Characterization and mechanism of anticoagulant action of a fibrin(ogen)olytic serine protease (Lunathrombase) purified from active fraction of *L. Indica* (AFLI)

5.1 Brief Introduction

Formation of endogenous thrombi in blood vessels is one of the leading causes of death. According to data provided by the World Health Organization (WHO) in 2017, heart diseases are responsible for 31% of the total mortality rate in the world. Further, higher levels of fibrinogen (hyperfibrinogenemia) have been reported to alter the hemodynamic properties of blood that subsequently enhance the intravascular fibrin deposition and pose as an independent risk factor for both arterial and venous thrombosis [1,2]. Higher levels of fibrinogen have also been reported to induce lipid proliferation that initiates the development of atherosclerosis, resulting in ischemic pathology [3]. Therefore, anticoagulant fibrinogenolytic enzymes capable of inhibiting thrombin have proven to be effective in preventing thrombosis [4-7] and treating hyperfibrinogenemia-associated disorders [8,9].

Till date proteases from snake venoms, leeches, annelids, insects, algae, caterpillars, microbes and other organisms have been isolated, characterized and shown to be useful for blood coagulation and fibrinolysis [7,10-12]. During recent years the research on traditional knowledge based drugs from plant origin has gained considerable importance due to low cost and less side effects in comparison with the first-generation thrombolytic agents (streptokinase and urokinase), and anticoagulants agents (warfarin and heparin). Despite these efforts, except for a few limited examples, fragmentary data is available on plant fibrin(ogen)olytic enzymes possessing anticoagulant activity [10,13-15]. Therefore, the isolation and characterization of highly specific fibrin(ogen)olytic protease from natural sources is a pre-requisite study in the treatment of thrombotic disorders. To the best of our knowledge, this is the first report on the biochemical and pharmacological characterization, and elucidation of the anticoagulant mechanism of a fibrin(ogen)olytic serine protease purified from the aqueous leaf extract of *L. indica*. This plant-derived fibrinogenolytic serine protease, named Lunathrombase, demonstrated dual inhibition of thrombin and FXa, and did not show *in vivo* toxicity in experimental animals which has never before been demonstrated for any protease, and the finding suggests its therapeutic application as an anticoagulant, antithrombotic drug

5.2 Results

5.2.1 Purification of major fibrinogenolytic protease from the leaves of *L. indica*

Fractionation of crude aqueous leaf extracts of *L. indica* through an anion exchange matrix resulted in separation of proteins into nine peaks (Fig. 5.1a). Peak1 (AEX_1) eluted with the equilibration buffer (unbound fractions) showed significant fibrinogenolytic and anticoagulant activities. Cation-exchange chromatography was used for the AEX_1 fraction, which was separated into eight fractions (CEX_1 to CEX_8) (Fig. 5.1b). The unbound peak CEX_1, which was eluted with the equilibration buffer demonstrated significant fibrinogenolytic and anticoagulant activities. HPLC gel filtration chromatography of the CEX_1 fraction showed a single peak (AF_GF1) (Fig. 5.1c). The SDS-PAGE (reduced) analysis of 20 µg of protein from the AF_GF1 peak proteins revealed a single, distinct band for a 35 kDa protein (Fig. 5.1d), which was named lunathrombase. The molecular mass of lunathrombase was determined by MALDI-ToF-MS to be 34767.52 Da (Fig. 5.1e).The summary of purification of lunathrombase is shown in Table 5.1.

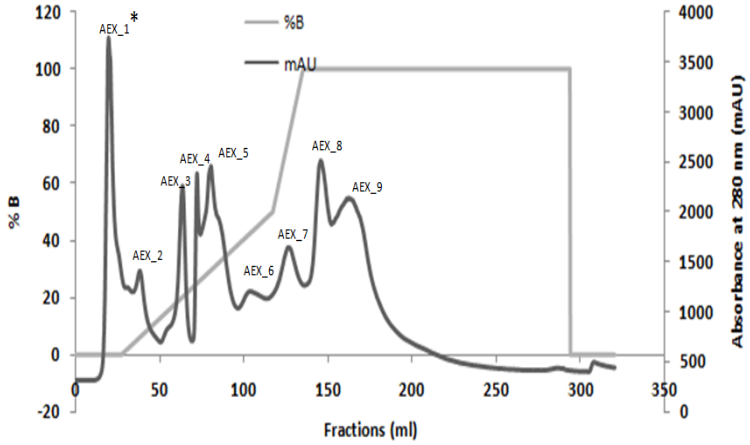

Fig. 5.1a. Fractionation of crude aqueous shade leave extract of *L. indica* on a PrepTM anion exchange DEAE-cellulose FF 16/10 column. After washing the column with two volume of equilibration buffer (20 mM K.P buffer, pH 7.4),the bound fraction were eluted with a linear gradient of 0.1 – 1.0 M NaCl in 20 mM K.P buffer at pH 7.4 at a flow rate of 1.0 ml/min. The elution profile was monitored at 280 nm. The first peak (AEX_1) corresponds to the elution of fraction showing highest anticoagulant and fibrin(ogeno)lytic activities.

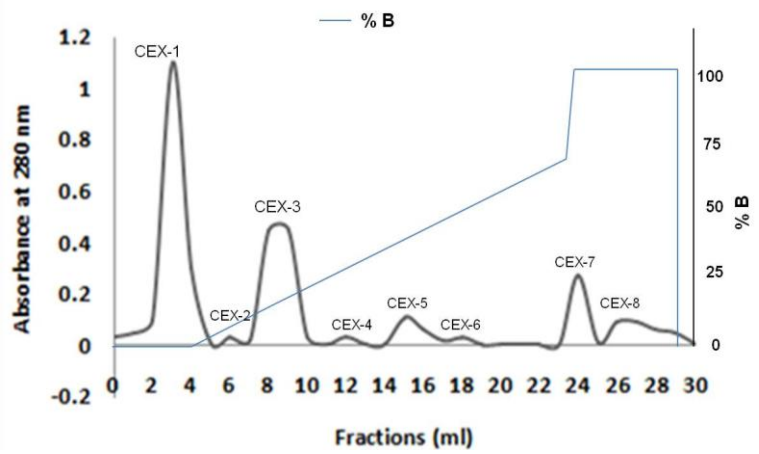

Fig. 5.1b. Fractionation of the anion-exchange unbound fraction (AEX_1 peak) on cation exchange CM-cellulose (20 mm X 60 mm) column. After washing the column with two volume of equilibration buffer (20 mM K.P buffer, pH 7.4), the bound fraction were eluted with a linear gradient of 0.1 – 1.0 M NaCl in 20 mM K.P buffer at pH 7.4 at a flow rate of 0.5 ml/min. The elution profile was monitored at 280 nm. The *L. indica* first peak (CEX_1) corresponds to the elution of fraction showing highest anticoagulant and fibrin(ogeno)lytic activities.

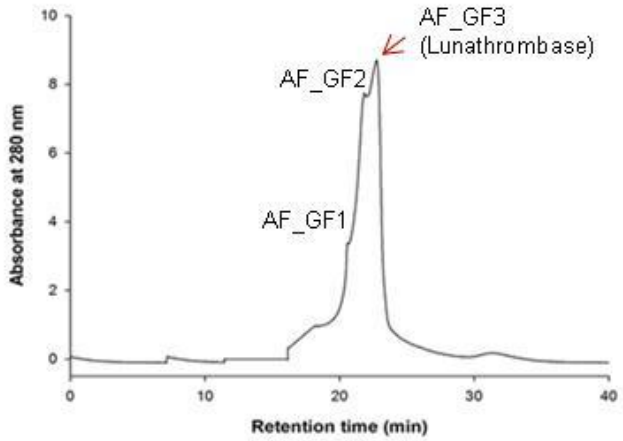

Fig. 5.1c. Gel filtration of the CEX_1 on Shodex KW-803 column (5 µm, 8 x 300 mm). After washing the column with two volume of equilibration buffer (20 mM K.P buffer, containing 150 mM NaCl pH 7.4). Elution was carried out with equilibration buffer at a flow rate of 0.5 ml/min. The red arrow indicates elution of lunathrombase

Fig. 5.1d. Determination of purity and molecular mass of AF_GF3 (lunathrombase) by 12.5% SDS-PAGE; Lane 1, protein molecular markers; lane 2, reduced lunathrombase (20.0 µg).

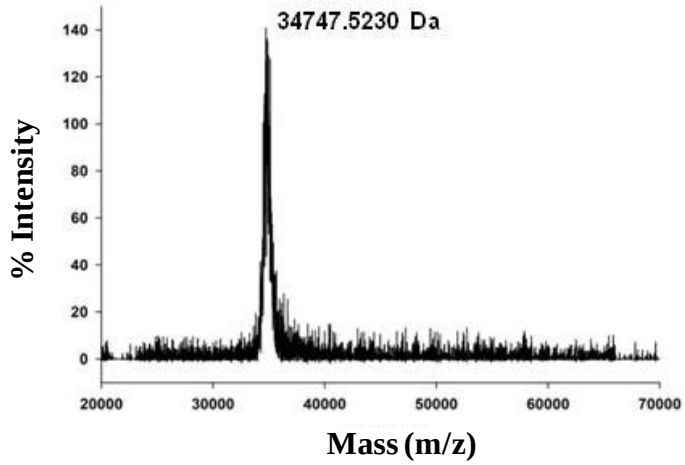

Fig. 5.1e. MALDI-ToF mass spectra of lunathrombase (5.0 µg). 1.0 µg of the lunathrombase was mixed with 1 µg of a-cyano-4 hydroxycinnamic acid matrix dissolved in 50% (v/v) acetonitrile containing 0.1 % (v/v) TFA. The mixture was then spotted onto an Opti-TOF-384 plate (ABSciex), dried, and analyzed in positive linear mode using an acceleration voltage of 25 kV and laser intensity of 3000. For experimental details see section 3.2.6.1.2.

Table 5.1. A summary of purification of lunathrombase from aqueous leaves extract of *L. indica*. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to crude aqueous extract, * $p < 0.01$

Fraction (1.0 µg/ml)	**Specific activity (Units/mg)**	
	Anticoagulant[a]	**Fibrinogenolytic[b]**
Crude aqueous leave extract	$30.5 \times 10^3 \pm 1.00$	8.10 ± 0.64
Anion exchange unbound fraction (AEX_1)	$34.06 \times 10^3 \pm 1.00$ *	7.90 ± 0.30
Cation exchange unbound fraction (CEX_1)	$46.4 \times 10^4 \pm 2.8$ *	12.13 ± 1.02 *
Gel filtrations		
AF_GF1	$15.5 \times 10^3 \pm 2.5$	4.20 ± 1.6
AF_GF2	$12.3 \times 10^3 \pm 1.5$	2.20 ± 1.3
AF_GF3 (lunathrombase)	$32.5 \times 10^3 \pm 3.5$	5.50 ± 1.4

[a]One unit of anticoagulant activity is defined as 1 s increase in clotting time of the control PPP in presence of sample

[b]One unit (U) of fibrinogenolytic activity is defined as 1.0 µg of tyrosine liberated for fibrinogen per min per ml of sample at 37 °C, pH 7.4

5.2.2 Peptide mass fingerprinting, *de novo* sequencing, amino acid composition, and secondary structure analyses of lunathrombase

Tandem mass spectrometry analysis and *de novo* sequencing of lunathrombase did not reveal its similarity with any plant protein, suggesting that it may be a new plant protein. Nevertheless, one of the MS-MS-derived tryptic fragments of lunathrombase (IITHPNFNGNTLDNDIMLIK) demonstrated a conserved domain belonging to the trypsin-like superfamily suggesting that lunathrombase may be a previously uncharacterized plant protease. The alignment of IITHPNFNGNTLDNDIMLIK with other trypsin (-like) enzymes is shown in Fig. 5.1f.

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1H9H|E              ------------------------IVGGYTCSAANSIPYQVSLNSGSHFCSGGSLINSQWV
NP_001156363.1      MNTFVLLALLGAAVAFPTDDDDKIVGGYTCA-ANSVPYQVSLNSGSHFCG-GSLINSQWV
1EPT|B              ------------------------------------------------------------
XP_020934119.1      MNTFVLLALLGAAVAFPTDDDDKIVGGYTCA-ANSIPYQVSLNSGSHFCG-GSLINSQWV
P00761.1            ----------------FPTDDDDKIVGGYTCA-ANSIPYQVSLNSGSHFCG-GSLINSQWV
Lunathrombase       ------------------------------------------------------------


1H9H|E              VSAAHCSYKSRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPA
NP_001156363.1      VSAAHC-YKSRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPA
1EPT|B              ---------SRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPA
XP_020934119.1      VSAAHC-YKSRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPA
P00761.1            VSAAHC-YKSRIQVRLGEHNIDVLEGNEQFINAAKIITHPNFNGNTLDNDIMLIKLSSPA
Lunathrombase       -----------------------------------IITHPNFNGNTLDNDIMLIK-----
                                                        ******************


1H9H|E              TLNSRVATVS-PRSCAAAGTECSLISGWGNTKSSGSSYPSLLQCSLKAPVLSDSSCKSSY
NP_001156363.1      TLNSRVATVSLPRSCAAAGTEC-LISGWGNTKSSGSSYPSLLQC-LKAPVLSDSSCKSSY
1EPT|B              TLNSRVATVSLPRSCAAAGTEC-LISGWGNTK----------------------------
XP_020934119.1      TLNSRVATVSLPRSCAAAGTEC-LISGWGNTKSSGSSYPSLLQC-LKAPVLSDSSCKSSY
P00761.1            TLNSRVATVSLPRSCAAAGTEC-LISGWGNTKSSGSSYPSLLQC-LKAPVLSDSSCKSSY
Lunathrombase       ------------------------------------------------------------


1H9H|E              PGQITGNMICVGFLEGGKDSCSQGDSGGPVVCSNGQLQGIVSWGYGCSAQKNKPGVYTKV
NP_001156363.1      PGQITGNMICVGFLEGGKDSC-QGDSGGPVVC-NGQLQGIVSWGYGCA-QKNKPGVYTKV
1EPT|B              ------------------------------------------------------------
XP_020934119.1      PGQITGNMICVGFLEGGKDSC-QGDSGGPVVC-NGQLQGIVSWGYGCA-QKNKPGVYTKV
P00761.1            PGQITGNMICVGFLEGGKDSC-QGDSGGPVVC-NGQLQGIVSWGYGCA-QKNKPGVYTKV
Lunathrombase       ------------------------------------------------------------


1H9H|E              CNYVNWIQQTIAAN
NP_001156363.1      CNYVNWIQQTIAAN
1EPT|B              --------------
XP_020934119.1      CNYVNWIQQTIAAN
P00761.1            CNYVNWIQQTIAAN
Lunathrombase       --------------
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Fig. 5.1f. Multiple sequence alignment of tryptic fragment of lunathrombase with homologous proteins deposited in the NCBI database. 1H9H/E, chain E, complex of Eeti-Ii with porcine trypsin; NP_001156363, trypsinogen precursor [*Susscrofa*]; 1EPT/B, chain B, refined 1.8 angstroms resolution crystal structure of porcine epsilon-trypsin; XP_020934119.1, trypsinogen isoform X1 [*Susscrofa*] ; P00761.1, full=trypsin, flags: precursor.

