DEPARTMENT OF BIOTECHNOLOGY
MINISTRY OF SCIENCE AND TECHNOLOGY, GOVT. OF INDIA
FUNDED RESEARCH PROJECT

National Bioscience Award for Career Development-2013

Project title

Discovering novel drug formulations from non-toxic, potent anticoagulant components of snake venom for the prevention and/or treatment of cardiovascular disease (CVD).

FINAL PROJECT REPORT

(25 November, 2014 to 31 March, 2018)

Submitted By

Prof. Ashis K. Mukherjee

Principal Investigator
Department of Molecular Biology and Biotechnology
Tezpur University, Tezpur-784 028, Assam

DEPARTMENT OF BIOTECHNOLOGY, NEW DELHI FUNDED RESEARCH PROJECT INFORMATION SHEET

Title of the project	Discovering novel drug formulations from non-toxic, potent anticoagulant components of snake venom for the prevention and/or treatment of cardiovascular disease (CVD).
DBT sanction no. and date	BT/HRD/NBA/34/01/2012-13 dated November 25, 2014
Name of the PI, designation and address where the project is implemented	Dr. A. K. Mukherjee, Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur – 784028, Assam
Category of the project	R & D under National Bioscience Award programme
Duration of project	3 years (due to non release of fund in time, project was extended up to 31.03.2018)
Effective date of starting the project	24 November, 2014
Total budget sanctioned	Rupees 15.0 lakhs
Project report No.	Third and Final
Duration of report	1st January, 2015 to 31st March, 2018.
Objectives of the project	**(i) First year:** Isolation of potent anticoagulant peptides from Indian Cobra (*Naja naja*, *N. kaouthia*) and Russell's Viper (*Daboia russelii*) venom samples, characterization of their biochemical properties and comparison of their anticoagulant potency with commercially available anticoagulant drugs. **(ii) Second year:** Pharmacological characterization, elucidation of mechanism of action and assessment of *in vitro* cytotoxicity of selected anticoagulant peptides (individual as well as their different combinations). **(iii) Third year:** Assessment of toxicity and dose-dependent *in vitro* and *in vivo* anticoagulant potency of anticoagulant drug formulations.

Dated: 31.7.2018

(A. K. Mukherjee)

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular Biology & Biotechnology
Tezpur University (Central)
Tezpur- 784028, Assam

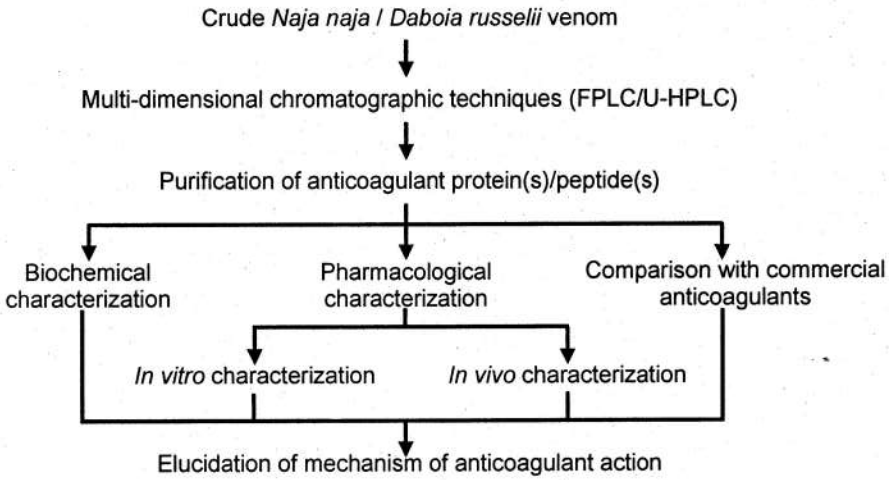
Annexure – I: Section-B: Scientific and Technical Progress:

Summary of objectives sanctioned vs objectives achieved

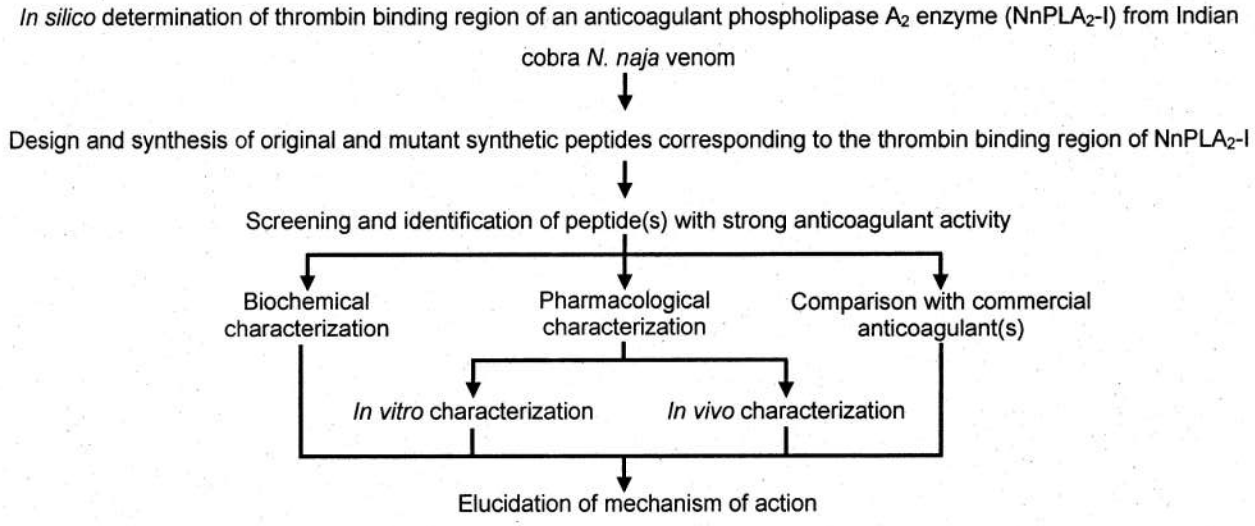
Period of study	Achievable targets	Targets achieved (Y/N)
0 – 12 months	Isolation of potent anticoagulant peptides from Indian Cobra (*Naja naja*, *N. kaouthia*) and Russell's Viper (*Daboia russelii*) venom samples, characterization of their biochemical properties and comparison of their anticoagulant potency with commercially available anticoagulant drugs.	Yes
13 – 24 months	Pharmacological characterization, elucidation of mechanism of action and assessment of *in vitro* cytotoxicity of selected anticoagulant peptides (individual as well as their different combinations).	Yes
25 – 36 months	Assessment of toxicity and dose-dependent *in vitro* and *in vivo* anticoagulant potency of anticoagulant drug formulations.	Yes

Flow chart of experiment scheme

1. Characterization of anticoagulant protein(s)/peptide(s) from snake venom



2. Synthesis and characterization of synthetic peptides derived from anticoagulant protein(s) of snake venom



B.1. Progress made against the approved objectives, targets, and timelines during the reporting period:

Brief outline of achievements in 1st year (Jan 2015 – Dec 2015)

1. Isolation and purification of an anticoagulant C-type lectin from *D. russelii* (RVSnaclec) venom.
2. Biochemical characterization and pharmacological (both *in vitro* and *in vivo*) characterization of RVSnaclec.
3. Elucidation of mechanism of anticoagulant action of RVSnaclec.

Brief outline of achievements in 2nd year (Jan 2016 – Dec 2016)

1. Isolation and purification of a thrombin-inhibiting anticoagulant PLA_2 enzyme from *N. naja* ($NnPLA_2$-I) venom.
2. Biochemical and *in vitro* pharmacological characterization of $NnPLA_2$-I and comparison of its anticoagulant activity with commercial anticoagulants.
3. Elucidation of mechanism of action of $NnPLA_2$-I and *in silico* determination of thrombin binding region of the purified protein.
4. Design, synthesis and characterization of a synthetic peptide corresponding to the thrombin binding region of $NnPLA_2$-I.
5. Isolation and purification of an anticoagulant Kunitz-type serine protease inhibitors (Rusvikunins) from *D. russelii* venom.
6. Biochemical and pharmacological characterization of Rusvikunins and their complex.

Brief outline of achievements in 3rd year (Jan 2017 – Mar 2018)

1. Design and synthesis of different mutant peptides corresponding to the thrombin binding region of $NnPLA_2$-I.
2. Screening of mutant peptides exhibiting potent *in vitro* anticoagulant activity.

3. *In vitro* and *in vivo* pharmacological characterization of the anticoagulant peptide and $NnPLA_2$-I and comparison with commercial anticoagulant drugs.
4. Provisional patent filing for the anticoagulant peptide.

Summary of Major Findings

1. RVsnaclec: A C-type lectin (RVsnaclec) from Russell's Viper (*Daboia russelii*) venom:

Snake venom C-type lectins (CTLs) or snaclec are non-enzymatic, Ca^{2+} dependent heteromeric proteins that are functionally diverse, target coagulation factors, membrane receptors and platelets receptors. Upon envenomation, these venom components cause disruption of haemostatic mechanisms of the victim/prey. RVsnaclec is the first report of an anticoagulant C-type lectin purified from Russell's Viper venom.

