100 µl of 1.0 mg/ml MTT was added to the cells and incubated for 3 h at 37°C. The cells were then treated with 100 µl DMSO and incubated in dark under shaking conditions. The background absorbance of multi-well plates was measured at 690 nm and subtracted from the 550 nm measurement. The percent cell viability was calculated as the ratio of treated cells to the control cells. All assays were done in triplicate and repeated at least three times[39].

3.2.8.6.2 Calcein-AM cell viability staining

HEK 293 cells (2 x 10^4 cells/well), cultured in 96-well plates in DMEM media supplemented with 10% FBS, were treated purified protein or medium (control) for 24 h. The cells were washed with 1X PBS, pH 7.4, stained with 5.0 µM calcein-AM (in 1X PBS, pH 7.4) and incubated for 5 min. The cells were washed with 1X PBS, pH 7.4, and visualized using an epi-fluorescence microscope at 40X magnification (Nikon ECLIPSE Ti-U, Tokyo, Japan). For the phase-contrast images, photomicrographs were captured using a Nikon ECLIPSE Ti-U (Tokyo, Japan) camera without filter [40,41].

3.2.8.6.3 Flow cytometry analysis to determine the cell cycle kinetics

The effect of active fractions/purified protein/component on the cell cycle of HEK 293 cells was determined by flow cytometry analysis using propidium iodide (PI) DNA staining dye[38]. The HEK 293 cells (1.5 x 10^5 cells per ml) were seeded in 96-well plates and allowed to adhere overnight at 37 ˚C. On the next day, the old medium was replaced with fresh media containing purified protein (2.0 µM) or only growth medium (control) and incubated for 24 h at 37 ˚C. Cells were collected by trypsinization and then fixed by adding chilled 70% ethanol before being stored at -20 ˚C until further analysis.

The fixed cells were centrifuged and washed with chilled 1X PBS, pH 7.4, following incubation with RNase at 37 ˚C for 1 h. Cells were then incubated with PI stain for 2 h before being analyzed by flow cytometry (FACscan, Becton Dickinson, Bedford, MA). The data was analyzed by ModFiT LT software.

3.2.8.6.4 Effect on the cytochrome P450 enzyme system

The potency of active fractions (0-20 µg/ml) to inhibit CYP1A, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, the cytochrome P450 (CYP) isoforms was determined with a commercial kit (Cytochrome p450 inhibition assay kit, Sigma-Aldrich, USA). Briefly, CYP isoforms (1.0 µg/ml) were pre-incubated with different concentrations active fractions (0-20 µg/ml) at 37 °C for 30 min. Then, isoforms-specific chromogenic substrates (0.2 mM) were added to the mixture in a final reaction volume of 100 µl adjusted with phosphate buffer saline (1X, pH 7.4). The release of pNA at 460 nM was determined after 10 min incubation at 37°C in a microplate reader

(MultiSkan GO, Thermo Scientific, USA). Residual activity of CYP isoforms due to the fractions was calculated by considering the activity of CYP isoforms (absorbance after 10 min of substrate hydrolysis) in the absence of inhibitor as 100%

3.2.8.7 *In vitro* blood clot lysing activity

For the measurement of *in vitro* blood clot lysis activity, 1.0 ml citrated blood was transferred to 2.0 ml pre-weighed microfuge tube to which 100.0 µl of $CaCl_2$ (250 mM) was added. The clot was allowed to form for 1 h at 37 °C. The clot was weighed and thereafter, a specified amount of the active fractions/component/purified protein/ clot bursting drug was added to the clot and incubated a 37°C for 90 min. The supernatant was removed by decanting the tube and the dry weight of the clot was measured. Clots incubated with buffer (20 mM potassium phosphate buffer, pH 7.4) and same concentration of thrombolytic drug (streptokinase, nattokinase or plasmin) served as negative and positive control, respectively. The *in vitro* thrombolytic activity was expressed as mg of blood clot (thrombus) lysed per µg of test samples, or commercial drugs (plasmin, nattokinase or streptokinase) as compared to the control (blood clot treated with buffer).

For determining the lysis of heat denatured clot, the blood clots were heated at 80°C for 30 min prior to addition of the active fractions/component/purified protein/ clot bursting drugs. Clot lysing property of human plasmin/streptokinase/nattokinase (1.0 µg) was assayed under identical conditions to serve as positive control [13].

3.2.13 *In vivo* toxicity assessment

Wistar strain rats (120-150 g) and Swiss albino mice (20-22 g) of both the sexes were obtained from Animal house experimentation facility of Institute of Advance Study in Science and Technology (IASST), Guwahati, Assam, CSIR-CIMAP, Lucknow and Defense Research Laboratory, Tezpur, Assam. All animals were pathogen free. General conditions of captivity were maintained in simulated atmospheric condition (temperature 25-32 °C, relative humidity ≥ 70%). The physiological status of the animals was supposed to be stabilized. The general conditions of captivity were applied as described above and animals were maintained in social groups before and after the experiments. Animal experiments were performed at IASST, Guwahati, Assam, CSIR-CIMAP, Lucknow and DRL, Tezpur, Assam by following the protocol of the

OECD/OCED guidelines 425 (2001). The animal experiment protocols were approved by the Tezpur University Animal Ethical Committee (Approval no: DORDPro/TUAEC/10-56/14/Res-10), Institutional Ethical Committee of IASST, Guwahati, Assam (Approval no: IASST/IAEC/2016-17/04), Institutional Ethical Committee of DRL, Tezpur, Assam (Approval no: DRL/02/Dec/2010-11/II) as well as Animal Ethical Committee of CSIR-CIMAP, Lucknow.