Analysis of the amino acid composition of lunathrombase using Swiss-prot and TrEMBL databases did not reveal any similarity with other proteins from plants (Table 5.2). The combined results indicate that lunathrombase is a novel protease from *L. indica*. In addition, analysis of the CD spectrum of lunathrombase revealed that it consists of 23.4% α-helix, 17% beta sheets, with a turn of 28.2% and 31% random coils (Fig. 5.1g).

Table 5.2. Amino acids composition of lunathrombase (% nmol). Values are mean of triplicate determinations.

Amino acids	% nM
ASP	4.2
HIS	5.0
GLU	23.1
SER	1.1
CYS	1.5
ARG	1.0
TYR	2.6
VAL	22.0
MET	3.0
PHE	2.0
ILE	1.3
LEU	1.8
LYS	30.0
PRO	1.4

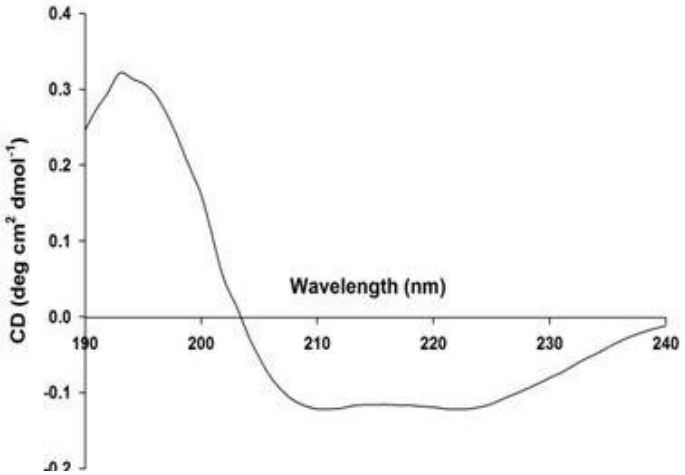


Fig. 5.1g. Circular dichroism (CD) spectra of lunathrombase. Native lunathrombase (0.3 mg/ml) was dissolved in 20 mM potassium phosphate buffer pH 7.0 and the far UV-CD spectra was recorded at room temperature (~25 °C) between 190 and 240 nm against the appropriate buffer (blank). For experimental details see section 3.2.6.3.

5.2.3 Lunathrombase demonstrated anticoagulant activity

Lunathrombase dose-dependently prolonged the Ca^{2+} clotting time of PPP, and at a concentration of 400 nM, saturation in anticoagulant activity was observed (Fig. 5.2a).

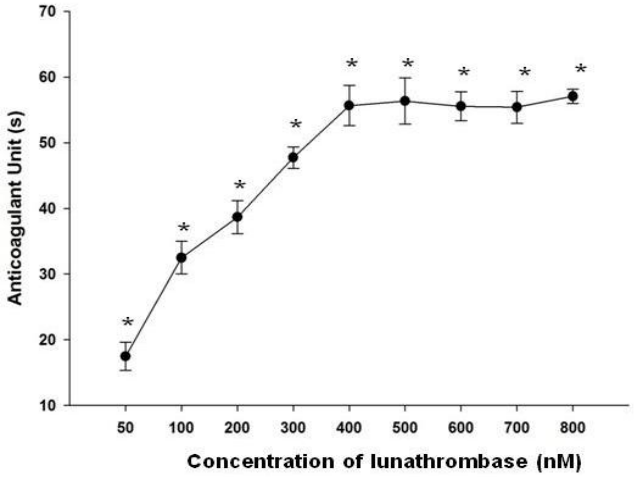


Fig. 5.2a. Dose- dependent *in vitro* anticoagulant activity of lunathrombase against human platelet-poor plasma. Ca- Clotting time of PPP under identical experimental conditions (control) was found to be 96.8 ± 1.8 s (mean ± S.D). Significance of difference with respect to control (without lunathrombase) * $p< 0.05$.

Lunathrombase demonstrated optimum anticoagulant activity at 10 min of pre-incubation with PPP (Fig. 5. 2b). At a concentration of 500 nM, lunathrombase did not affect APTT, though it significantly ($p<0.05$) enhanced the PT of PPP (Fig. 5.2c).

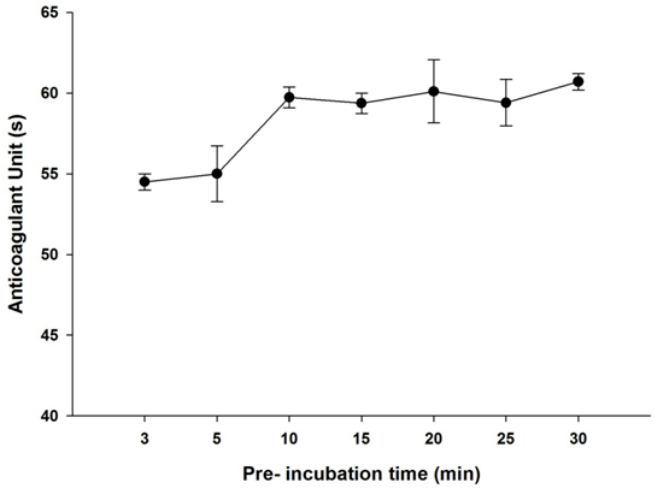


Fig. 5.2b. Time- dependent anticoagulant activity by lunathrombase (400 nM) against human PPP. Ca- Clotting time of PPP under identical experimental conditions (control)

was found to be 96.8 ± 1.8 s (mean ± S.D). All values are means ± S.D. of three independent experiments. * P< 0.05.

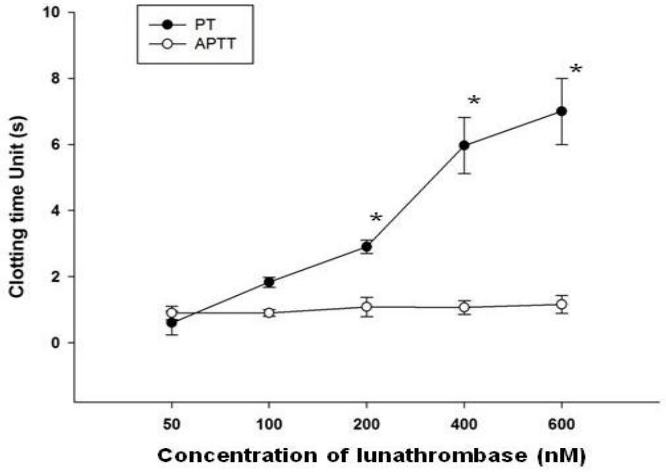


Fig. 5.2c. Effect of lunathrombase (50-600 nM) on APTT and PT of PPP isolated from human blood. APTT and PT of PPP under identical experimental conditions (control) was found to be 28.2 ±1.1 and 14.4 ± 1.2 s (mean ± S.D), respectively. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control (without lunathrombase) * p< 0.05.

5.2.4 Fibrin(ogen)olytic activity of lunathrombase

Lunathrombase showed dose- and time-dependent fibrin(ogen)olytic activity. The kinetics of fibrinogen/fibrin degradation indicated that lunathrombase preferentially degraded the Aα chain of fibrinogen/fibrin (Fig. 5.3a). With an increase in incubation time to 120 min, the Bβ chain was also removed; however, the γ-chain of fibrinogen/fibrin remained intact after 2 h of incubation at 37°C (Fig. 5.3a).

The fibrinogenolytic activity of lunathrombase was found to be superior to nattokinase and plasmin (Fig. 5.3b); whereas, the fibrinolytic activity of lunathrombase surpassed that of nattokinase, streptokinase, and plasmin under identical experimental conditions (Fig. 5.3c). The *Km and Vmax* values of lunathrombase towards fibrinogen were determined to be 52.64 ± 9.8 µM and 52.33 ± 5.7 µM/min (mean ± SD), respectively (Fig. 5.3d) whereas the *Km and Vmax* values of nattokinase towards fibrinogen were determined to be 2.88 ± 1.1 µM and 1.97 ± 0.5 µM/min, respectively (Fig. 5.3e).

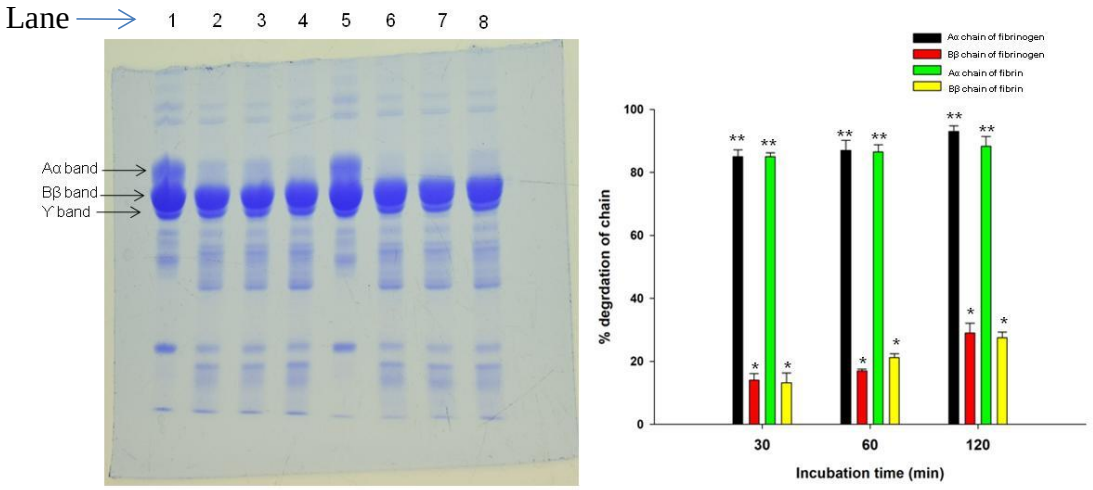


Fig. 5.3a. Kinetics of fibrinogenolytic activity of lunathrombase. The degradation products were separated by 12.5% SDS-PAGE (reducing conditions). Lane 1, control human fibrinogen (0.25% w/v in 20 mM K-phosphate buffer, 150 mMNaCl, pH 7.4); lanes 2-4, human fibrinogen treated with lunathrombase (0.2 µM) for 30, 60 and 120 min, respectively, at 37 ºC, pH 7.4. Kinetics of fibrinolytic activity of lunathrombase. Lane 5, control human fibrin; lanes 6 - 8, human fibrin treated with 0.2 µM lunathrombase for 30, 60 and 120 min, respectively, at 37 ºC, pH 7.4. **Inset**. Densitometry analysis to determine the percent degradation of Aα- and Bβ- chains of fibrinogen/fibrin. Significance of difference with respect to control Aα chain of fibrinogen/fibrin (0% degradation), ** p< 0.01; Significance of difference with respect to control Bβ chain of fibrinogen/fibrin (0% degradation) * p< 0.05.

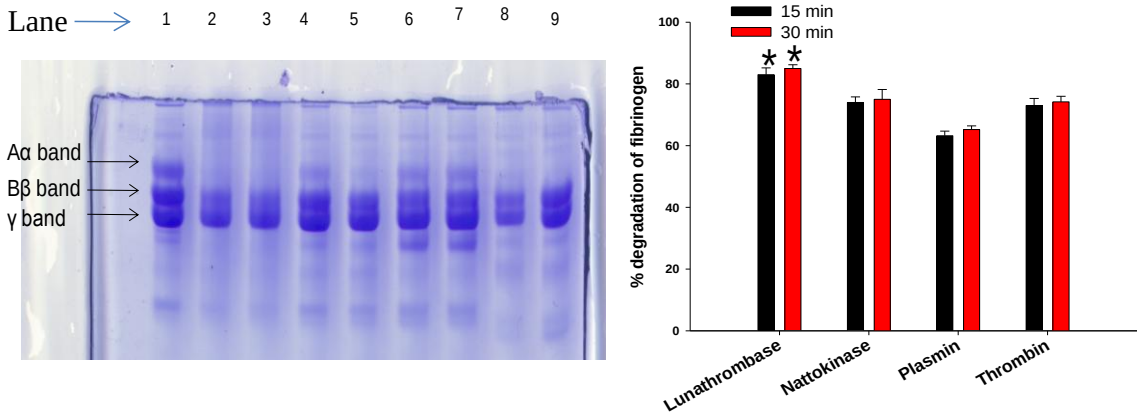


Fig. 5.3b. A comparison of fibrinogenolytic activity among lunathrombase, nattokinase, plasmin, and thrombin under identical conditions by 12.5% SDS-PAGE analysis. Lane 1, control human fibrinogen (0.25% w/v in 20 mM K-phosphate buffer, 150 mM NaCl, pH 7.4); lanes 2- 3, human fibrinogen treated with lunathrombase (0.2 µM); lanes 4- 5,

human fibrinogen treated with nattokinase (0.2 µM); lanes 6-7, human fibrinogen treated with plasmin (0.2 µM); lanes 8-9, human fibrinogen treated with thrombin (0.2 µM) for 15, 30 min, respectively, at 37 ºC, pH 7.4. **Inset.** Densitometry analysis to determine the percent degradation of fibrinogen. All values are means ± S.D. of three independent experiments. Significance of difference with respect to nattokinase, plasmin and thrombin, * p< 0.05.

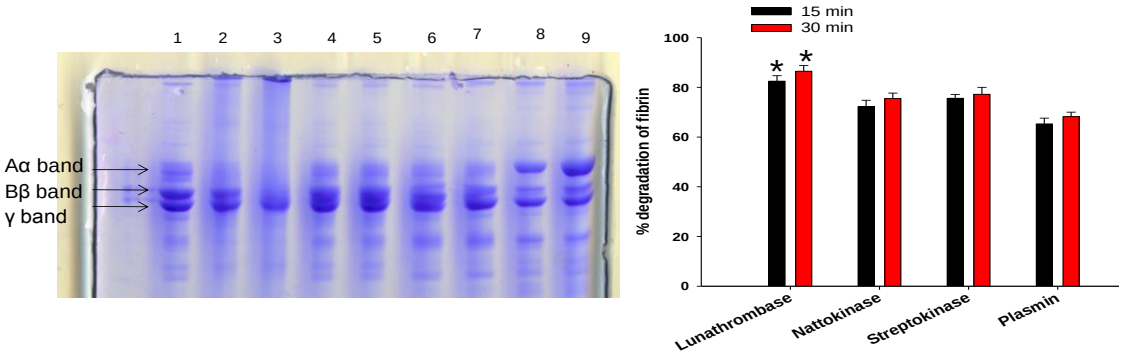


Fig. 5.3c. Determination of fibrinolytic activity of lunathrombase, nattokinase, streptokinase, and plasmin under identical experimental conditions by 12.5% SDS-PAGE analysis. Lane 1, control human fibrin (0.25% w/v in 20 mM K-phosphate buffer, 150 mM NaCl, pH 7.4); lanes 2- 3, human fibrin degradation by lunathrombase (0.2 µM); lanes 4- 5, human fibrin degradation by nattokinase (0.2 µM); lanes 6-7, human fibrin degradation by streptokinase (0.2 µM); lanes 8-9, human fibrin degradation by plasmin (0.2 µM) at 15, 30 min of incubation, respectively, at 37 ºC, pH 7.4. **Inset.** Densitometry analysis to determine the percent degradation of fibrin. All values are means ± S.D. of three independent experiments. Significance of difference with respect to nattokinase, streptokinase and plasmin, * p< 0.05.