1.1. Purification and characterization of RVsnaclec – an anticoagulant C-type lectin from Russell's Viper (*Daboia russelii*) venom:

RV snaclec was purified by a combination of multidimensional chromatographic techniques wherein lyophilized *D. russelli* venom was fractionated on a Bio Gel P-100 size exclusion column (2.8 X 80 cm) (Mukherjee and Mackessey, 2013) which resolved into 12 distinct peaks (Fig 1A). The GFC peak 1 fractions were pooled, desalted, lyophilized and further subjected to anion exchange chromatography on a Tricorn Mono Q5/50GL column connected to an FPLC system (ÄKTA Purifier 10 Fast Protein Liquid Chromatography System, GE Healthcare). The anion-exchange peak (Fig. 1B) with the strongest anticoagulant activity was subjected to further study. The homogeneity of the preparation was analysed by 12% SDS-PAGE (inset of Fig. 1B), which displayed a single band with an apparent molecular weight of 66.3 kDa under non-reducing conditions, and under reducing conditions, it showed two distinct bands corresponding to of 15.1 and 9.0 kDa, respectively.

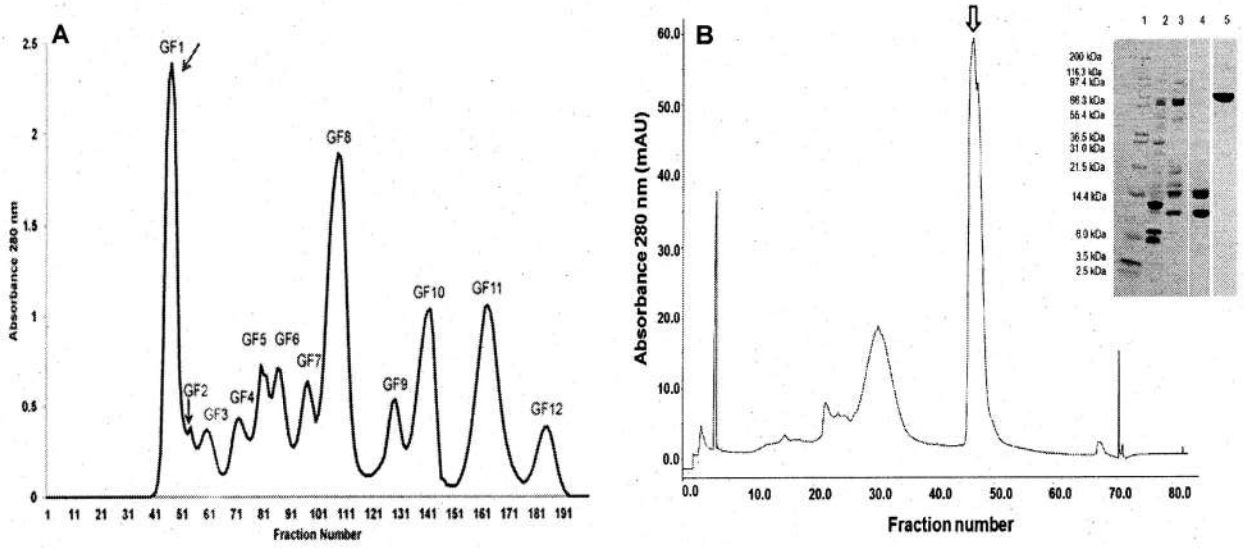


Fig. 1. A. Fractionation of Russell's Viper (*D. russelii*) venom (200 mg dry weight) on size-exclusion BioGel P-100 column (2.8 × 80 cm). The flow rate was 6 ml/h at 4 °C. **B.** FPLC cation exchange fractionation of gel-filtration fraction 1 of *D. r. russelii* using a Tricorn Mono Q 5/50 GL column. **Inset figure-** determination of purity and molecular mass of RVsnaclec by 12.5% SDS-PAGE: Lane 1, protein molecular markers; lane 2, crude RVV - reduced (20 µg); lane 3, gel-filtration fraction - reduced (12 µg); lanes 3 and 4, reduced and non-reduced RVsnaclec (8.0 µg), respectively.

The LC-MS/MS analysis of RVsnaclec showed the presence of putative conserved domains [tryptic peptide sequences K.GSHLLSLHNIAEADFVLK.K (m/z 982.5356), and M.GLNDVWNEC (+57.02) NWGWTDGAK.L (m/z 1061.459)] of C-type lectins (CTL) or the carbohydrate-recognition domain (CRD), a typical feature of snaclecs. Taken together, RVsnaclec is the first example of a snaclec from *D. russelii*, and it represents a new C-type lectin-like protein from snake venom.

RVsnaclec dose-dependently increased the Ca-clotting time (Fig. 2A) and prothrombin time (Fig. 2B) of mammalian platelet-poor plasma (PPP); however, it did not affect the partial thromboplastin time (APTT) or thrombin time of PPP. One unit of anticoagulant activity was defined as an RVsnaclec-induced one second increase in clotting time of PPP compared with the clotting time of control plasma.

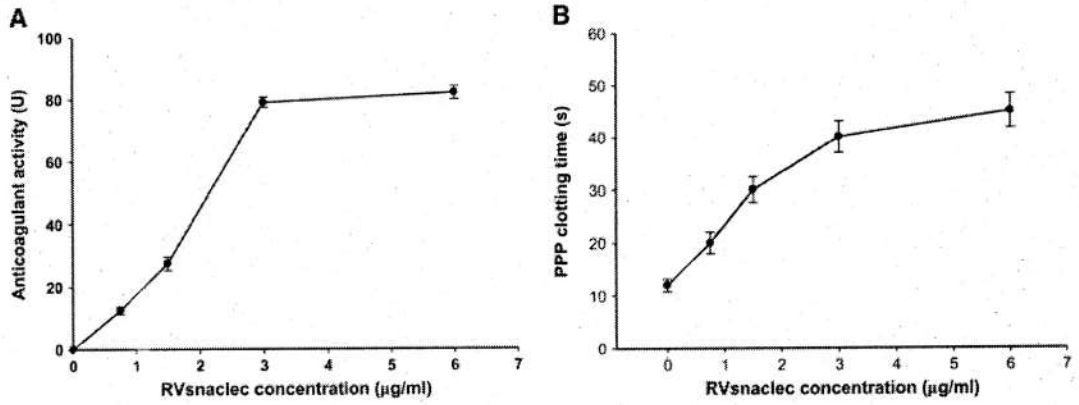


Fig. 2. A. Dose-dependent *in vitro* anticoagulant activity of RVsnaclec. Different doses of RVsnaclec were pre-incubated for 3 min with 300 µl of goat platelet-poor plasma (PPP) at 37 °C and then 40 µl of 250 mM $CaCl_2$ was added to initiate the fibrin clot formation. **B.** Effect of different concentrations of RVsnaclec on prothrombin time of PPP. RVsnaclec was pre-incubated for 3 min with 100 µl of PPP at 37 °C and then 200 µl liquiplastin reagent (pre-warmed at 37 °C) was added and re-incubated for 3 min at 37 °C before assay of clotting activity. Values are means ± SD of triplicate determinations.

RVsnaclec dose-dependently inhibited the amidolytic activity of FXa which was also observed in presence of 0.5 mM EDTA by a reversible, uncompetitive mechanism (Fig. 3A). The *Ki* value for inhibition of amidolytic activity of FXa by RVsnaclec was determined at 0.52 ± 0.1 nmol. RVsnaclec also inhibited the prothrombin activating property of FXa in a dose-dependent manner (Fig. 3B). The presence of 0.25 mM Ca^{2+} increased RVsnaclec mediated inhibition of FXa (Fig. 3B).

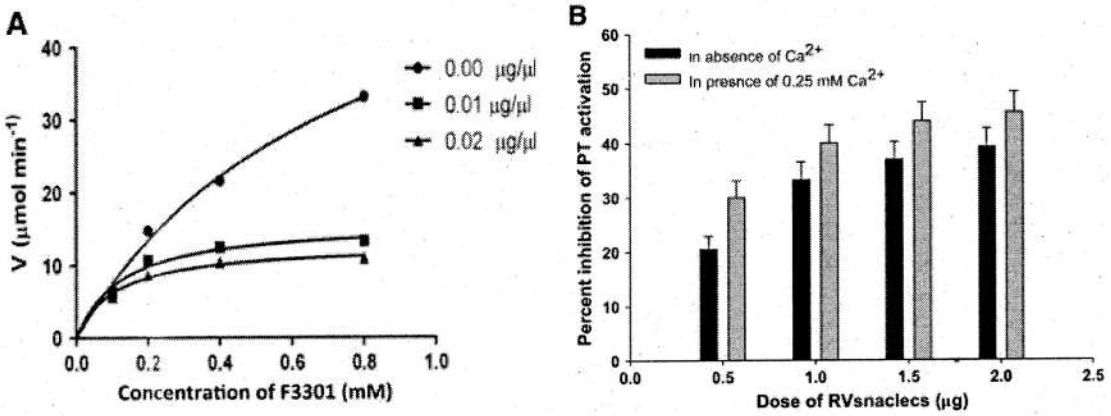


Fig. 3. A. Michelis-Menten plot showing dose-dependent inhibition of amidolytic activity of FXa (20 nM) by RVsnaclec (0 – 0.02 µg/ml). The release of pNA was determined at 405 nm. **B.** Dose-dependent inhibition of prothrombin activation of FXa (0.1 µg/ml) by RVsnaclec (1.0 µg/ml) in the absence and presence of calcium (0.25 mM). Values are means ± SD of triplicate determinations.