3.2.13.1 Assessment of acute *in vivo* toxicity

M. charantia are non toxic to human and is well documented so the toxicity study of active fraction of *M. charantia* was not performed [42,43]. The *in vivo* toxicity of the active fraction of *L. indica* (2000 mg/kg, oral administration) and purified protein (10.0 mg/kg, intraperitoneal injection) was determined by analyzing the biochemical and hematological changes.. Briefly, Wistar strain rats (n=6) were divided in two groups; the experimental group of rats were administered active fraction (orally, 500 µl, dissolved in 1X PBS, pH 7.4)/ purified protein (*i.p,* 100 µl, dissolved in 1X PBS, pH 7.4) whereas the control (placebo) group of rats (n=6) received same volume of normal saline. The animals were observed at regular intervals up to 72 h and 7 days post administration of purified protein and active fraction, respectively, for any behavioral changes viz. body weight, food and water intake, defecation and urination, grip strength, ear twist, rectal temperature, effect on circulatory system, and/or death.

Whole blood clotting time was measured at regular intervals by capillary glass method [44] or tail bleeding method [45]. Briefly, the tails of treated as well as control rat were transected at 2 mm from their tips after 6 h. Bleeding time was defined as the time elapsed until bleeding stopped [44].Alternatively, blood from the transected tail was taken in capillary tube and the tubes were carefully broken at regular intervals to check the formation of fibrin clot [45]. The blood was collected immediately by cardiac puncture and all hematological parameters such as WBC, RBC, lymphocyte and monocyte count, Hb, MeV, MCR and MCHC were analyzed by Hematology Auto Analyzer MS4-S (Melet Schloesing Laboratories, France). The plasma obtained from the control as well as treated groups of rats were analyzed for different biochemical parameters viz. total protein, glucose, uric acid, creatinine, cholesterol and urea levels as well as for different enzyme activity viz. alkaline phosphatase (ALP), creatine phosphokinase (CPK), plasma glutamic oxaloacetic transaminase (SGOT) and plasma

glutamic pyruvic transaminase (SGPT) by using commercial diagnostic kits and following the instructions of the manufacturer. All biochemical parameters were analyzed on an auto analyzer (Biochemical Systems International SRL Model 3000 evolution, Italy).

3.2.13.2 Histopathological studies

The treated rats as well as the control group of rats were sacrificed with an overdose of sodium pentobarbital after 48 h of treatment. Various organs such as the liver, kidney and heart tissues were dissected out immediately, cut into 5 mm^3 sizes and washed with PBS (1X, pH 7.4) to remove the adhered blood. The tissues were fixed in 10.0% neutral buffered formalin and then processed by dehydrating with increasing grades of ethanol, and finally embedded in paraffin (ParaplastTM resin). Paraffin sections (5.0 µm) were processed for light microscopic observation (Leica DM 3000) by hematoxylin eosin staining [46].

3.2.14 Determination of *in vivo* defibrinogenating activity and anticoagulant potency

For determination of *in vivo* defibrinogenating and anticoagulant activities, different doses of purified protein (0.125, 0.25 and 0.5 mg/kg body weights of rats, n=6) was administered via i.p injection and active fractions (12.5-50 mg/kg weight of mice, n=6)/component (12.5-50 mg/kg body weight, mice, n=6) were administered orally and blood was withdrawn 5 h after injection by retro orbital bleeding.

The PT, APTT, tail bleeding time, and plasma Ca-clotting time of control and treated groups of rats were determined as described in sections 3.2.8.1, 3.2.8.2, 3.2.8.3, respectively. For quantitative determination of plasma fibrinogen, FIBRO-TEK® (r2 diagnostic, India) kit was used. In a pre-warmed test tube (at 37 °C) 0.2 ml of PPP was taken and incubated at 37 °C for 2 min. Further, reagent 1 pre-warmed at 37 °C was added to the reaction mixture. The plasma fibrinogen concentration in active fraction/protein treated and control rodent plasma was measured with respect to standard calibration curve and expressed in terms of mg/dl [4,47]. At the end of experiments, the rats were euthanized with an over-dose of Na-pentobarbital as per the recommendations of the CPCSEA.

3.2.15 Determination of *in vivo* antithrombotic activity

The in vivo antithrombotic activity of active fractions/components was assessed in carrageenan-induced thrombosis in rodent (rat/mice) model [48] with a slight modification as described by Majumdar et al. [49]. A total of 30 animals were randomly subdivided into 5 groups, each group containing 6 animals (males and females). The animal in group 1 was treated with 1X PBS, pH 7.4 (the control, placebo). The animal in groups 2, 3, and 4 were orally treated with graded concentrations (12.5, 25, and 50 mg/kg in 1X PBS, pH 7.4, respectively) of active fractions/component in a total volume of 200 µl for a week. The animal in group 5 (positive control) was orally treated with a 50 mg/kg of Nattokinase for a week. Thirty minutes after the last treatment with active fractions/component and nattokinase, the tails were ligated and 0.9 mg/kg body weight of k-carrageenan was given by intravenous injection [49,50]. Ligatures were removed after 15 min of injection. The length of the infarcted region was measured, and the appearance of the wine-colored thrombus formation in the tails was photographed after 48 h of treatment by the above-mentioned thrombolytic agents. The thrombus lengths in the tails were measured at 24, 48, and 72 h [49].

3.3. Statistical analysis

Data are expressed either as means or as mean ± standard deviation (S.D.) of three experiments or unless otherwise indicated. The statistical analysis of the data was done using the student's t-testone way ANOVA using the SigmaPlot 10.0 for Windows (version 7.0) software and Graph Pad software, respectively. Values of $p < 0.05$ and 0.01 were considered to be statistically significant.

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