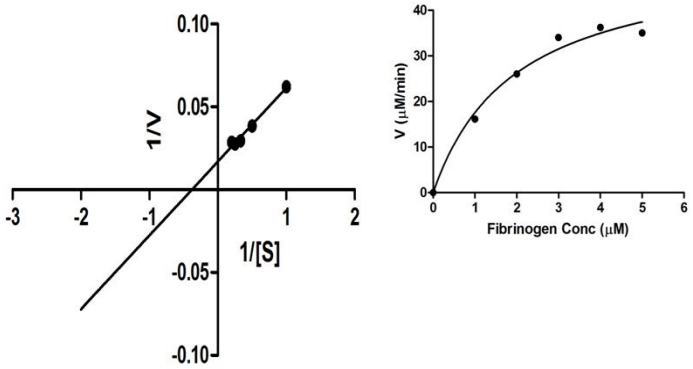


Fig. 5.3d. Determination of *Km* and *Vmax* values of lunathrombase against fibrinogen by Lineweaver-Burk plot and Michaelis-Menton plot (inset).

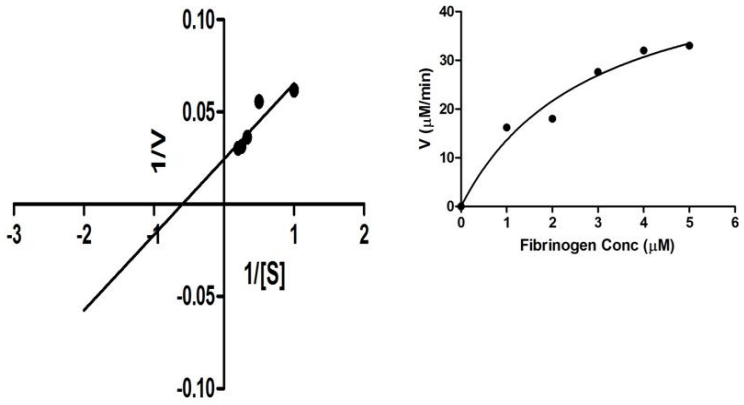


Fig. 5.3e. Determination of *Km* and *Vmax* values of nattokinase against fibrinogen by Lineweaver-Burk plot and Michaelis-Menton plot (inset).

5.2.5 RP-UHPLC analysis of fibrinogen degradation by lunathrombase and nattokinase

The RP-HPLC analysis indicated that lunathrombase and nattokinase cleave different sites of fibrinogen/fibrin resulting in different elution profiles of the fibrinogen/fibrin degradation products from the RP-HPLC column (Fig. 5. 3f).

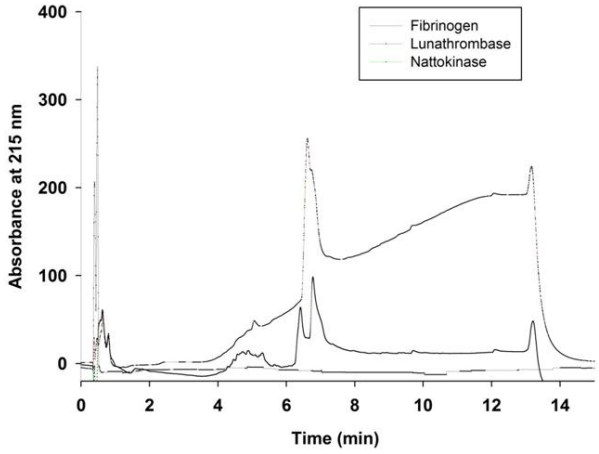


Fig. 5.3f. A comparison of fibrinogen degradation under identical experimental conditions between nattokinase and lunathrombase by RP-UHPLC analysis. Human fibrinogen solution (2.5 mg/ml in 20 mM potassium phosphate buffer containing 100 mM NaCl, pH 7.4) was incubated with 0.2 µM of lunathrombase or nattokinase for 15 min at 37 °C. For experimental details see section 3.2.7.5.

5.2.6 FPA and FPB did not influenced the fibrinogenolytic activity of lunathrombase

The lunathrombase-mediated degradation of fibrinogen in the presence of FPA or FPB, or with both FPA and FPB, did not result in the inhibition of fibrin(ogeno)lytic activity, when compared to controls (without fibrinopeptides) (Table 5.3).

Table 5.3. Effect of FPA and FPB on fibrinogenolytic activity of lunathrombase. Values are mean ± SD of triplicate determinations.

	Fibrinogenolytic activity (U/mg)
Lunathrombase	5.80 ± 1.2
Lunathrombase + FPA	5.45 ± 2.1
Lunathrombase + FPB	5.55 ± 1.8
Lunathrombase + FPA +FPB	5.30 ± 1.2

5.2.7 Substrate specificity and effect of temperature and pH on catalytic activity of lunathrombase

Lunathrombase demonstrated preferential hydrolysis of bovine plasma fibrinogen followed by fibrin; however, lunathrombase displayed poor hydrolytic activity against casein or globulin or albumin (Table 5.4). It showed optimum fibrin(ogen)olytic activity at 35-37°C (Fig. 5.4a) and at pH 7.0-7.4 (Fig. 5.4b). The SDS-PAGE analysis also suggested that lunathrombase was unable to degrade any of the tested extracellular matrix proteins, namely Type-IV collagen, laminin, and fibronectin at physiological conditions (37°C, pH 7.4) (Fig. 5.5c).

Table 5.4. The substrate specificity of lunathrombase at 37 °C, pH 7.4.Values represents mean ± SD of triplicate determinations. Significance of difference with respect to fibrinogenolytic activity $^{*}p < 0.001$.

Substrate (1mg/ml)*	**Activity (U/mg)**
Bovine plasma fibrinogen	5.50 ± 1.4
Fibrin	3.70 ± 0.4
Casein	0.20 ± 0.01*
Bovine serum γ-globulin	0.3 ± 0.04*
Bovine serum albumin	0.01± 0.001*

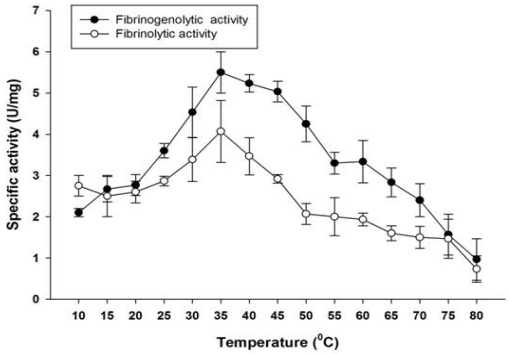


Fig. 5.4a. Effect of temperature (10-80 ºC) on fibrin(ogen)oytic activity of lunathrombase. The values are mean ± S.D. of triplicate determinations.

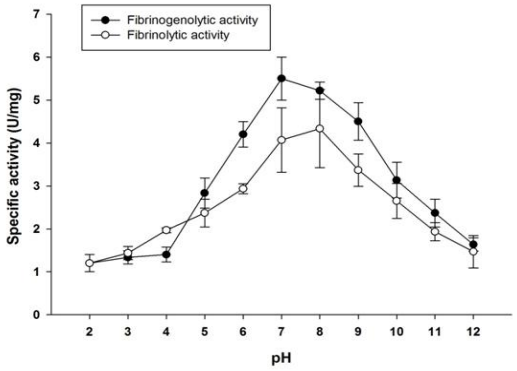


Fig. 5.4b. Influence of pH (2-12) on fibrin(ogen)oytic activity of lunathrombase. The values are mean ± S.D. of triplicate determinations.

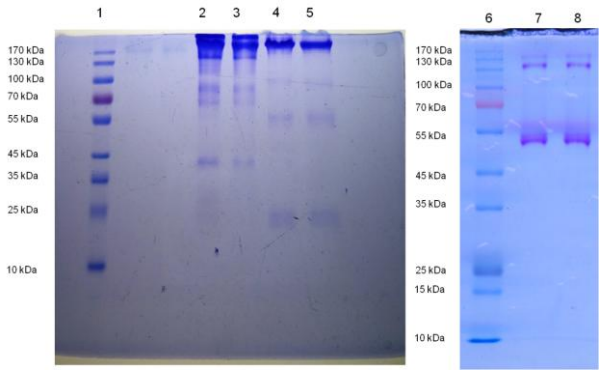


Fig. 5.4c. Degradation of flbronectin, laminin, and type-IV collagen by lunathrombase. ECM proteins (laminin, type-IV colIagen, and fibronectin) were incubated with lunathrombase in a substrate: enzyme ratio of 15: I (w/w) at 37 °C, pH 7.4 for 12 h and the degradation products were analyzed by 10% SDS-PAGE (reduced conditions). Lanes 1 and 6, protein molecular markers; lanes 2, 4, and 7 control laminin, type-IV collagen, and fibrinonectin respectively; lanes 3, 5, and 8 laminin, type-IV collagen, and fibronectin, respectively treated with lunathrombase (0.2 µM).

5.2.8 Lunathrombase is devoid of N- or O-linked oligosaccharides

Lunathrombase contained 7.0% of neutral sugar, though it did not contain N- or O-linked oligosaccharides (Fig. 5.5).

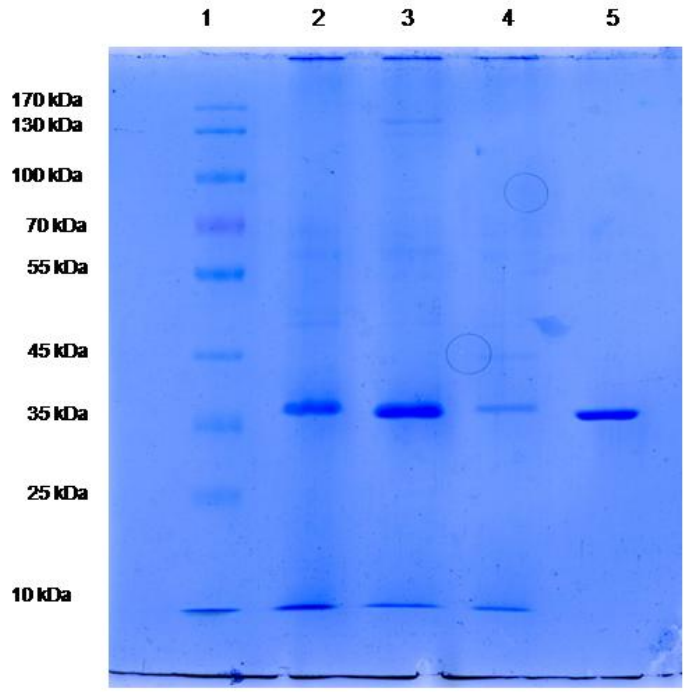


Fig. 5.5. Determination of glycosylation in lunathrombase. Lane 1, protein marker; lane 2, denatured (heated at 100 °C for 10 min)lunathrombase (20 µg,) incubated at 37 °C for 4 h; Lanes 3 & 4, denatured lunathrombase (20 µg) treated with PNGase (500 units) and neuraminidase, respectively, for 4 h at 37 °C; Lane 5, native lunathrombase (20 µg).

5.2.9 Inhibitor study shows lunathrombase is a serine protease and its activity is not influenced by the endogenous protease inhibitors of plasma

The fibrin(ogen)olytic activity of lunathrombase was not affected ($p>0.05$) by any of the tested metal ions (Fig. 5.6a), though itwas inhibited by PMSF (a serine protease inhibitor), iodoacetamide (a cysteine protease inhibitor), and pBPB (a histidine inhibitor) (Table 5.6). Nevertheless, EDTA (a metalloprotease inhibitor) and DTT (a disulfide bond reducing agent) failed to inhibit the fibrin(ogen)olytic activity of lunathrombase (Table 5.6).

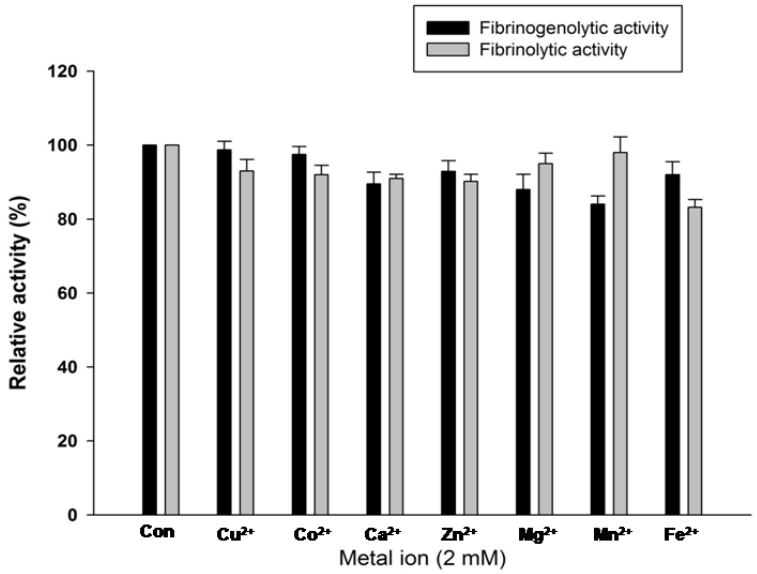


Fig. 5.6a. Effect of metal ions (2 mM) on fibrin(ogen)oytic activity of lunathrombase. The values are mean ± S.D. of triplicate determinations.

Table 5.5. Effect of inhibitors on fibrinogenolytic activity of lunathrombase. Values represent mean ± SD of three determinations. Significance of difference with respect to control. * $p< 0.01$, ** $p < 0.05$

Inhibitors	**Relative activity (%)**
Control (without inhibitor)	100
Inhibitors (concentrations)	
PMSF (2 mM)	23.69 ± 1.3 **
PMSF (4 mM)	18.81 ± 2.2 *
IAA (2 mM)	48.15 ± 2.7 **
IAA (4 mM)	38.40 ± 3.1 *
pBPB (2 mM)	53.38 ± 2.1 **
pBPB (4 mM)	43.12 ± 1.7**
TPCK (100 µM)	87.20 ± 1.1
TLCK (100 µM)	85.23 ± 2.3
DTT (4 mM)	91.51 ± 3.5
EDTA (4 mM)	98.24 ± 5.1
α_2 macroglobulin	97.50 ± 3.2
antiplasmin	96.42 ± 4.5

The inhibitory effects of PMSF, iodoacetamide, and pBPB against fibrin(ogen)olytic activity of lunathrombase were also confirmed by SDS-PAGE analysis of fibrinogen degradation, and in the presence of these inhibitors, the Aα- and Bβ-chains of

fibrinogen were not degraded by lunathrombase (Fig. 5.6b). Further, the endogenous protease inhibitors, α_2 macroglobulin and antiplasmin, did not inhibit the fibrin(ogen)olytic activity of lunathrombase (Table 5.6).