RVsnaclec was devoid of hemolytic activity or cytotoxicity against several human cancer cell lines, and demonstrated concentration-dependent aggregation and deaggregation of human platelet poor plasma (PRP) collected from healthy volunteers (Fig. 4).

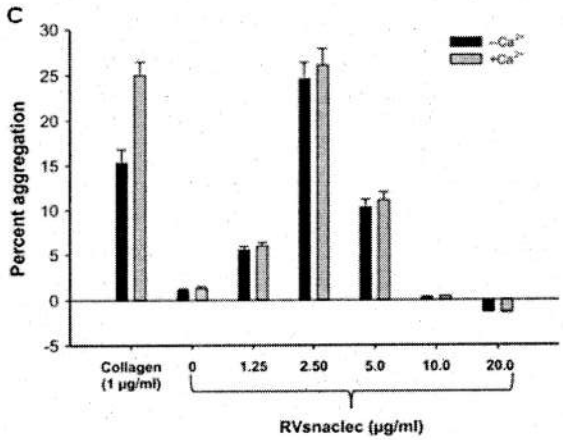


Fig. 4. Concentration-dependent aggregation and deaggregation of human PRP by RVsnaclec.

RVsnaclec (i.p.) at a dose of 5.0 mg/kg body weight was non-lethal to NSA mice, and no behavioral changes or detrimental effects were observed in the treated animals. Six hours after i.p administration, RVsnaclec significantly ($p < 0.05$) prolonged the *in vivo* blood coagulation time of treated mice (310.0 ± 12.0 s, n=3) as compared with coagulation time of control mice (67 ± 5.0 s, n =3). This *in vivo* anticoagulation activity of RVsnaclec is likely correlated with its FX binding property. The *in vitro* and *in vivo* anticoagulant activity of RVsnaclec is correlated to its binding and subsequent uncompetitive inhibition of FXa (K_i =0.52 µM) in a Ca^{2+}-independent manner. The anticoagulant potency of RVsnaclec was found to be superior to the commercial anticoagulant drugs heparin and warfarin and comparable to that of argabatron.

2. Purification, characterization, and mechanism of anticoagulant action of a Kunitz-type serine protease inhibitor (KSPI) from Russell's viper (*D. russelii*) venom

Kunitz-type serine protease inhibitors (KSPI) are low molecular mass snake venom peptides which consist of 50–60 amino acid residues and exhibit a wide variety of biological functions, such as inhibition of one or more serine proteases, blocking of ion-channels, interference with blood coagulation, inflammation and fibrinolysis. The KSPI Rusvikunin was eluted as a complex (Rusvikunin complex) when the pooled, desalted and concentrated low molecular mass gel-filtration fractions (tubes 131-135) of *D. russelii* venom (described above, Fig. 5A) was subjected to separation on a Mono S 5/50 GL cation exchange column coupled to an AKTA Purifier Fast Protein Liquid Chromatography System (GE Healthcare). The anticoagulant Rusvikunin complex eluted as a sharp symmetrical peak at a 0.3 M NaCl gradient (Fig. 5B) and was further subjected to reverse-phase high pressure liquid chromatography (U-HPLC) on a Jupiter C_{18} (250 mm x 4.6 mm) column pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The bound proteins were eluted with a linear gradient over 75 min from 0 to 40% (v/v) acetonitrile (ACN) containing 0.1% (v/v) TFA. Rusvikunin eluted as a small

peak at retention time 44.1 min (RP-44), while another component of the complex, Rusvikunin-II, eluted as a major peak at 34.2 min (RP-34) (Fig 5C).

The molecular weight and purity of preparations were determined by both 12.5% SDS-PAGE (NuPAGENovex® Bis-Tris Mini Gels; under both non-reduced and reduced conditions) and MALDI-TOF mass spectrometry (Bruker Ultraflex) analysis. RP-44 displayed a single band on SDS-PAGE in both reduced and non-reduced conditions and its molecular mass was determined as ~7 kDa (Fig. 5D) which is in concordance with the molecular mass determined by MALDI-TOF-MS (6936.89 Da) (Fig. 5E). The N-terminal sequence alignment and peptide mass fingerprinting analyses of purified RP-44 showed presence of putative conserved domains which were significantly similar to venom basic protease inhibitors, Kunitz-type protease inhibitors and trypsin inhibitors from Viperidae venoms (Table 1).

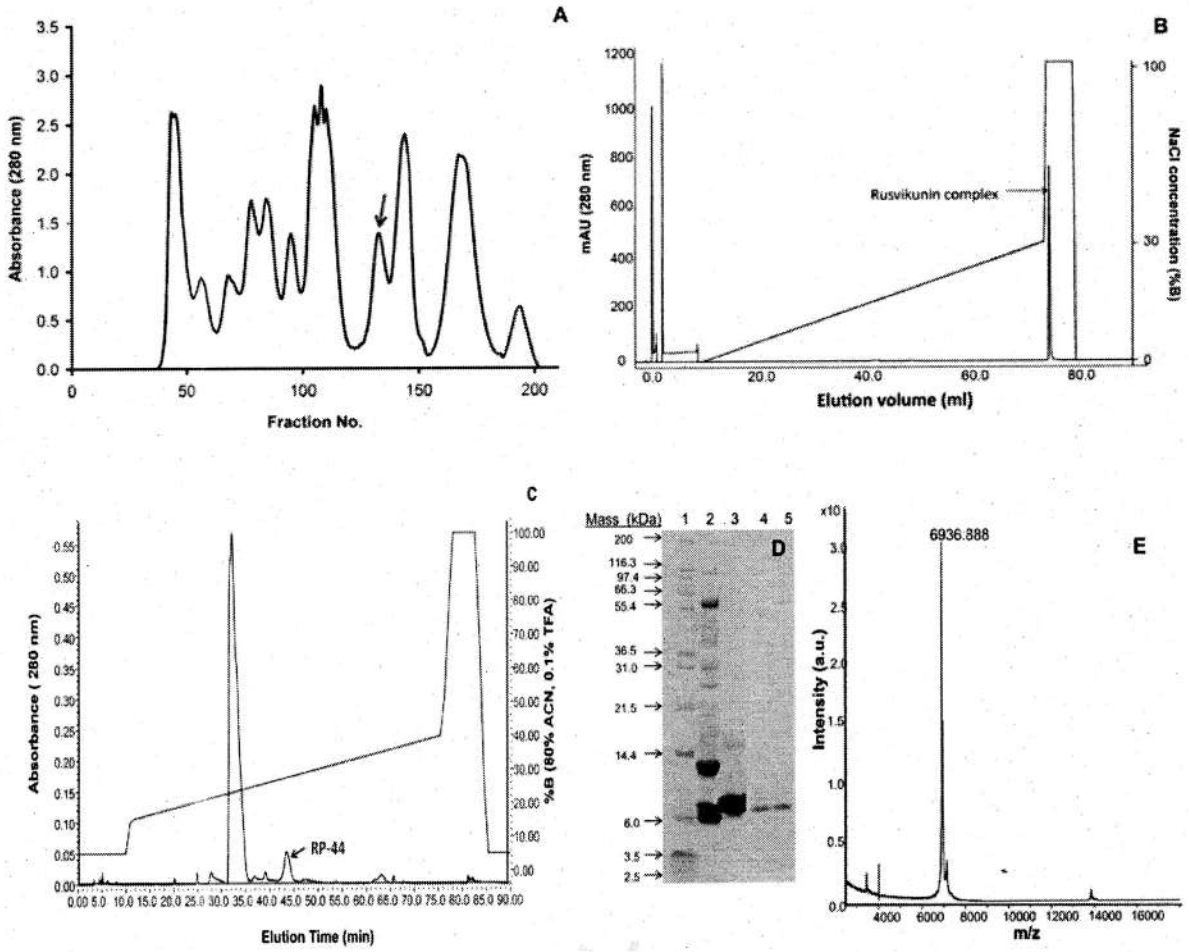


Fig. 5. A. Fractionation of crude *D. russelii russelii* venom on size-exclusion BioGel P-100 column (2.8 × 80 cm). The arrow indicates elution of proteins (RVGF 131-135) showing anticoagulant activity. **B.** FPLC cation exchange of anticoagulant protein peak using a Tricorn MonoS 5/50 column. **C.** Fractionation of FPLC-cation exchange eluted protein complex on a C_{18} RP-HPLC column. One protein eluted at 44.2 min (RP-44), and the other eluted at 34.2 min (RP-34). **D.** Determination of purity and molecular mass of RP-44 by SDS-PAGE; Lane 1, protein molecular markers; lane, 2 reduced crude RVV (20 µg); lane 3, reduced gel-filtration

fraction (8 µg); lanes 4 and 7, reduced and non-reduced RP-44 (3.0 µg), respectively. **E.** MALDI-TOF-MS of RP-44 (~2 µg).

Table 1. Partial sequence of RP-44 protein aligned with homologous sequences from snake venom using CLUSTALO1.0.

Inhibitors	Sequence	
Protease inhibitor C5	MSSGGLLLLLALLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNPDSNKCE-VF60	
Protease inhibitor C1	MSSGGLLLLLGLLTLWAELTPISGQDRPKFCNLAPESGRCRGHLRRIYYNPDSNKCE-VF60	
Kunitz-type prot.Inhi.	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCK-VF60	
Protease inhibitor B3	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCE-VF60	
Protease inhibitor B2	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCN-VF60	
Protease inhibitor 1	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCK-VF60	
Protease inhibitor	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRAHLRRIYYNLESNKCE-VF60	
Protease inhibitor B4	MSSGGLLLLLGLLTLWAELTPISGHDRPTFCNLAPESGRCRGHLRRIYYNLESNKCE-VF60	
RP-44	----------HDRPTFCNLAPESGR------RIYYNPDSNKCEYVF36	
	:***.********** ***** :****: **	
Protease inhibitor C5	FYGGCGGNDNNFETRKKCRQTCGAPRKGRPT	90
Protease inhibitor C1	FYGGCGGNDNNFETRKKCRQTCGAPRKGRPT	90
Kunitz-type prot.Inhi.	FYGGCGGNDNNFETRDECRQTCG----GK--	84
Protease inhibitor B3	FYGGCGGNDNNFSTRDECRHTCV----GK--	84
Protease inhibitor B2	FYGGCGGNDNNFETRDECRQTCG----GK--	84
Protease inhibitor 1	FYGGCGGNANNFETRDECRQTCG----GK--	84
Protease inhibitor	FYGGCGGNDNNFSTWDECRHTCV----GK--	84
Protease inhibitor B4	FYGGCGGNDNNFSTWDECRHTCV----GK--	84
RP-44	FYGGCGGNDNNFETR----QTCGAPR-----	67
	******** ***.* :**	

*Indicates identical residues in all sequences; (:) = highly conserved; (.) = moderately conserved. The GenBank accession number of aligned sequences were: Rusvikunin (present study), Protease inhibitor C5 (gi|239977257), Protease inhibitor C1 (gi|239977246), Kunitz-type protease inhibitor (gi|380842421), Protease inhibitor B3 (gi|239977251), Protease inhibitor B2 (gi|239977248), Protease inhibitor 1 (gi|123913156), Protease inhibitor (gi|377657518), Protease inhibitor B4 (gi|239977254).

RP-44 dose-dependently prolonged the Ca-clotting time of citrated goat plasma (Fig. 6A) and demonstrated antiplasmin activity by significantly inhibiting the plasmin-mediated degradation of fibrin (Fig. 6B). Inhibitory activity of RP-44 against a panel of serine proteases (trypsin, chymotrypsin, thrombin, plasmin, factor Xa, t-PA) was determined by amidolytic assays with their respective chromogenic substrates. RP-44 showed significant inhibition of trypsin towards its chromogenic substrate BApNA with an IC_{50} value of 50 nmol/l (Fig. 7A), followed by plasmin (IC_{50} = 1.1 µmol/l) (Fig. 7B). RP-44 also dose-dependently inhibited the fibrinogen clotting and plasma clotting activity of thrombin (Fig. 7C) with an IC_{50} value of ~1.3 µmol/l.

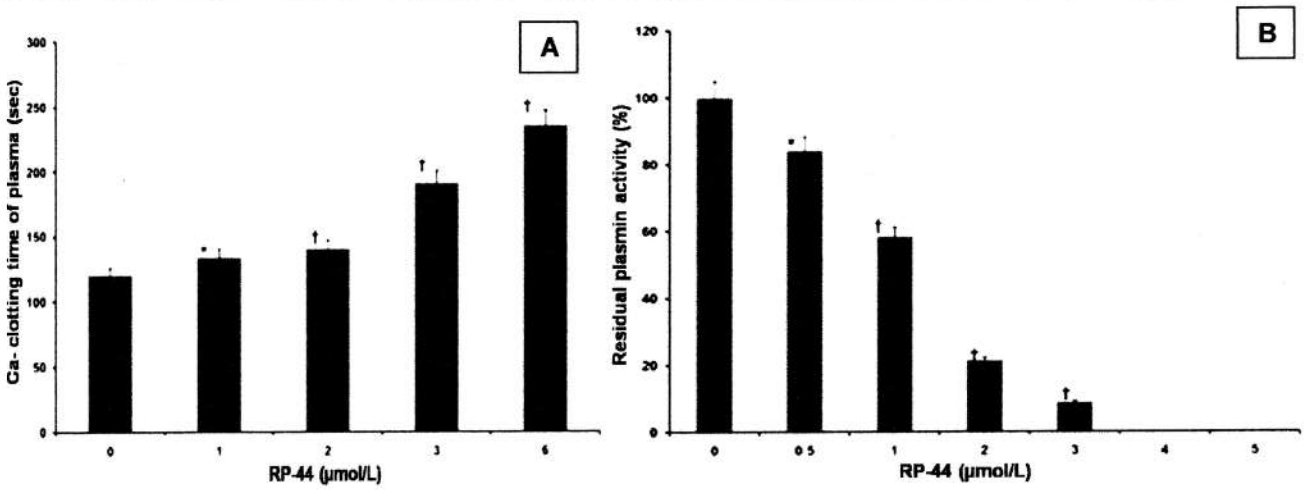


Fig. 6. A. Dose-dependent *in vitro* anticoagulant activity of RP-44. **B.** Dose-dependent inhibition of fibrin degrading activity of plasmin by RP-44. Values are mean ±S.D. of triplicate determinations. Significance of difference with respect to control ($^{*}p < 0.05$, $^{\dagger}p < 0.001$).

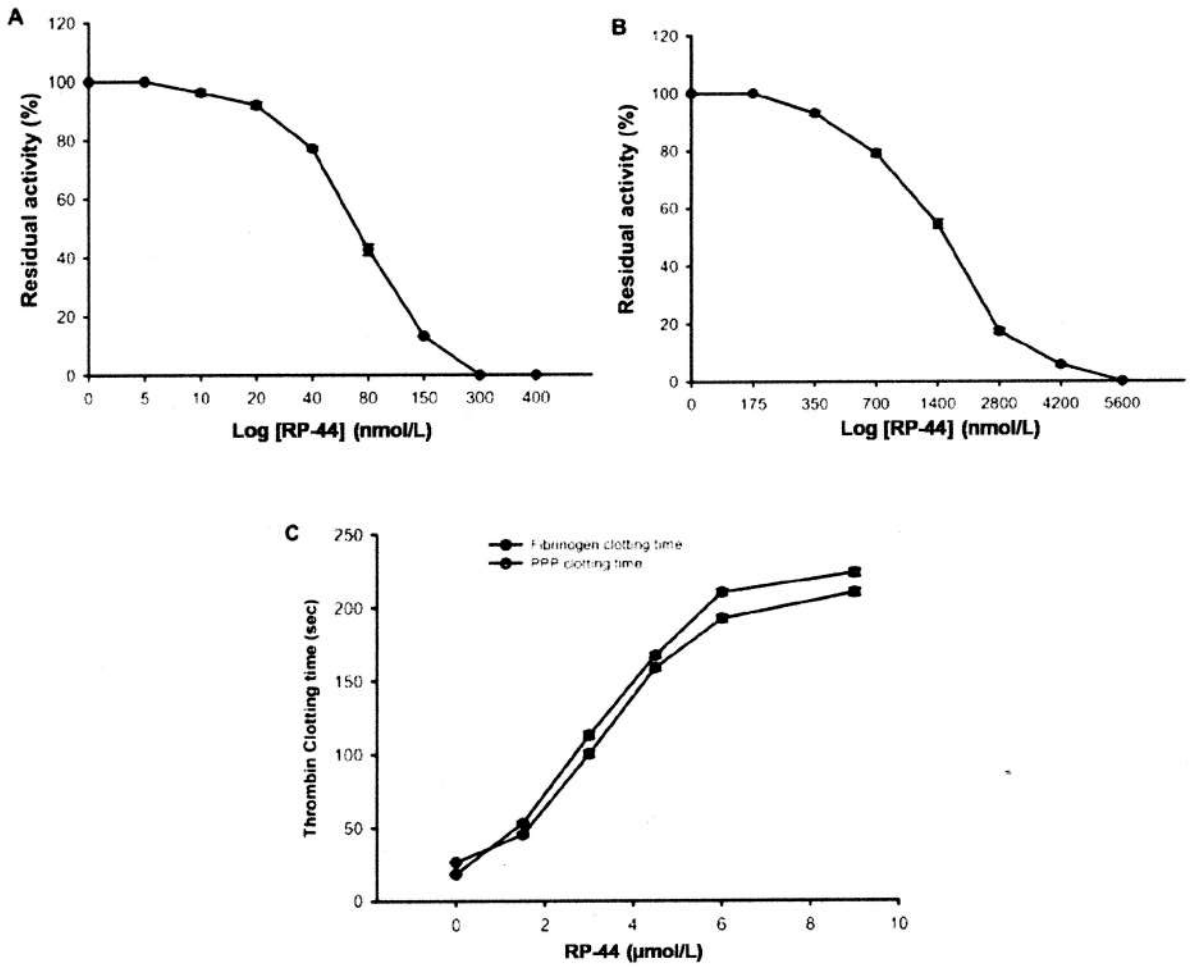


Fig. 7. Dose-dependent inhibitory activity of RP-44 against **(A)** amidolytic activity of trypsin (5 µM), **(B)** amidolytic activity of plasmin (0.5 µM), and **(C)** fibrinogen clotting and PPP clotting activity of thrombin (0.5 µM). Values are mean ±S.D. of triplicate determinations.