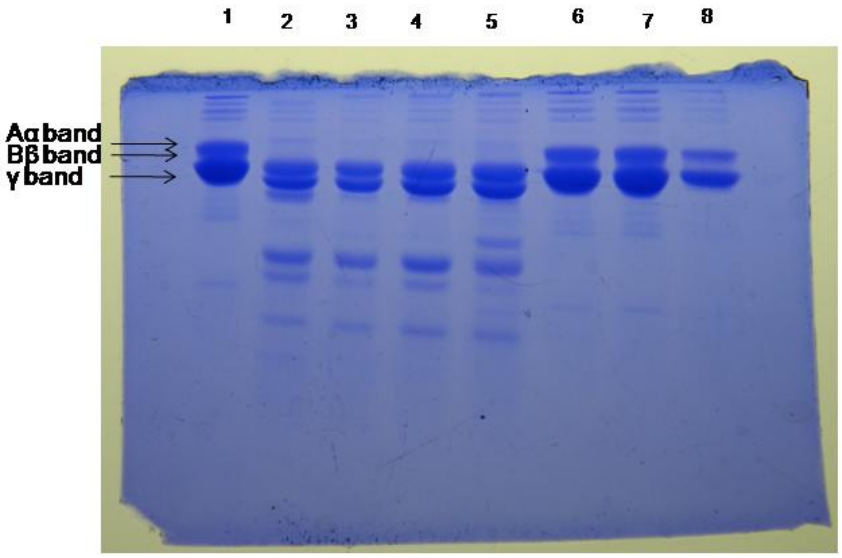


Fig. 5.6b. The effect of inhibitors on fibrinogenolytic activity of lunathrombase. The degradation products were separated by 12.5% SDS-PAGE (reducing conditions). Lane 1, control human fibrinogen (0.25% w/v in 20 mM K-phosphate buffer, 150 mMNaCl, pH 7.4); lanes 2-8, human fibrinogen degradation by lunathrombase (0.2 µM) pre-incubated with 4 mM DTT, EDTA, TPCK, TLCK, PMSF, IAA and pBPB, respectively, for 60 min at 37 ºC, pH 7.4.

5.2.10 Lunathrombase inhibits the pharmacological activity of thrombin and FXa- the key components of blood coagulation

Lunathrombase significantly inhibited the amidolytic activity of thrombin (Fig. 5.7a) and FXa (Fig. 5.7c). The *Ki* value for the inhibition of amidolytic activity of thrombin and FXa by lunathrombase was determined to be 26.90 ± 0.9 nM and 10.35 ± 1.8 nM (mean ± SD, n=3), respectively (Figs 5.7b,d). Lunathrombase dose-dependently prolonged the fibrinogen clotting time of thrombin and the saturated thrombin inhibition was observed at a 200 nM concentration of lunathrombase (Fig. 5.7e). The optimum inhibition was observed at 10 min of pre-incubation with thrombin and 200 nM lunathrombase (Fig. 5.7f). Further, lunathrombase (0.2 µM) completely (100%) inhibited the prothrombin activation by FXa (Fig. 5.7g).

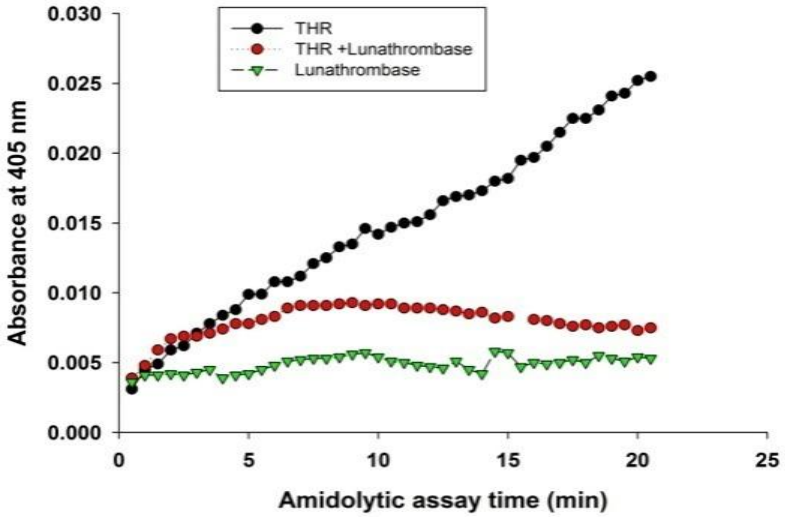


Fig. 5.7a. Effect of lunathrombase (0.2 µM) on amidolytic activity of thrombin (36.6 nM) against its chromogenic substrate T1637 (0.2 mM). The plots are the means of 3 independent measurements.

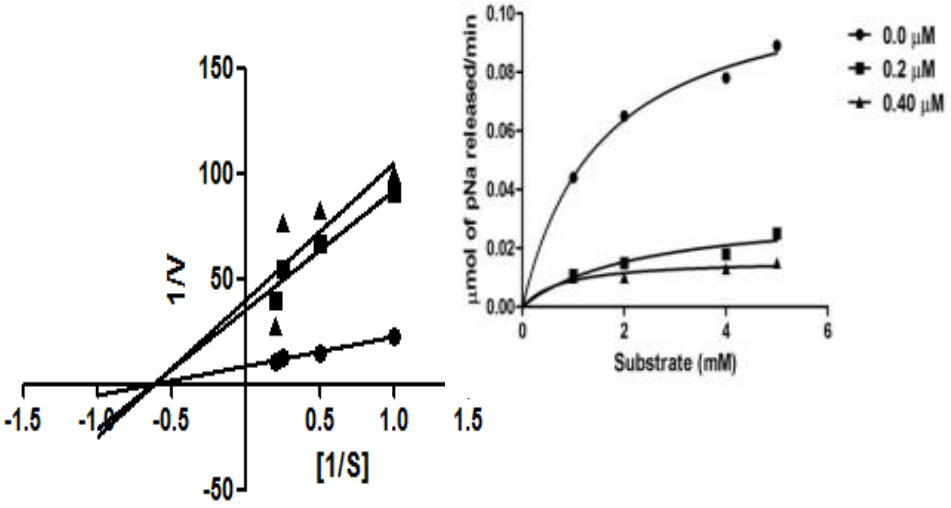


Fig. 5.7b. Lineweaver-Burk and Michaelis-Menton (inset) plot showing inhibition of amidolytic activity of thrombin towards T1637 (0.2mM) by lunathrombase (0- 0.4 µM). The values are mean of triplicate determinations.

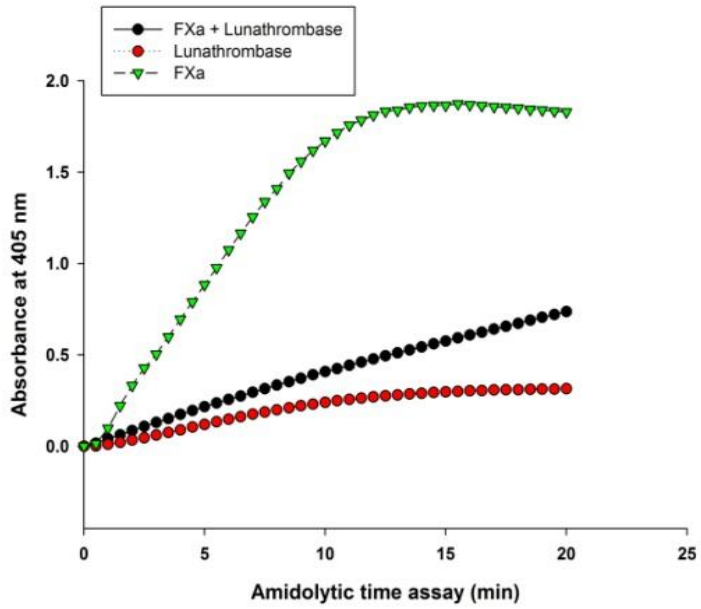


Fig. 5.7c. Effect of lunathrombase (0.2 µM) on amidolytic activity of FXa (0.13 µM) against its chromogenic substrate F3301 (0.2 mM). The plots are the means of 3 independent measurements.

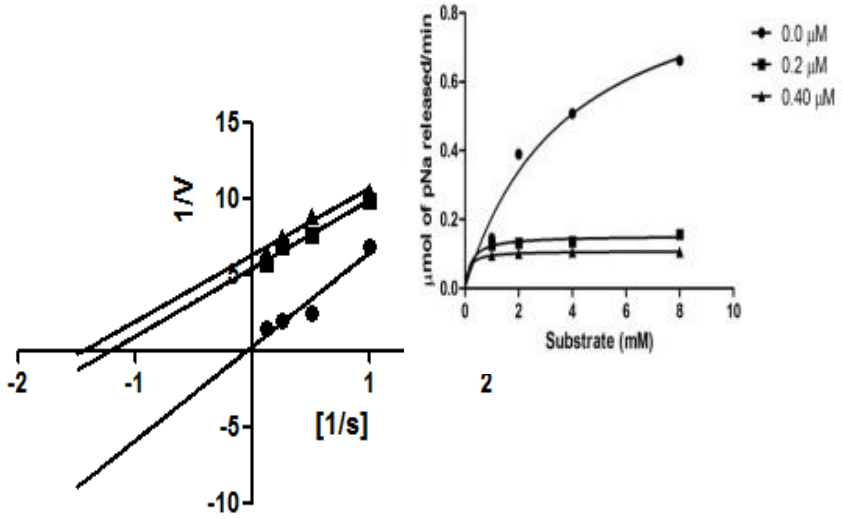


Fig. 5.7d. Lineweaver-Burk and Michaelis-Menton (inset) plot showing inhibition of amidolytic activity of FXa towards F3301 (0.2 mM) by lunathrombase (0- 0.4 µM). The values are mean of triplicate determinations.

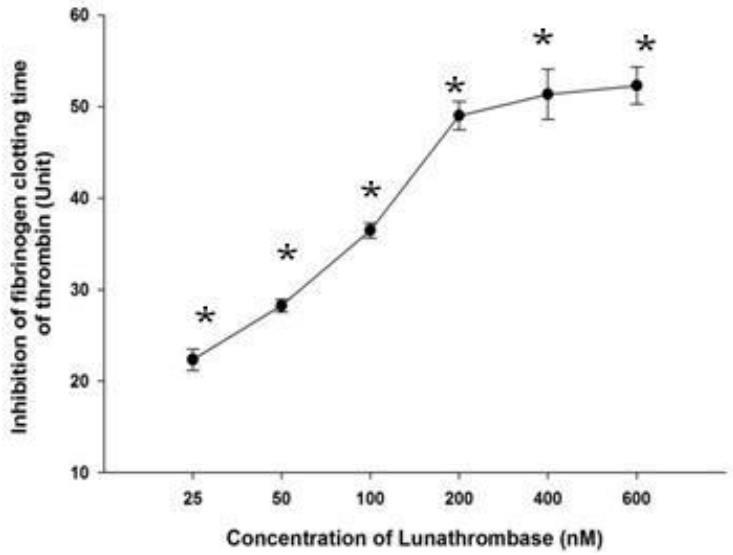


Fig. 5.7e. Dose-dependent (25-600 nM) inhibition of fibrinogen clotting activity of thrombin by lunathrombase at 37 °C, pH 7.4. The values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control (without lunathrombase), * $p < 0.05$.

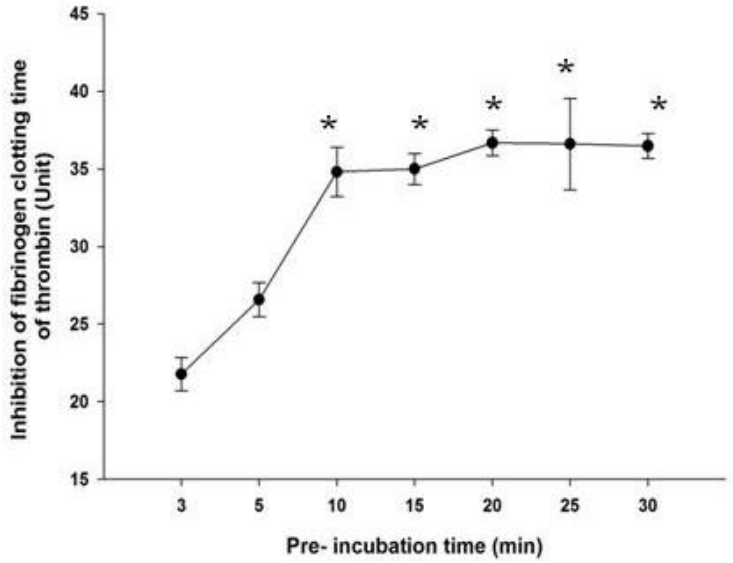


Fig. 5.7f. Time- dependent inhibition of fibrinogen clotting time of thrombin by lunathrombase (100 nM) at 37 °C, pH 7.4. The fibrinogen clotting time of thrombin under identical experimental conditions (control) was found to be 39.17 ± 1.54 s. The values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control (without lunathrombase) * $p < 0.05$.

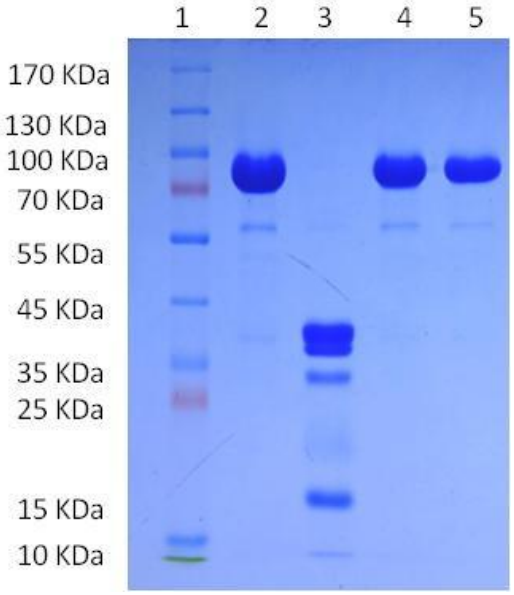


Fig. 5.7g. Inhibition of prothrombin activation property of FXa by lunathrombase. After reduction with β-mercaptoethanol, degradation products were separated by 12.5% SDS-PAGE. Lane 1, protein molecular markers; lane 2, 1.4 µM PTH; lane 3, PTH (1.4 µM) incubated with FXa (0.13 µM) for 30 min at 37 ºC, pH 7.4; lane 4, (0.13 µM FXa pre-incubated with 0.2 µM of lunathrombase for 15 min) + PTH (1.4 µM); lane 5, PTH + lunathrombase. The experiment was repeated three times to assure the reproducibility.