3. Purification and characterization of NnPLA$_2$-I: an anticoagulant phospholipase A$_2$ from India cobra (*Naja naja*) venom

Phospholipase A_2 is an unambiguously present component of all snake venoms and has been extensively studied owing to its crucial role in inducing various pharmacological effects in victims. The *N. naja* venom resolved into 6 major peaks (NnCM1-6) (Fig. 8A) when subjected to cation exchange chromatography in a HiPrep CM FF 16/10 column of which NnCM1 showed the highest anticoagulant as well as phospholipase activities. This protein peak was further subjected to gel-filtration chromatography in a Sephadex G-50 gel-filtration column (1 x 64 cm^2) with a 20 mM Tris-HCl buffer, pH 7.4. The NnCM1 fraction was resolved into 8 (NnCM1GF1-8) major peaks when subjected to GFC. The peak NnCM1GF6 demonstrated significant PLA_2 as well as anticoagulant activities and was considered for further analyses. The purified protein, NnPLA$_2$-I, constitutes only 3.4% of the total venom proteins The homogeneity and molecular weight of the purified protein NnPLA$_2$-I was determined using a 12.5% SDS-PAGE, with or without reduction of the protein, wherein NnPLA$_2$-I showed a single band of ~15.2 kDa under reduced condition, whereas under non-reduction it displayed a diffused band of ~21 kDa (Fig. 8B). By MALDI-TOF-MS analysis, the NnPLA$_2$-I demonstrated a doubly charged [MH^{2+}], low intensity peak at m/z 7092.5, and an [MH^+] peak at m/z 14186.0 (data not shown). Furthermore, Lc-MS/MS analysis of NnPLA$_2$-I confirmed the presence of putative conserved domains of PLA_2-like superfamily.

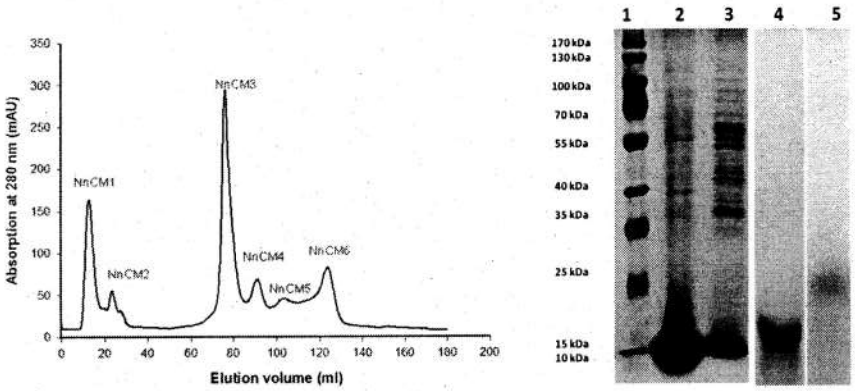


Fig. 8. A. Fractionation of crude *N. naja* venom on cation exchange Hiprep CM FF 16/10 column (16 X 100 mm). The first peak corresponds to the elution of proteins showing phospholipid hydrolysis and anticoagulant activity. **B.** Determination of purity and molecular mass of NnPLA$_2$-I by 12.5% SDS-PAGE; Lane 1, protein molecular markers; lane 2, reduced crude *N. naja* venom (30 µg); lane 3, reduced cation exchange fraction (30 µg); lanes 4 and 5,reduced and non-reduced NnPLA$_2$-I (40 µg), respectively.

NnPLA$_2$-I demonstrated dose-dependent PLA_2 (Fig 9A) and anticoagulant activities (Fig 9B). NnPLA$_2$-I demonstrated significantly higher ($p<0.05$) anticoagulant activity compared to commercial anticoagulants (Fig

9B) and interfered with the intrinsic pathway of blood coagulation, which was confirmed by its effect on activated partial thromboplastin time (APTT) (Fig 9C).

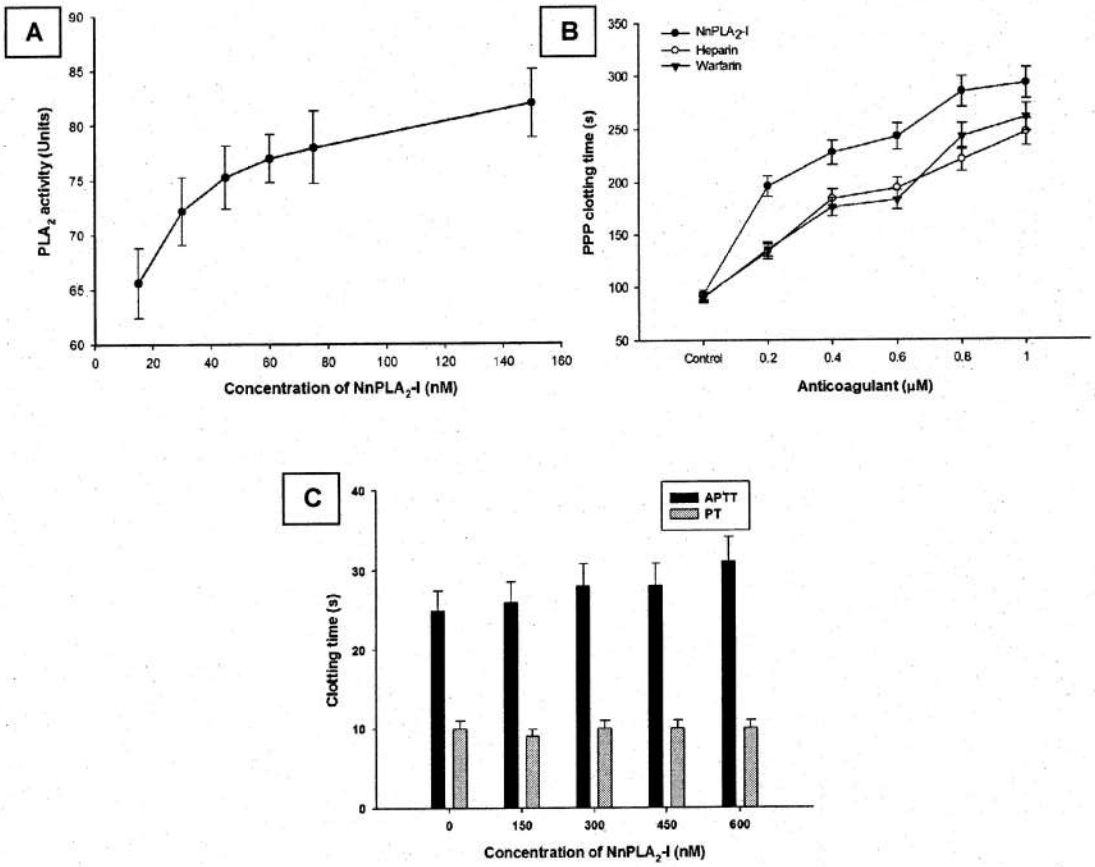


Fig. 9 A. Dose dependent phospholipid hydrolytic activity of $NnPLA_2$-I. **B.** Comparison of the dose-dependent anticoagulant activity of $NnPLA_2$-I (●), heparin (○) and warfarin (▼). **C.** Effect of different concentrations of $NnPLA_2$-I on APTT and PT of PPP. Values are mean ± S.D. of triplicate determinations.

$NnPLA_2$-I inhibited thrombin in a dose-dependent (Fig 10A) as well as time-dependent manner (Fig 10B). This result unambiguously indicated that $NnPLA_2$-I is a thrombin inhibitor. The mode of thrombin inhibition exhibited by $NnPLA_2$-I is a mixed inhibition (Fig 10C), with a *Ki* and α value of 9.3 ± 0.01 (mean ± SD) nM and 7.4 ± 0.7 (mean ± SD), respectively (Table 2). This suggests a high affinity of $NnPLA_2$-I towards thrombin.

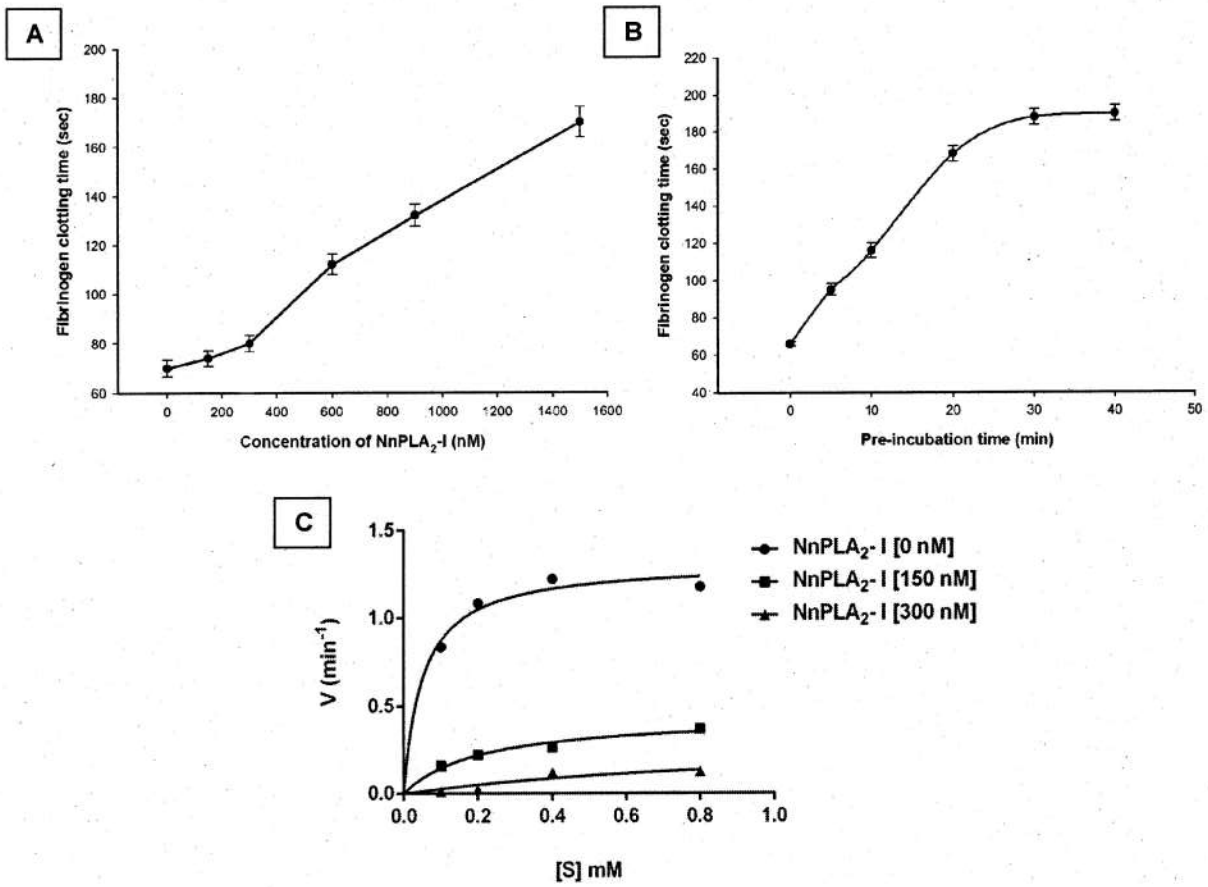


Fig. 10 A. Dose – dependent thrombin inhibition by $NnPLA_2$-I (in terms of fibrinogen clotting assay). The control (fibrinogen and thrombin) showed a clotting time of 70 ± 3.6 s. **B.** Time – dependent thrombin inhibition (determined by fibrinogen clotting activity assay) by a fixed dose of $NnPLA_2$-I (0.5 µM). **C.** Michaelis-Menten plot for studying the kinetics of thrombin inhibition (by amidolytic activity assay) in two different inhibitor concentrations (150 nm and 300 nm) of $NnPLA_2$-I.

Table 3. Kinetics of thrombin inhibition by $NnPLA_2$-I. The kinetic parameters (*Km and Vmax*) were determined from Michaelis-Menten plot as described in the text. The values are mean ± SD of triplicate determinations.

Kinetic parameters	**Concentration of $NnPLA_2$-I (nM)**		
	0	**150**	**300**
Vmax (nmol pNA min^{-1})	1.31 ± 0.08	0.44 ± 0.05	0.31 ± 0.38
Km (nM)	0.05 ± 0.02	0.20 ± 0.07	1.03 ± 1.9
Kcat (min^{-1})	7.9 ± 0.6	2.7 ± 0.2	1.9 ± 0.1

Furthermore, it was observed that $NnPLA_2$-I showed dose-dependent anti-platelet effect when tested against platelet poor plasma (PRP) (Fig. 11A) but it did not show any effect against washed platelets (Fig. 11B). However, supplementation of the reaction mixture with PPP or purified phospholipids PC and/or PS to washed platelets resulted in significant increase in deaggregation property of $NnPLA_2$-I (Fig. 11B) suggesting that the anti-platelet property of $NnPLA_2$-I is by virtue of its catalytic activity. It also inhibited the collagen-induced aggregation of PRP in a dose-dependent manner with an IC_{50} value of 4.9 nM (Fig. 11C). Similar observations were made for thrombin-induced aggregation of human platelets (Fig. 11D). Nevertheless, $NnPLA_2$-I, at a dose of 500 nM, did not exhibit hemolysis, antibacterial or cytotoxicity against mammalian erythrocytes, bacterial strains or U87MG cells, respectively (data not shown). These data highlights the safety of $NnPLA_2$-I for future therapeutic application as potent anticoagulant drug to treat thrombosis-associated cardiovascular disorders.

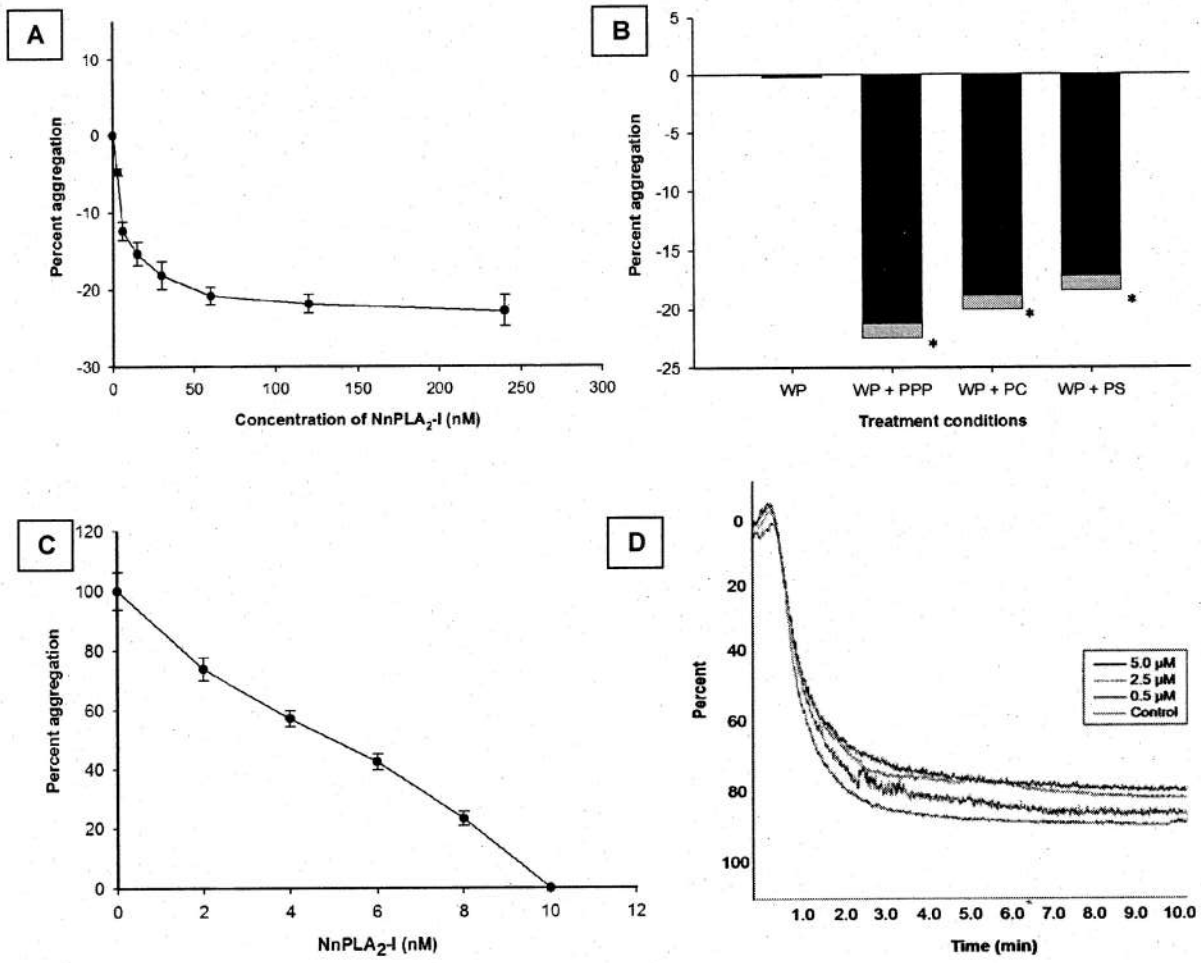


Fig.11. (A) Dose-dependent (3-240 nM) platelet deaggregation property of $NnPLA_2$-I.**(B)** Effect of $NnPLA_2$-I (60 nM) on washed platelets in absence and presence of PPP/PC/PS. **(C)** Inhibition of collagen (1.0 µg/ml) -induced aggregation of PRP by different concentrations (2-10 nM) of$NnPLA_2$-I. **(D)** Dose-dependent (0.5 to 5.0 µM) inhibition of thrombin (0.20 µg/ml) -induced platelet aggregation by $NnPLA_2$-I. Values are mean ± S.D. of three independent experiments. Significance of difference with respect to control, *$p<0.05$.