5.2.11 Spectrofluorometric analysis shows the interaction of lunathrombase with thrombin/ fibrinogen/ FXa

A steady decrease in the fluorescence intensity of thrombin was observed in the presence of lunathrombase (Fig. 5.8a). The interaction between lunathrombase and FXa (Fig. 5.8b), or lunathrombase and fibrinogen (Fig. 5.8c) resulted in an increase in the fluorescence intensity, compared to the fluorescence intensity of individual proteins. The dissociation constant (*Kd*) for the binding of lunathrombase to thrombin, FXa, and fibrinogen was calculated to be 0.2492 µM, 1.908 µM, and 0.5516 µM, respectively (Figs. 5a-c).

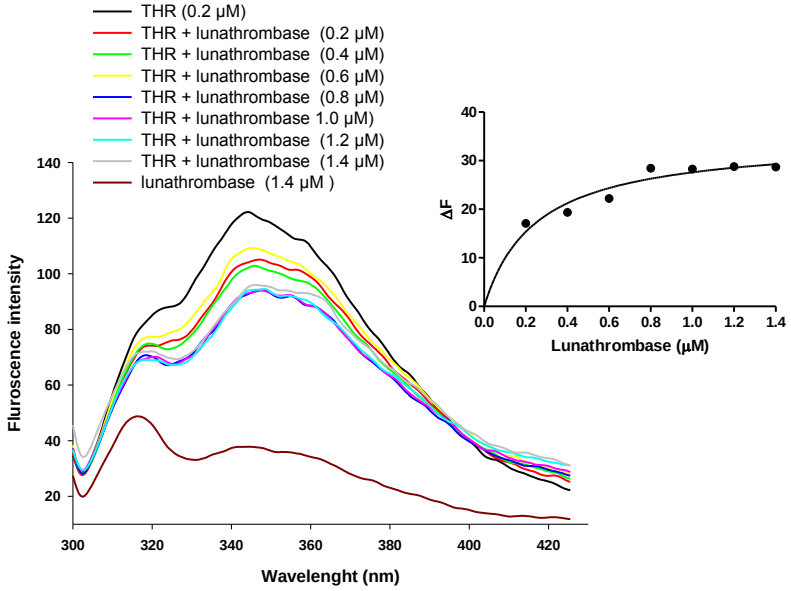


Fig. 5.8a. Fluorescence spectra showing interaction of thrombin (0.2 µM) with different concentrations of lunathrombase (0.2-1.4 µM). Inset. One site saturation binding curve of lunathrombase for thrombin.

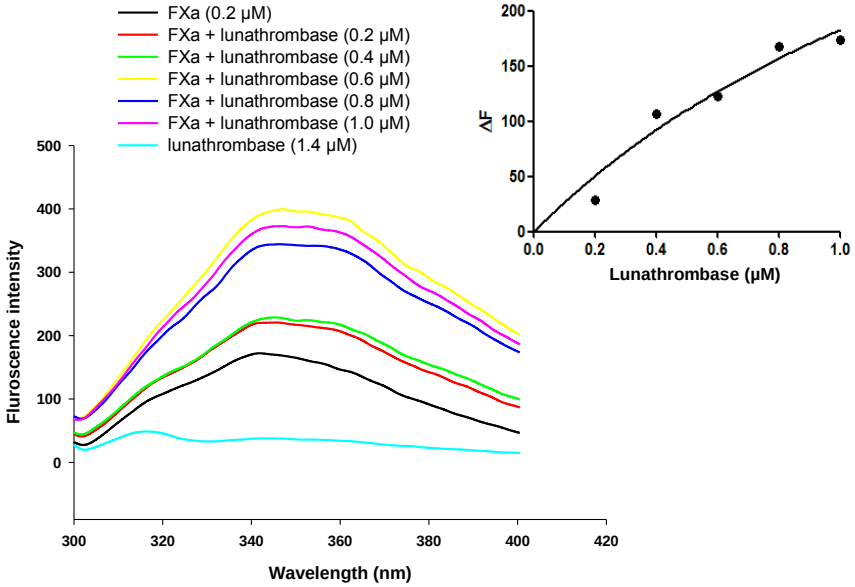


Fig. 5.8b. Fluorescence spectra showing interaction of FXa (0.2 µM) with different concentrations of lunathrombase (0.2-1.4 µM). Inset. One site saturation binding curve of lunathrombase for FXa.

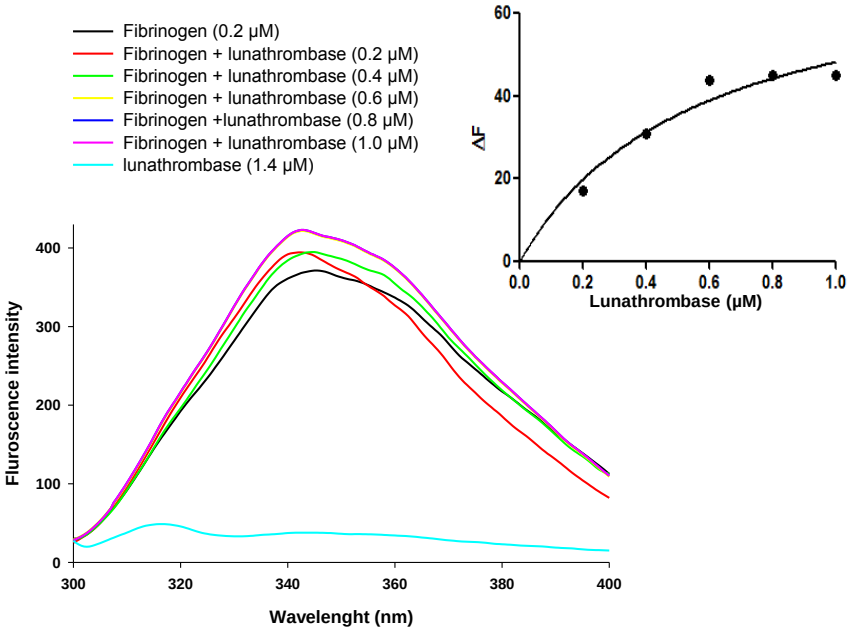


Fig. 5.8c. Fluorescence spectra showing interaction of fibrinogen (0.2 µM) with different concentrations of lunathrombase (0.2-1.4 µM). Inset. One site saturation binding curve of lunathrombase for fibrinogen**.**

5.2.12 Isothermal calorimetry analysis of the interaction between lunathrombase and thrombin /fibrinogen

The titration of thrombin or fibrinogen with lunathrombase resulted in a strong endothermic generation of heat with a clear sigmoidal saturation curve indicating the direct binding interactions (Figs. 5.9a, b). The best fit for the titration curve was obtained with a sequential binding site model with a binding constant (*Ka*) of 9.7 x 10^{-4} M^{-1}, ΔH = 2.07 x 10^{-5}, ΔS= 717 cal/mol/deg for the interaction between lunathrombase and thrombin, and 1.02 x 10^{-5} M^{-1}, ΔH = 2.3 x 10^{-5}, ΔS= 816 cal/mol/deg for the interaction between lunathrombase and fibrinogen.

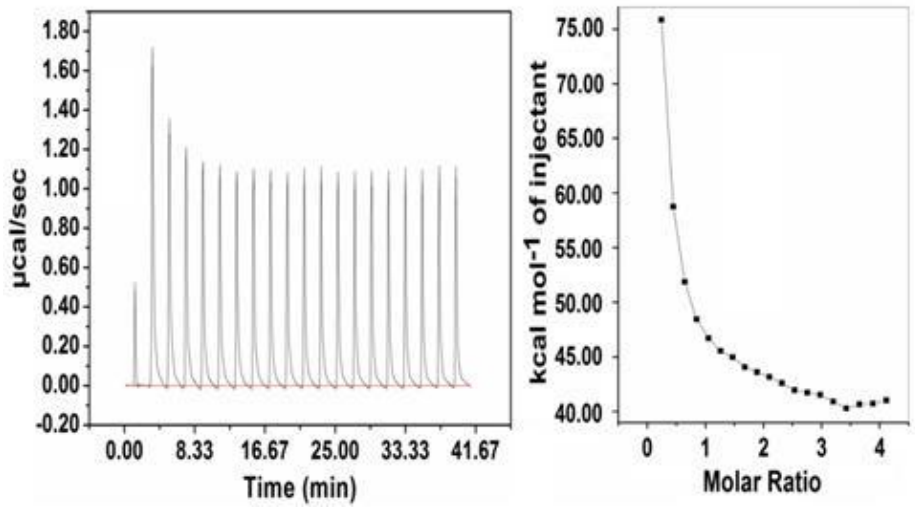


Fig.5.9a. ITC profile for lunathrombase (10 µM) binding to thrombin (200 µM). Left panel shows heat change upon ligand addition; right panel shows an integrated ITC isotherm and is best fit to a sequential binding site model.

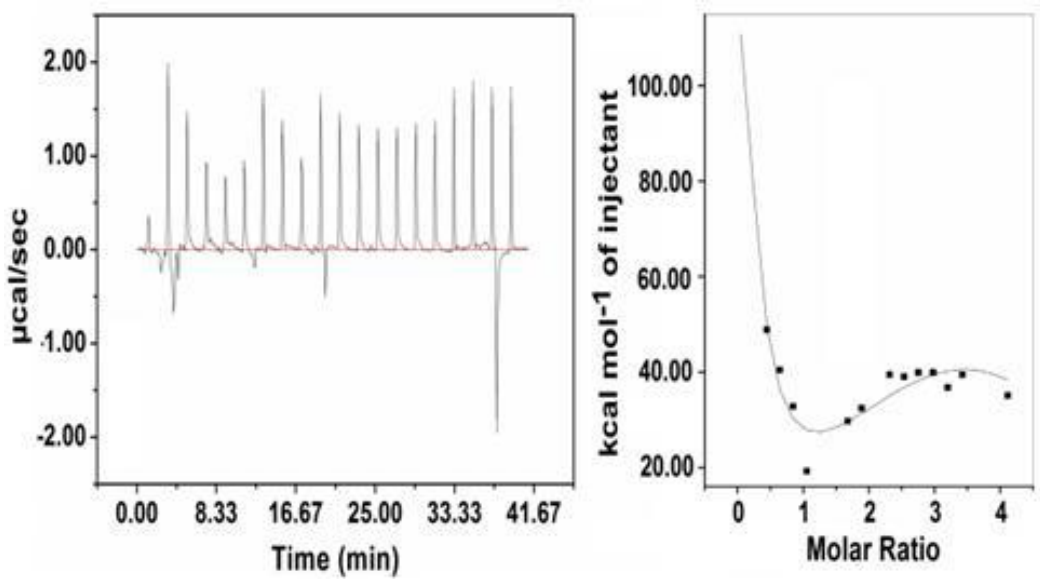


Fig. 5.9b. ITC profile for lunathrombase (10 µM) binding to fibrinogen (200 µM). Left panel shows heat change upon ligand addition; right panel shows an integrated ITC isotherm and is best fit to a sequential binding site model.

5.2.13 Lunathrombase has *in vitro* thrombolytic potency

The *in vitro* thrombolytic potency of lunathrombase and commercial thrombolytic agents (streptokinase, plasmin, nattokinase) is shown in Fig. 5.10. The *in vitro* thrombolytic activity of equimolar concentrations of streptokinase, plasmin, nattokinase, and lunathrombase was found to be identical. Nevertheless, the

thrombolytic potency of lunathrombase, nattokinase, and plasmin towards a heat-treated blood clot was reduced to 80%, 75%, and 60%, respectively, of the original activity to dissolve an unheated blood clot (Fig. 5.10). Streptokinase showed negligible activity (<1%) in dissolving a heated blood clot (Fig. 5.10).

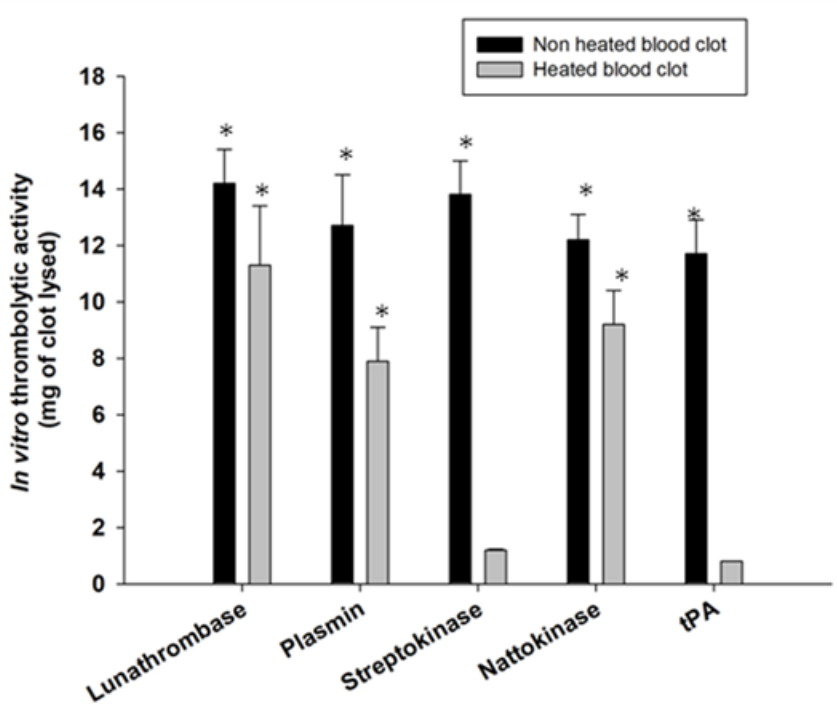


Fig. 5.10. A comparison of *in vitro* thrombolytic activity of lunathrombase (1 µM), streptokinase (1 µM), plasmin (1 µM), nattokinase (1 µM) and tissue plasminogen activator (tPA) (1 µM) under identical experimental conditions on heated and non-heated blood clots. Values are means ± S.D. of triplicate determinations. Significance of difference with between heated and non-heated blood clot dissolving property by lunathrombase, *p< 0.05.

5.2.14 Lunathrombase is devoid of hemolytic activity and cytotoxicity against mammalian cells

Lunathrombase did not show *in vitro* hemolysis against washed erythrocytes (Fig. 5.11a) or cytotoxicity against HEK 293 cells (Fig. 5.11b). The fluorescence microscopic study indicated that at 24 h of treatment, lunathrombase did not change the cell morphology or membrane integrity of treated-HEK 293 cells, in comparison to control cells (Fig. 5.11c).