4. *In silico* study to determine the interaction of $NnPLA_2$-I with thrombin

In another approach to determine the binding of $NnPLA_2$-I with thrombin, an *in silico* analysis was performed. The sequence of $NnPLA_2$-I was determined by PMF analysis, LC-MS/MS and *de novo* sequencing of the purified protein (Thakur et al., 2015; Dutta et al., 2017). The derived sequence of $NnPLA_2$-I is-

NLYQFKNMIKCTVPSRSWWDFADYGCYCGRGGSGTPVDDLDRCCQVHDNCYNEAEKISGCWPYFKTYSYECSQGTLTCKGDN
NACAASVCDCDRLAAICFAGAPYNDNNYNIDLKARCQ

I-TASSER predicted the most favourable structure of $NnPLA_2$-I (Fig. 12A) with an estimated TM-score and RMSD value of 0.93 ± 0.06 and 1.5 ± 1.4 Å, respectively. The best model for interaction of the two molecules ($NnPLA_2$-I and human thrombin) was analysed using PatchDock and FireDock softwares (Fig. 12B). The best model (having the highest score) was submitted to PDBSum software, wherein it was demonstrated that a total of 22 surface residues of $NnPLA_2$-I interacted with 26 surface residues of the heavy chain of thrombin through 369 non-bonded contacts and two H-bonds (Figs. 12C,D).

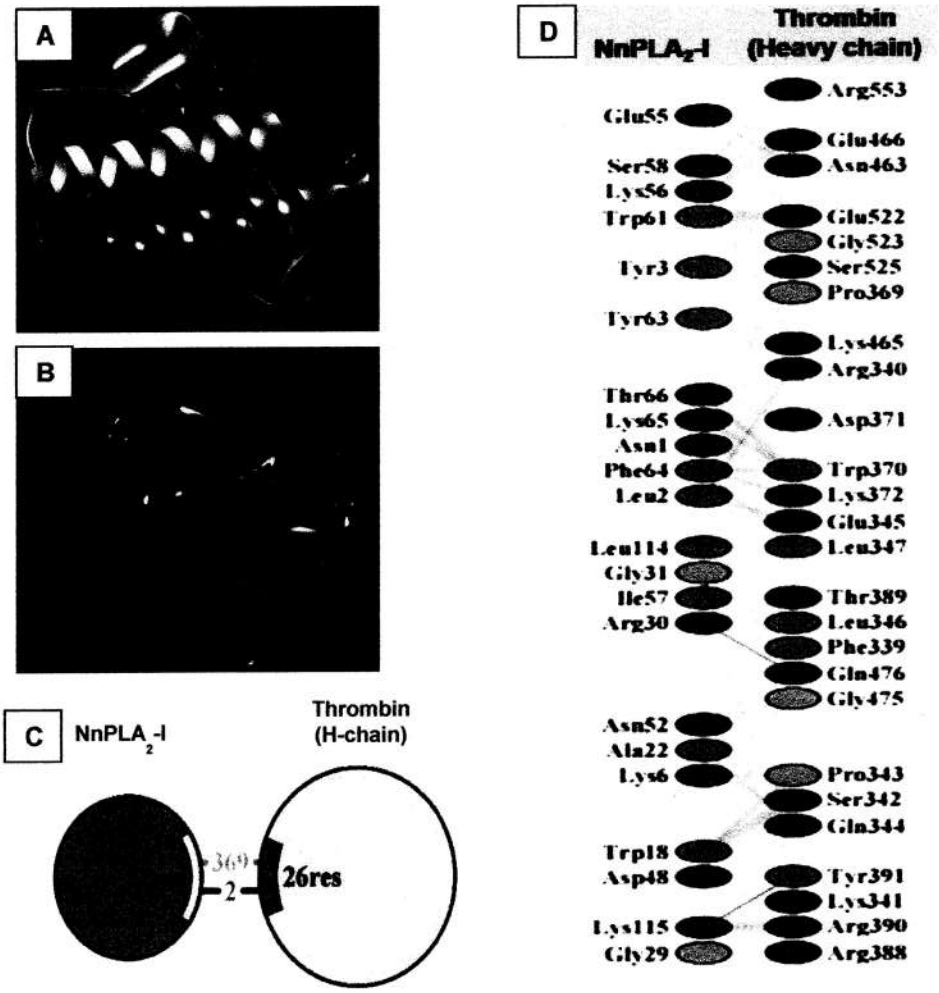


Fig. 12. A. Three-dimensional structure of $NnPLA_2$-I as predicted by I-TASSER software; **B.** The best docking predicted model to study the interaction of $NnPLA_2$-I with human thrombin as predicted by Patchdock and Firedock softwares; **C.** Schematic representation of the interaction between $NnPLA_2$-I (orchid) and heavy chain of human thrombin (yellow) as obtained after analysis by PDBSum software. **D.** Schematic representation of the residue-to-residue interaction between $NnPLA_2$-I and heavy chain of human thrombin as analyzed by PDBSum server.

5. A small 7- amino acid anticoagulant synthetic peptide: Designing, synthesis and characterization

Based on the *in silico* study of $NnPLA_2$-I –thrombin interaction, 12 numbers of anticoagulant region peptides (ACR 1 to 12) were synthesized which correspond to the thrombin binding region of $NnPLA_2$-I, with or without modifications. Among them only one peptide corresponding to formula X_1-A_1-A_2-A_3-A_4-A_5-A_6-A_7-X_2 (M_r = 775.85 Da) showed the highest anticoagulant activity higher than that of heparin and $NnPLA_2$-I (Fig 13) at the tested dose of 3 µg/ml. Therefore, this 7-mer peptide (named as ACR 9) was considered for further characterization.

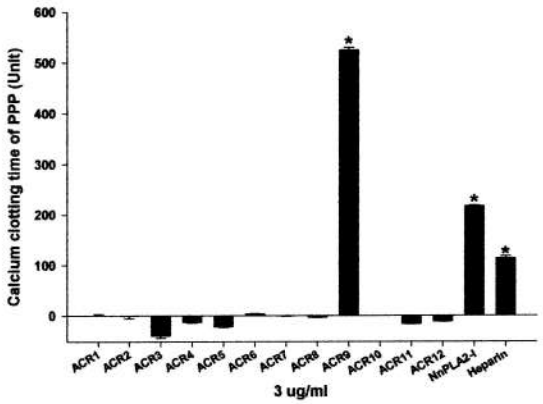


Fig. 13. Comparison of re-calcification time of mammalian PPP in the presence of anticoagulant region peptides (ACR 1 to 12), $NnPLA_2$-I, and heparin at a fixed dose of 3 µg/ml. The clotting tome of control plasma was recorded to be 118.5 ± 2.2 s and other values were compared to that. Values are mean ± SD of triplicate determinations; *$p<0.05$ as compared to control.

It was observed that ACR 9 exhibited higher anticoagulant activity as compared to argatroban, a commercial anticoagulant direct inhibitor of thrombin (Fig. 14A). ACR 9 dose-dependently pro-longed the PT and APTT of mammalian PPP (Fig. 14B), suggesting it interferes with the common pathway of the blood coagulation cascade. Therefore, the effect of the peptide on the amidolytic activity of thrombin and factor Xa, two important components of the common pathway of the coagulation cascade was studied. It was evident that ACR 9 inhibited the amidolytic activity of both the coagulation factors significantly in a dose-dependent manner (Fig 14C,D). Although the thrombin inhibition was comparatively higher with respect to argatroban (commercial thrombin inhibitor) (Fig. 14C), while the FXa inhibition by ACR 9 was comparable to that of fondaparinax (commercial FXa inhibitor) (Fig. 14D).