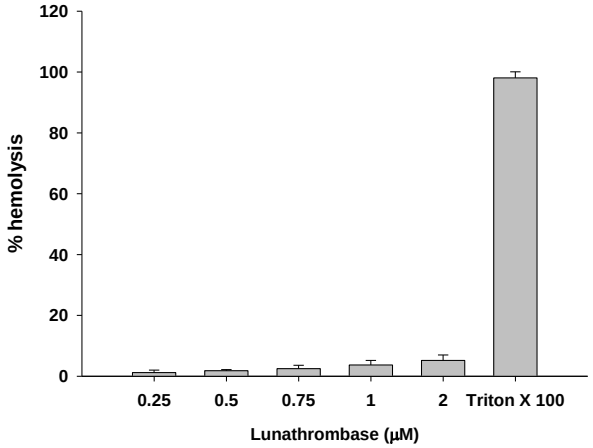


Fig. 5.11a. *In vitro* blood hemolysis assay. The human erythrocytes (5%, v/v) were treated with lunathrombase (0.25-2.0 μM) for 90 min 37˚C. All values are mean ± SD of triplicate determinations.

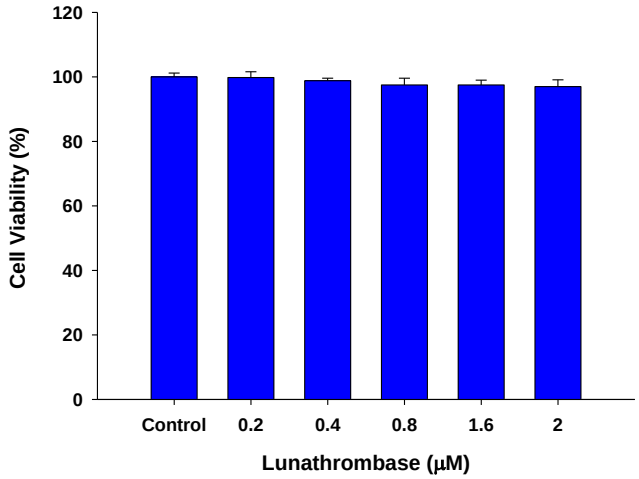


Fig. 5.11b. *In vitro* cell viability by MTT assay. HEK 293 cells (1.0 x 10^5 cells per ml) were treated with lunathrombase (0- 2.0 μM) for 24 h at 37 ˚C. All values are mean ± SD of triplicate determinations.

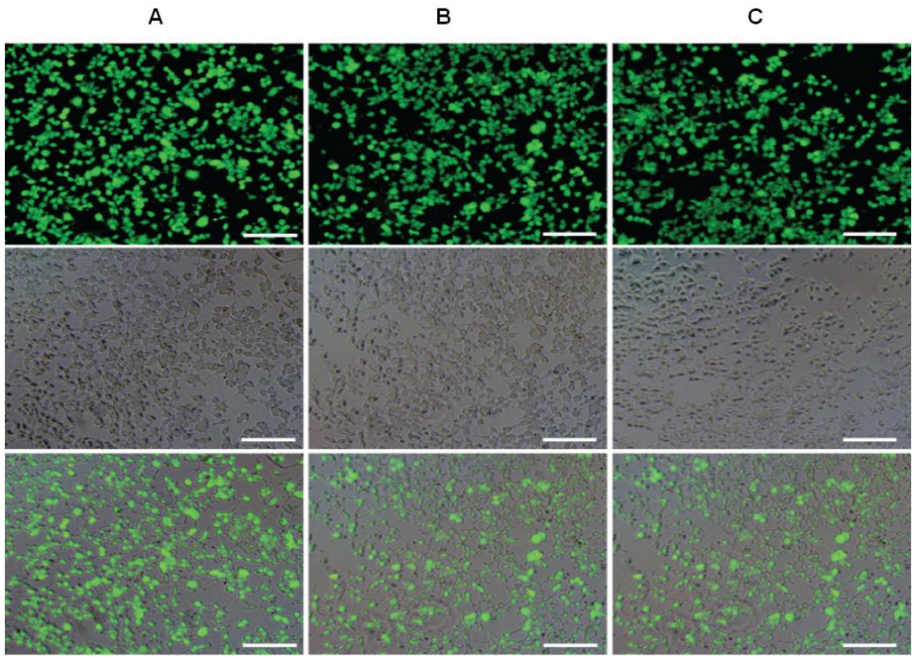


Fig. 5.11c. Calcein-AM staining of HEK 293 cells. **A.** HEK 293 cells treated with control media. **B.** HEK 293 cells treated with 1X PBS, pH 7.4 (without lunathrombase), and **C.** HEK 293 cells treated with lunathrombase (2.0 µM) for 24 h at 37 ºC and then stained with Calcein-AM for 5 min. Green fluoresces indicating live cells.

Further, no significance difference ($p>0.05$) was found in G1, S, and G2 phases of lunathrombase-treated cells, compared to control HEK 293 cells when analyzed by flow cytometry (Fig. 5.11d).

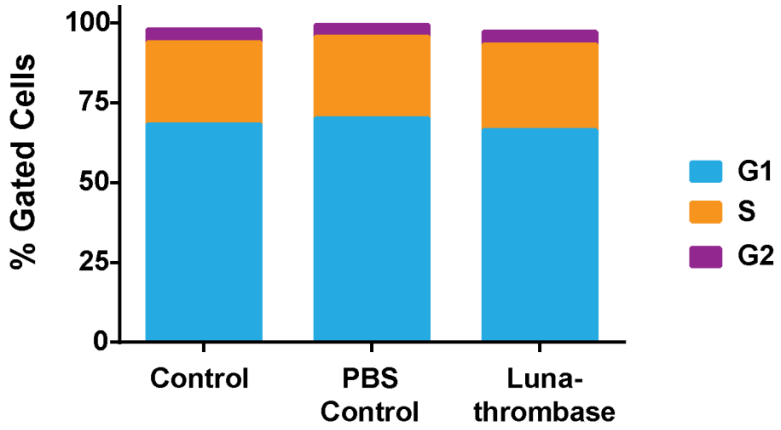


Fig. 5.11d. Cell cycle analysis using propidium iodide (PI) staining and flow cytometry. HEK 293 cells (1.5 x 10^5 cells per ml) were treated for 24 h at 37 ˚C with or without lunathrombase (2.0 µM). Cells were harvested using trypsinization and stained with PI for 2 h and analyzed by flow cytometry.

5.2.15 Lunathrombase showed antiplatelet activity by inhibiting collagen/ADP/arachidonic acid-induced platelet aggregation

A comparable dose-dependent platelet de-aggregation (antiplatelet) property was displayed by equimolar concentrations of lunathrombase and aspirin (Fig. 5.12a). Lunathrombase also exhibited a dose-dependent inhibition of the collagen/ADP/arachidonic acid-induced aggregation of PRP (Fig. 5.12b). The concentration at which lunathrombase demonstrated 50% inhibition of collagen/ADP/arachidonic acid-induced platelet aggregation (IC_{50}) was determined at 152.82 nM, 181.26 nM, and 159.89 nM, respectively (Fig. 5.12b).

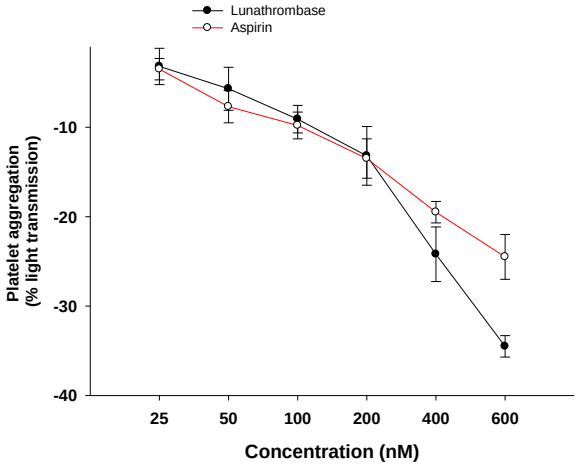


Fig. 5.12a. Dose-dependent platelet deaggregation by lunathrombase / aspirin. Different concentrations of (0–600 nM) of lunathrombase or aspirin were incubated with platelet rich plasma at 37 °C and absorbance was recorded at 540 nm. Data represent mean ± SD of triplicate experiments.

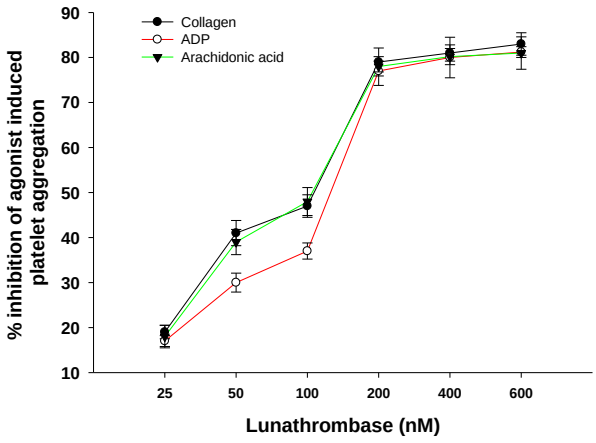


Fig. 5.12b. Dose-dependent inhibition of collagen / ADP / arachidonic acid -induced platelet aggregation by lunathrombase. Different concentrations of lunathrombase(0-600

nM) were incubated with PRP at 37 °C for 10 min and then collagen (6.2 nM) / ADP (30 µM) / arachidonic acid (15 µM) was added in the reaction mixture. The percent platelet aggregation by collagen/ADP/arachidonic acid in absence of lunathrombase was considered as 100% activity and other values were compared to this. The IC_{50} value of lunathrombase (that showed 50% inhibition of collagen / ADP / arachidonic acid - induced platelet aggregation) was determined from the regression analysis of inhibition curve. Data represent mean ± SD of triplicate experiments.

The platelet deaggregation property of catalytically inactive lunathrombase was reduced to ~30% of its original activity exhibited by the catalytically active lunathrombase (Fig. 5.12c). No difference was seen between the catalytically inactive and active lunathrombase in inhibiting the collagen / ADP / arachidonic acid-induced platelet aggregation (Fig. 5.12c).

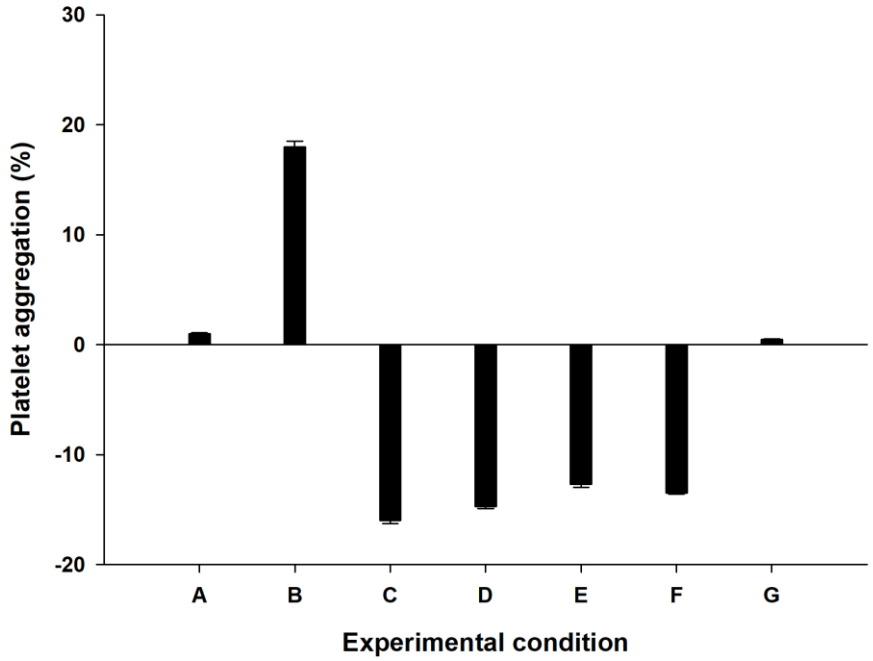


Fig. 5.12c. Percent aggregation / deaggregation of chymotrypsin-treated or untreated washed platelets (1 x 10^6 cells). Effect of **(A)** fibrinogen (0.2 µM), **(C)** lunathrombase (0.2 µM), and **(E)** catalytically inactive lunathrombase (0.2 µM) on washed untreated (control) platelets. Effect of **(B)** fibrinogen (0.2 µM), **(D)**lunathrombase (0.2 µM), **(F)** catalytically inactive lunathrombase (0.2 µM) and **(G)** BSA (0.2 µM) on washed platelets pre-treated with α-chymotrypsin for 15 min. Values are mean ± SD of three determinations.

Fibrinogen-induced aggregation of chymotrypsin-treated platelets but did not show aggregation of control (untreated) platelets (Fig. 5.12d). On the contrary, native and PMSF-treated lunathrombase caused deaggregation of α-chymotrypsin-treated and control platelets.

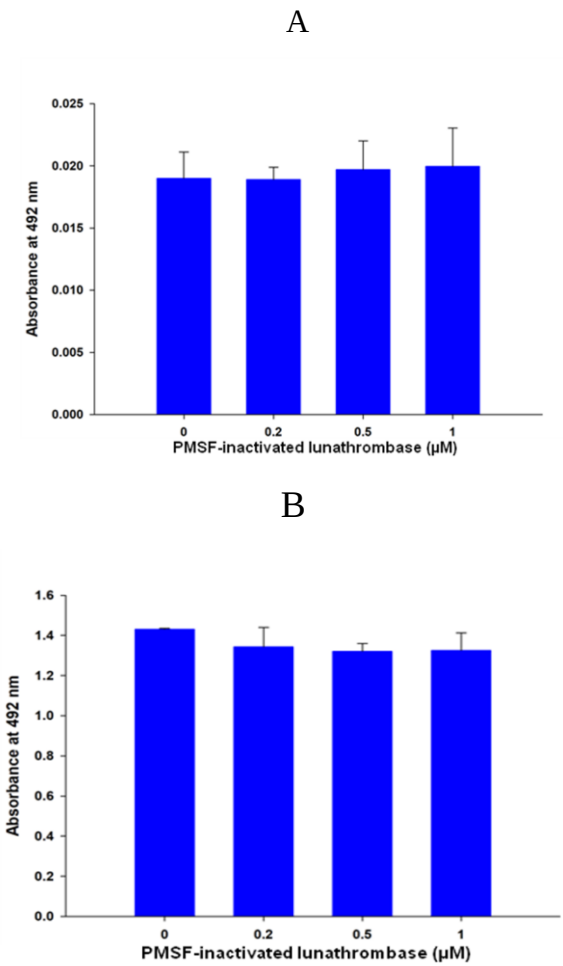


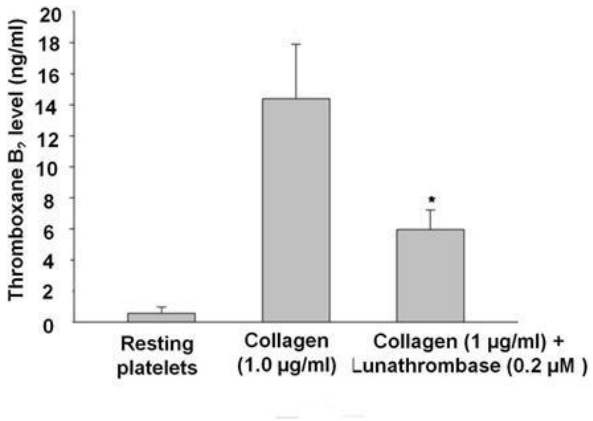
Fig. 5.12d. Binding of PMSF-inactivated lunathrombase (0.2 -1.0 µM) to **A.** human fibrinogen (1000 ng), and **B.** human platelet GPIIb/IIIa receptor. Values are mean ± SD of triplicate determinations.

5.2.16 Lunathrombase increased cAMP level and inhibited COX-1 to exert its antiplatelet effect

Resting platelets produced a negligible quantity of TxB_2 compared to that produced by collagen-activated platelets. Lunathrombase (0.2 µM) significantly inhibited the collagen (1 µg/ml) -stimulated TxB_2 formation in platelets (Fig. 5.13a). The addition of lunathrombase (0.2 µM) increased the endogenous cAMP level of platelets (Fig. 5.13b).

Furthermore, lunathrombase inhibited the COX-1 activity of collagen-treated platelets (Fig. 5.13c). Lunathrombase non-competitively inhibited the COX-1 enzyme with a *Ki* value of 5.947 ± 0.97 µM. The *Km and Vmax* values of lunathrombase towards COX-1 enzyme were determined to be 1.5 ± 0.16 µM and 138.5 ± 5.7 µM/min (mean ± SD), respectively (Fig. 5.13d).

a



b

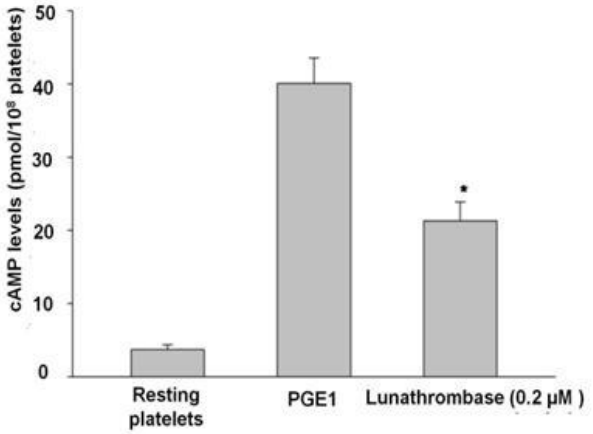


Fig. 5.13a. Effect of lunathrombase on thromboxane B_2 and **b)** cAMP formation in activated platelets. Washed platelets were pre-incubated with lunathrombase (0.2 µM) or 0.5% DMSO on intraplatelet levels of cAMP formation in human platelets. Platelets were incubated with PGE1 (0.2 µM, positive control) or lunathrombase (0.2 µM) for measurement of cAMP formations. Values are means ± S.D. of triplicate determinations. Significance of difference with respect to resting platelets, * $p< 0.05$.

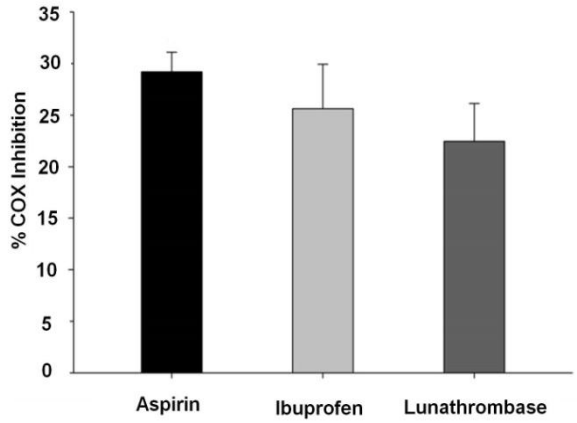


Fig. 5.13c. Effect of lunathrombase on COX-1 activity. The COX-1 enzyme was pre-incubated with 0.2 μM of lunathrombase or aspirin (positive control) or ibuprofen (positive control) for 30 min at 37 °C. The activity of control (without drugs) was considered as 100% and other values were compared with that. All values are means ± S.D. of triplicate determinations. Significance of difference with respect to control (without lunathrombase), * $p < 0.05$.

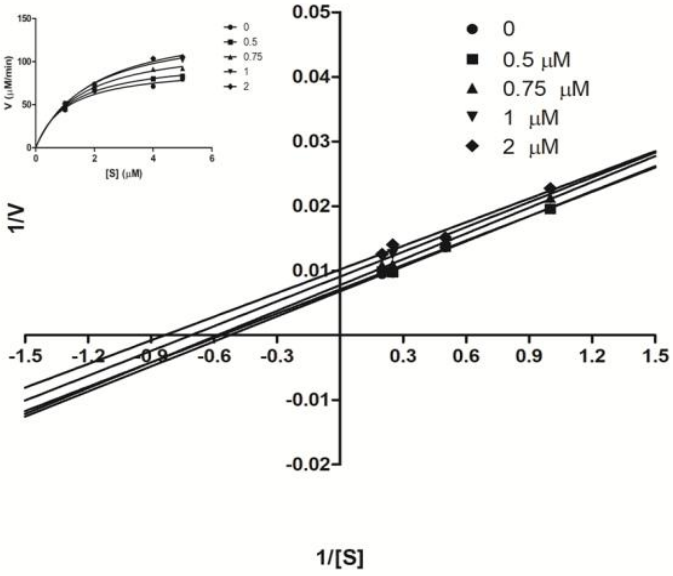


Fig. 5.13d. Determination of *Km* and *Vmax* values of lunathrombase against COX-1 enzyme**.** Lineweaver-Burk plot and Michaelis-Menton plot (inset) of lunathrombase against COX-1.

5.2.17 Determination of *in vivo* toxicity of lunathrombase in Wister strain rats

Lunathrombase at a dose of 10.0 mg/kg was found to be non-toxic to rats and showed no adverse effects and/or behavioral changes in treated-ratsup to 72 h of *i.p* injection (Table 5.7).

Table 5.6. Behavioral and physical changes in lunathrombase treated rats (10 mg/kg) after *i.p* injection

Parameters	**Control**	**lunathrombase-treated (10 mg/kg)**
Survivality	No death	No death
Grip Strength (s)	25.5 ± 2.1	24.0 ± 1.8
Rectal temperature	35.3 ºC	35.7 ºC
Drooping of eyelids	Not observed	Not observed
Movement inability	Not observed	Not observed
Changes in body weight	Not observed	Not observed
Food and water intake	Normal	Normal

5.2.17.1 Effect on hematological, serum biochemical and immunoglobulin parameters

The hematological parameters of blood from lunathrombase-treated rats (72 h post-treatment) did not show any significant deviation compared to the control group of rats; however, a minor increase in neutrophil content was found in the blood of the treated group of rats compared to control rats, which was within the normal range (Table 5.8). In addition, none of the serum parameters of the treated rats were found to be significantly altered ($p>0.05$), in comparison to the control group of rats (Table 5.9). Plasma IgG, IgA, and IgE contents of lunathrombase-treated rats did not differ significantly from those of the control group (Table 5.10).

Table 5.7. Serum hematological parameters of lunathrombase-treated (10 mg/kg) and control group of rats after 72 hours of *i.p.* injection. Values are mean ± SD of 6 rats. There was no significant difference of values ($p>0.05$) between control and lunathrombase-treated groups of rats.

	Values	
Parameters (Unit)	**Control**	**lunathrombase-treated (10 mg/kg)**
WBC(m/mm^3)	5.06 ± 0.36	4.85 ± 0.98
Lymphocytes(%)	36.17 ± 3.00	30.60 ± 0.61
Monocytes(%)	5.67 ± 0.20	5.13 ± 0.06
Neutrophils(%)	50.63 ± 6.62	61.33 ± 0.31
Eosinophils(%)	10.63 ± 0.26	12.20 ± 0.10
Basophils(%)	0.10 ± 0.00	0.17 ± 0.12
Total RBC(m/mm^3)	8.77 ± 0.12	7.65 ± 0.53
MCV(fl)	44.23 ± 2.60	48.77 ± 1.99
HCt(%)	36.20 ± 0.40	35.17 ± 1.77
MCH(pg)	15.77 ± 0.63	18.60 ± 2.12
MCHC(g/dl)	37.13 ± 0.66	31.17 ± 4.80
RDW	15.73 ± 0.16	12.70 ± 2.00
Hb(g/dl)	12.73 ± 0.25	12.67 ± 0.64
MPV(fl)	6.93 ± 0.10	6.80 ± 0.17
PCt(%)	0.31 ± 0.11	0.29 ± 0.03
PDW	8.37 ± 0.16	8.07 ± 0.25

Table 5.8. Some biochemical properties of serum of control and lunathrombase-treated (10 mg/kg) rats after 72 hours of *i.p*. injection. Values are mean ± SD of 6 rats. There was no significant difference of values ($p>0.05$) between control and lunathrombase-treated groups of rats.

	Values	
Parameters (Unit)	**Control**	**lunathrombase-treated**
LDL(mg/dl)	13.30 ± 0.60	13.20 ± 0.50
HDL(mg/dl)	18.80 ± 0.40	16.60 ± 1.10
Glucose(mg/dl)	70.00 ± 5.20	67.00 ± 5.50
Urea(mg/dl)	53.80 ± 3.20	53.00 ± 3.30
Total protein(g/dl)	6.30 ± 0.40	6.10 ± 0.30
Uric acid(mg/dl)	1.30 ± 0.40	1.30 ± 0.20
Triglycerides	52.10 ± 2.60	50.40 ± 3.20
Cholesterol (mg/dl)	46.80 ± 3.40	48.20 ± 2.10
Creatinine(mg/dl)	1.00 ± 0.20	1.40 ± 0.20
SGPT(U/L)	91.30 ± 6.60	95.40 ± 1.70
SGOT(U/L)	162.30 ± 4.90	166.50 ± 3.10

Table 5.9. Serum immunoglobulin parameters of the lunathrombase-treated (10.0 mg/kg) rats after 72 hours of *i.p*. injection. Values are mean ± SD of 6 rats. There was no significant difference of values ($p>0.05$) between control and lunathrombase-treated groups of rats.

Parameters (mg/ml)	**Vehicle (control)**	**Lunathrombase-treated (10.0 mg/kg) rats**
IgG	1.8 ± 0.34	1.7 ± 0.6
IgA	0.9 ± 0.03	1.2 ± 0.08
IgM	1.3 ± 0.2	1.1± 0.35

5.2.17.2 Histopathological study

Light microscopic examination of the liver, kidney, and cardiac tissues of the lunathrombase-treated rats did not show any morphological alterations or

pathophysiological symptoms as compared to the tissues of control group of rats (Fig. 14).

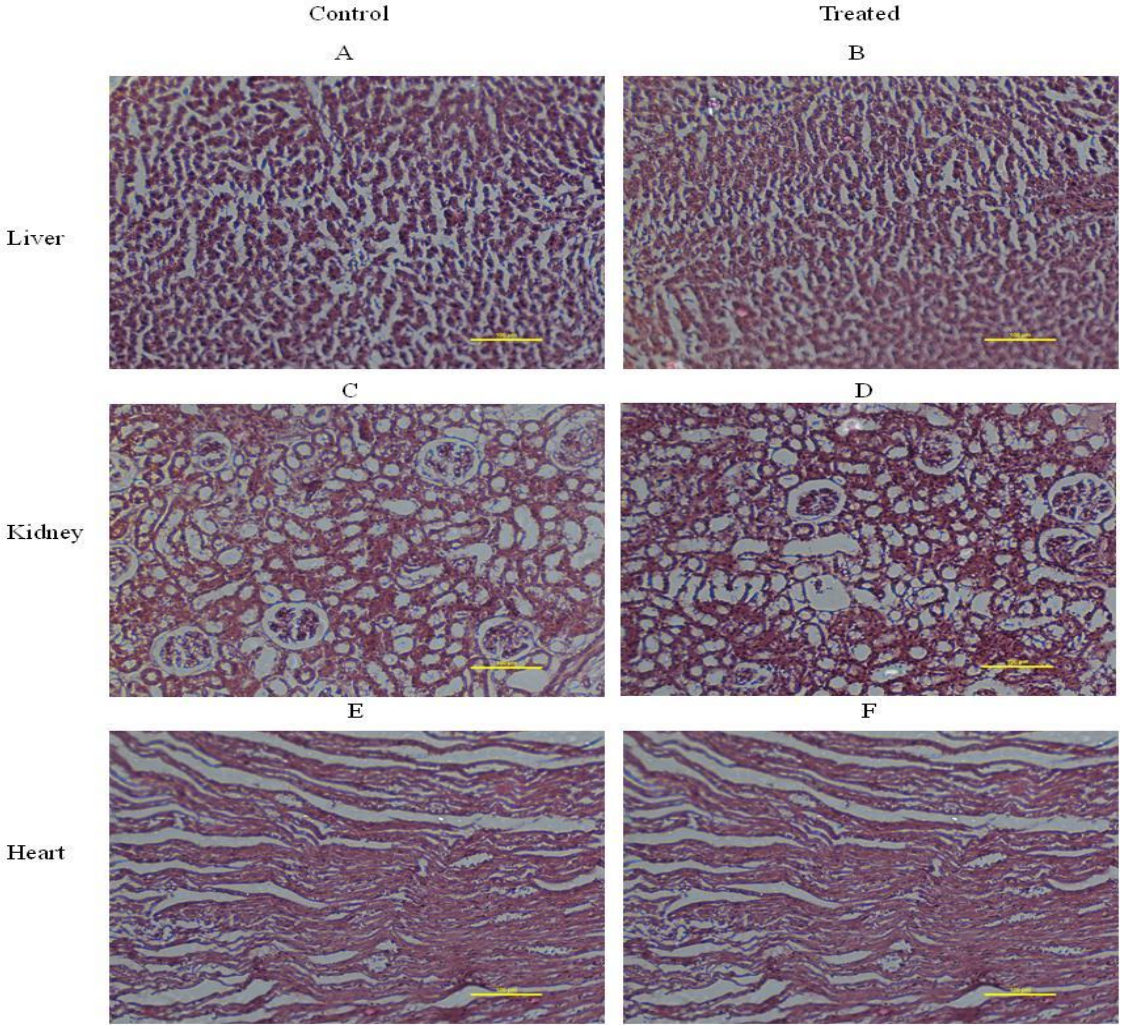


Fig. 5.14. Light microscopic observation of the effect of lunathrombase on liver, kidney, and heart tissues of Wistar rats. The tissues were processed for microscopic observation by hematoxylene-eosin (H & E) staining as described insection3.2.13.2. Tissues obtained from rats treated with 1X PBS served as control. (A) Control liver tissue, (B) lunathrombase-treated liver tissue, (C) Control kidney tissue, (D) lunathrombase-treated kidney tissue, (E) Control heart tissue, and (F) lunathrombase-treated heart tissue. Magnification l0X: Bar = 100 µm.

5.2.18 Lunathrombase demonstrated *in vivo* anticoagulant and defibrinogenating activity

Lunathrombase demonstrated dose-dependent *in vivo* defibrinogenation of rat plasma (Fig. 5.15) with a corresponding dose-dependent increase in the *in vitro* tail bleeding

time, Ca-clotting time and PT of PPP in the treated group of rats, compared to the control group (Table 5.11).

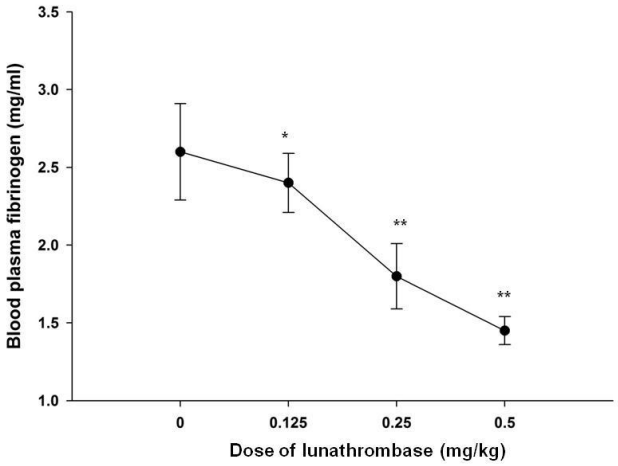


Fig. 5.15. Dose- dependent *in vivo* defibrinogenating activity of lunathrombase 5 h after *i.p*. injection in rats (n=6). Values are means ± S.D. of triplicate determinations. Significance of difference with respect to control, *p< 0.05, **p<0.01.

Table 5.10. A comparison of *in vivo* anticoagulant activity of lunathrombase, heparin and Nattokinase treated Wister strain rats (n=6). The blood was withdrawn 5 h after *i.p.* injection of lunathrombase (0.5 mg/kg) or heparin (0.5 mg/kg) or Nattokinase (0.5 mg/kg).Values represent mean ± SD of six determinations. Significance of difference with respect to control, * $p < 0.01$. INR = ($prothrombin_{test}$ / $prothrombin_{control}$).

	PT (s)	**PT (INR)**	**APTT (s)**	**APTT (INR)**	**Tail bleeding time (s)**	**Plasma clotting time (s)**
Control rats	14.8 ± 0.77	1.0	28.3 ± 1.4	1.0	45.3 ± 2.5	175.5 ± 9.1
Lunathrombase- treated rats (0.5 mg/kg)	28.1 ± 2.9*	1.89	30.2 ± 2.1	1.06	110.0 ± 1.4*	216.3 ± 8.8*
Heparin-treated rats (0.5 mg/kg)	21.4 ± 2.6*	1.44	32.7 ± 2.0	1.16	137.5 ± 4.9*	202.5 ± 10.3*
Nattokinase- treated rats (0.5 mg/kg)	20.1 ± 0.2*	1.35	30.3 ± 4.1	1.06	116.0 ± 8.0	201.3 ± 13.1*

5.3 Discussion

The present study is the first to report on the purification and characterization of a fibrin(ogen)olytic serine protease showing strong anticoagulant, antithrombotic, and thrombolytic activities from the leaves of *L. indica*. Although several proteases have been isolated and characterized from the plant [16-18], their interference with hemostasis and possible biomedical application(s) have not been well documented.

The anticoagulant and fibrinogenolytic activity of all the gel filtration fractions were found to be lower as compared to CEX_1 fraction which was due to other low molecular mass phytochemicals present in this fraction (CEX_1) that contributed to anticoagulant activity. Further, the combined fibrinogenolytic activity of all the three gel filtration fractions results in higher specific activity of cation exchange fraction CEX_1.

The molecular mass of lunathrombase (35 kDa) is consistent with the reported molecular masses of plant serine proteases (26-110 kDa) [16,19]; however, the proteomics and amino acid composition analyses suggest that lunathrombase is a previously uncharacterized novel plant protease. On the basis of their specificity towards the α-chain and β-chain of fibrin/fibrinogen, fibrin(ogen)olytic enzymes are classified as α and/or β fibrinogenases [20]. The α-fibrinogenase from different medicinal plant sources, such as *Costaria costata* [21], *Codium fragile* [22], *Aster yomena* [23], *Paecilomyces tenuipes* [24], and *Perenniporia fraxinea* [25] were reported to hydrolyse the Aα subunits. Lunathrombase is considered an αβ-fibrinogenase, because it can degrade both α- and β-chains of fibrinogen, demonstrating a fibrinogen degradation pattern that differs from other plant proteases.

Inhibition studies with group-specific reagents have been used to identify the nature of the active site in enzymes and the cofactor requirements during proteolysis [11,26,27]. The significant inhibition of the enzymatic activity of lunathrombase by PMSF, IAA, and pBPB (but not by EDTA), unambiguously demonstrates that lunathrombase is a serine protease. Furthermore, the lack of inhibition by DTT also suggests the absence of intramolecular and intermolecular disulfide linkage(s) in lunathrombase. Failure to inhibit the protease activity of lunathrombase by α_2MG or antiplasmin suggests that this enzyme may also exert its activity *in vivo*.

Lunathrombase significantly prolonged the *in vitro* Ca^{2+} clotting time of PPP indicating that it is a potent anticoagulant enzyme. Defibrinogenation, inhibition of platelet aggregation,

and/or interference with components of the blood coagulation cascade are some of the key mechanisms by which proteolytic enzymes exert their anticoagulant effect [6,7,22,27,28].

Our study suggests that the anticoagulant action of lunathrombase is due to its fibrinogenolytic property as substantiated by its potency in inhibiting thrombin and FXa and its antiplatelet effect. The prolongation of PT by lunathrombase and the inhibition of the extrinsic and/or common pathways of coagulation and the marginal effect of lunathrombase on APTT suggest that it does not influence the intrinsic pathway of the coagulation cascade.

Due to the great sensitivity and selectivity of fluorescence spectroscopy, the powerful tool can be used to study protein-protein interactions [29,30]. Our previous studies have also shown that fluorescence spectroscopy is a reliable tool to study protein-protein interactions due to its great selectivity and sensitivity [30-32]. Furthermore, this technique has been proven to be useful in determining the stoichiometry of binding [29]. The three aromatic amino acids viz., tyrosine, tryptophan and phenylalanine, which have distinct absorption and emission wavelengths, contribute to intrinsic fluorescence of a protein. However, the tryptophan residue is excited at 280 nm and shows emission in 340-345 nm range, and it has stronger fluorescence and higher quantum yield compared to tyrosine and phenylalanine residues [33]. Depending on the nature of the protein molecules, their interaction may result in either a decrease in yield owing to quenching of fluorescence of tyrosine and/or phenylalanine residues by a nearby tryptophan residue [29] or it can lead to an increase in fluorescence intensity of the protein complex compared to the individual components of the complex [6,34]. Therefore, the nature of protein-protein interactions may lead to an increase or decrease in the fluorescence intensity [30].

In our spectrofluorometric analysis, a steady decrease in the fluorescence intensity of thrombin was seen in the presence of lunathrombase to suggest the quenching of the tryptophan residues of thrombin when bound to lunathrombase [29,30,35]. Likewise, the spectrofluorometric analysis demonstrated interactions between lunathrombase and FXa/fibrinogen that led to an increase in fluorescence, compared to the fluorescence intensity of individual proteins. Binding affinity is the strength of the binding interaction between a single biomolecule to its ligand/binding. Binding affinity is typically measured and reported by the equilibrium dissociation constant (*Kd*), which is used to evaluate and

rank order strengths of bimolecular interactions [36,37]. Binding affinity is influenced by non-covalent intermolecular interactions such as hydrogen bonding, electrostatic interactions, hydrophobic and Van der Waals forces between the two molecules [38]. In addition, binding affinity between a ligand and its target molecule may be affected by the presence of other molecules. The higher *Kd* value indicated stronger interactions between lunathrombase and thrombin/FXa compared to those between lunathrombase and fibrinogen. Nevertheless, lunathrombase exerts its catalytic activity only on fibrinogen and would therefore inhibit thrombin/FXa by a non-enzymatic mechanism. The lower *km* value for lunathrombase and fibrinogen, compared to lunathrombase and nattokinase indicates its higher specificity for the physiological substrate fibrinogen, instead of nattokinase.

The interaction between lunathrombase and thrombin/fibrinogen was also ascertained by ITC titration, which is considered a sensitive and convenient method for detecting bimolecular interactions by measuring the heat that is generated or absorbed by protein-protein or protein-ligand binding [39,40]. It provides the complete thermodynamic picture of a binding reaction [41]. The ITC experiments considered direct interactions between lunathrombase and thrombin/fibrinogen, where higher ΔH values suggest that the interactions are enthalpy-driven with the primary contributions to the complex stabilization likely resulting from electrostatic interactions and/or hydrogen bonds [40]. The *Kd* value is inversely proportional to the *Ka* value; the higher the *Ka* value for lunathrombase towards thrombin, vs. fibrinogen, indicates its higher affinity for thrombin, which is consistent with the spectrofluorometric analysis.

The anticoagulant mechanism of lunathrombase, appears to differ from that of the currently available anticoagulant drugs, such as heparin and warfarin that act via the indirect inhibition of thrombin and by vitamin K antagonism, respectively [42,43]. The dual inhibition of thrombin and FXa by lunathrombase may lead to its consideration as an alternative new drug to the traditional cardiovascular drugs.

A close association exists between platelet aggregation and the initiation of thrombus formation [44]. A great number of agonists, for example ADP or collagen, bind to the purinergic receptors P2Y1 and P2cyc, GPVI receptors, Integrin αIIbβ3, and the fibrinogen receptor, on the platelet surface to induce platelet aggregation and adhesion [45,46]. Different clinical conditions can mediate abnormal platelet activation and favor

pathological thrombosis and CVD [45-47]. Therefore, the precise control of platelet function is an obligatory requirement for preventing thrombotic events [46]. Although inhibitors of platelet aggregation, like aspirin, have widely been used to prevent thrombus formation [47] several complications have been reported for the prolonged use of aspirin [48].

While platelets are activated by different activators via complex signal pathways, the inhibitory effects of lunathrombase were detected from the stimulation by a broad range of agonists (e.g., ADP, collagen, and AA). The inhibition of platelet aggregation by lunathrombase was corroborated by a significant increase in the intraplatelet cAMP level. The activation of human platelets is inhibited by intracellular cAMP- and cGMP-mediated pathways, indicating the well established importance of cAMP and cGMP in modulating platelet reactivity [49]. An increase in the intraplatelet levels of cAMP has been shown to downregulate the expression of the P2Y1R ADP-receptor, which is necessary for shape change [50], maintaining GPVI (collagen receptor) in a monomeric form, keeping platelets in a resting state [51,52], and inhibiting the release of sCD40L from platelets via the HSP27/p38 MAP kinase pathway [53]. The COX-1 isoenzyme is involved in the synthesis of prostaglandin that participates in platelet aggregation via the prostaglandin derivative, thromboxane B_2 [54,55], and in platelets, the inhibition of COX-1 leads to inhibition of thromboxane B_2 synthesis, which results in the inhibition of platelet aggregation [54]. Lunathrombase inhibits platelet aggregation via COX-1 inhibition and by upregulation of the cAMP level. The exact mechanism of COX-1 inhibition by lunathrombase remains to be further explored.

The results suggest that lunathrombase disaggregates platelets by both enzymatic and non-enzymatic mechanisms, though the latter mechanism predominates. The equipotent inhibition of collagen / ADP/ arachidonic acid-induced platelet aggregation by native (untreated) and catalytically inactive lunathrombase indicates that these inhibitions are independent of the enzymatic activity of lunathrombase.

Limited proteolysis with α-chymotrypsin exposes glycoproteins GPIIb and GPIIIa, the two subunits of the platelet fibrinogen receptor GPIIb/IIIa complex at the surface of platelets, without interfering with cell activation or granular secretion [56]. Subsequently, fibrinogen binds to α-chymotrypsin-treated platelets to induce their aggregation [56]. Nevertheless,

platelet deaggregation of α-chymotrypsin-treated platelets, caused by both native and PMSF-treated (catalytically inactive) lunathrombase suggests that this protease does not hydrolyze the fibrinogen receptor GPIIb/IIIa complex of platelets to exert the antiplatelet activity. The exact mechanism of the anti-platelet activity of lunathrombase remains to be explored.

To the best of our knowledge, lunathrombase is the first example of a plant-derived fibrin(ogen)olytic serine protease that shows potent antiplatelet activity and is capable of inhibiting the collagen and ADP-induced platelet aggregation, possibly by binding and/or degrading their receptors on the platelet surface. This result shows great promise for the therapeutic use of lunathrombase as an antiplatelet agent. Further, the characterization of the clot bursting activity of lunathrombase against non-heated blood provides a fair indication that lunathrombase is a plasmin-like, direct-acting fibrinolytic enzyme. Moreover; the thrombolytic potency of lunathrombase, which is comparable to that of streptokinase, plasmin, or nattokinase at physiological conditions (37°C, pH 7.4) reinforces its possible therapeutic application as a thrombolytic agent.

Lunathrombase, at a concentration of 2.0 µM, which is 40 times greater than its *in vitro* minimum anticoagulant dose (0.05 µM) did not show any adverse effects on the viability of HEK 293 cells; thus indicating its lack of appreciable cytotoxicity against mammalian cells. Lunathrombase also lacks haemolytic activity. Further, when lunathrombase was given at a dose of 10.0 mg/kg, which is approximately 90 times greater than its anticoagulant dose (0.125 mg/kg), it did not produce acute toxicity or adverse pharmacological effects in rats, indicating its preclinical safety and high therapeutic index.

Hyperfibrinogenemia in blood is associated with increased risk of cardiovascular disorders, such as thrombosis [57]. Moreover, elevated levels of fibrinogen can also promote the growth of lung and prostate cancer cells through interactions with fibroblast growth factor 2 [58]. Abnormally high levels of fibrinogen may increase the risk of morbidity due to various disease states, thus emphasizing the need for defibrinogenating drugs. Lunathrombase, at the tested dose of 0.125 mg/kg also demonstrated an *in vivo* defibrinogenation potential. The data, when taken together, leads us to anticipate possible therapeutic applications of lunathrombase for treating and/or preventing cardiovascular diseases, and the need for clinical trials.

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