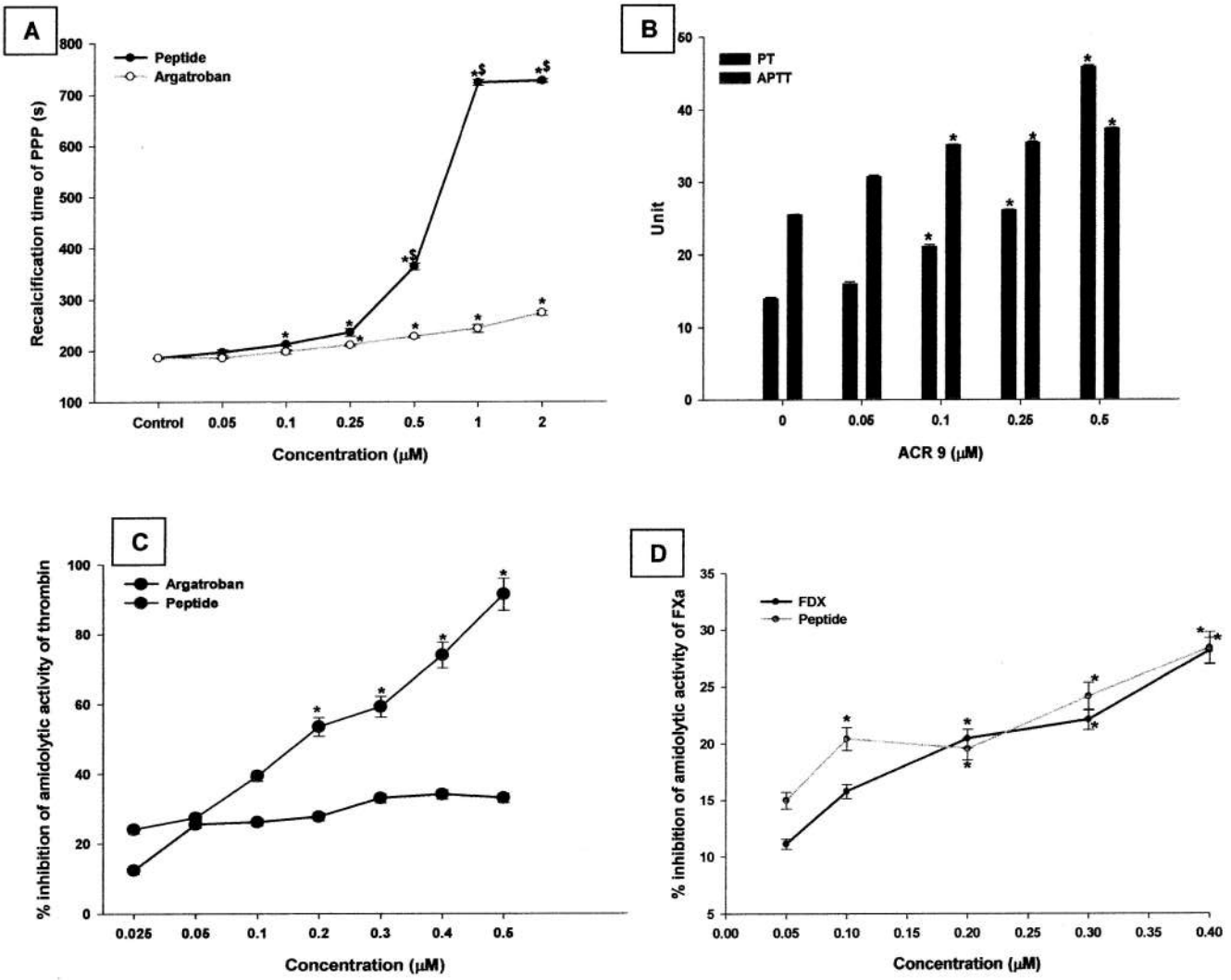


Fig 14. A. Dose-dependent effect of ACR 9 on re-calcification time of mammalian PPP and its comparison to argatroban; **B.** Dose-dependent effect of ACR 9 on prothrombin time (PT) and activated partial thromboplastin time (APTT) of mammalian PPP; **C.** Dose-dependent effect of ACR 9 on amidolytic activity of thrombin and comparison to that of argatroban; **D.** Dose-dependent effect of ACR 9 on amidolytic activity of factor Xa and comparison to that of fondaparinax (FDX). Values are mean ± SD of triplicate determinations; *$p<0.05$ as compared to control, $^{\$}p<0.05$ as compared to positive control.

6. Assessment of *in vivo* anticoagulant activity of synthetic peptide (ACR-9) in rat model

Animal experimental protocols were approved by the Tezpur University Animal Ethical Committee (Approval no: DORDPro/TUAEC/10-56/14/Res-10), and Defense Research Laboratory, Tezpur (Approval no: DRL/02/Dec/2010-11/II). For *in vivo* studies, wistar rats (either sex, 120 g) were injected intravenously with 0.4 mg/kg of argatroban (positive control), heparin (positive control), ACR 9 and $NnPLA_2$-I. To the placebo group, equivalent amount of 1x PBS was administered. After 60 min of injection, blood was withdrawn from the retro-orbital capillary and the toxicity and therapeutic parameters were assessed. Neither ACR 9 or $NnPLA_2$-I were

found to be toxic to the animals, however both showed significantly higher anticoagulant activity as compared to argatroban (Table 3). The effect of ACR 9 and $NnPLA_2$-I on the different parameters of anticoagulation have been summarized in Table 3. The ACR 9, owing to its dual inhibition of thrombin and FXa, showed the highest anticoagulant activity suggesting it can be developed in to a potential anticoagulant drug for treatment of thrombosis associated disorders.

Table 3: Summary of *in vivo* anticoagulant activity of ACR 9 and $NnPLA_2$-I at the tested dose of 0.4 mg/kg, and its comparison to that of argatroban at the same dose. Values are mean ± SD of three independent experiments; significance of difference with respect to control, *$p<0.05$. PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; INR, international normalized ratio.

Sample	PT	INR	APTT	INR	TT	INR	Ca^{2+} clotting time	Tail bleeding time
Control	17.20 ± 0.4	1.0	30.32 ± 0.1	1.0	63.01 ± 0.1	1.0	79.93 ± 1.9	41.11 ± 5.3
ACR 9 (0.4 mg/kg)	38.13 ± 1.1*	2.22	39.61 ± 0.7*	1.31	574.18 ± 5.8*	9.11	246.94 ± 8.8*	206.40 ± 10.6*
$NnPLA_2$-I (0.4 mg/kg)	31.84 ± 0.8*	1.98	44.04 ± 0.1*	1.45	507.45 ± 3.4*	8.05	225.86 ± 7.6*	178.19 ± 9.3*
Argatroban (0.4 mg/kg)	27.81 ± 0.8*	1.62	37.91 ± 0.4*	1.25	106.18 ± 0.6*	1.69	107.43 ± 2.8*	154.56 ± 10.2*
Heparin (0.4 mg/kg)	23.84 ± 0.6*	1.39	34.73 ± 1.0	1.15	93.22 ± 3.7*	1.48	97.48 ± 2.9*	120.13 ± 6.5*

B.2. Summary and conclusions of the findings:

Snake venoms are a rich source of pharmacologically active proteins and peptides. These venoms are known to possess a number of naturally occurring anticoagulant proteins and peptides, such as PLA_2s, snake venom proteases, KSPIs and C-type lectins. The present study showed the purification and characterization of two anticoagulant proteins from *D. russelii* venom and one from *N. naja* venom.

Low toxicity anticoagulants are in great demand for use in the prevention and treatment of occlusive thrombosis, and compounds such as RVsnaclec may hold promise in the design of effective peptide-based cardiovascular drugs. Our results here underscore the utility of investigating natural compounds such as snake venom as a source of human therapeutics, and highly coagulopathic venoms, such as Russell's Viper venom, represent a fertile source for new anticoagulants such as RVsnaclec. On the other hand, Rusvikunin, isolated from the same venom, is the first anticoagulant thrombin inhibiting Kunitz-type protease inhibitor (KSPI) to be reported. Clinical application of Rusvikunin as a peptide-based drug for the treatment of cardiovascular disorders, such as ocular thrombosis, trypsin-mediated inflammatory reactions and anti-bleeding disorders due to antiplasmin deficiencies, can be suggested.

$NnPLA_2$-I is a non-toxic anticoagulant phospholipase A_2 purified from the venom of *N. naja* which exhibits a strong anticoagulant activity, under both *in vitro* and *in vivo* conditions. The anticoagulant mechanism of the

purified PLA_2 is partially by its catalytic activity (~20%) and primarily by non-enzymatic thrombin inhibition (~80%). It demonstrates superior anticoagulant activity when compared to classical anticoagulant drugs-heparin and warfarin, which are known to exhibit several side effects, for example bleeding complications when administered in patient. $NnPLA_2$-I showed a non-enzymatic, mixed inhibition of thrombin with a *Ki* value of 9.3 nM, which is comparable to other thrombin inhibitors isolated from similar sources (Mukherjee et al., 2014; Zingali et al., 1993).

The 7-mer peptide ACR 9 (M_r = 775.85 Da) derived from anticoagulant region of $NnPLA_2$-I, is a strong anticoagulant peptide, capable of future therapeutic application in thrombosis owing to its non-toxic nature. Another potential advantage of the peptide is that due to a low molecular weight (comparable to that of argatroban), it would be cleared quite rapidly from the circulation, without causing any residual effect. Therefore, the application of this peptide as a future cardiovascular therapeutic agent is suggested.

Acknowledgements:

I thank Prof. Stephen P. Mackessey, University of Northern Colorado, USA for providing laboratory facility for purification of RVSnaclec and Rusvikunin. I also thank the project staff Dr. R. Thakur and Mr. A. Chanda for helping in the analysis. Special thanks to Ms. S. Dutta DST-INSPIRE SRF purification and characterization of $NnPLA_2$-I and 7-mer peptide. Dr. Pronobesh Chattopadhyay, Defense Research Laboratory, Solmara has provided us with the animal experimentation facility for *in vivo* experiments of $NnPLA_2$-I and peptide.

B.4. Details of New Leads Obtained, if any:

1. Purification, characterization and elucidation of mechanism of action of two non-toxic, anticoagulant proteins from Russell's viper venom and one non-toxic anticoagulant protein from Indian cobra venom. In this study, for the first time, a C-type lectin like anticoagulant protein (RVsnaclec) and anti-thrombin KSPI (Rusvikunin) have been purified from *Daboia russelii* venom. Also this is the first report of a thrombin inhibitor PLA_2 ($NnPLA_2$-I) from *N. naja* venom.
2. Designing, synthesis and characterization of an anticoagulant 7-mer peptide which is a dual inhibitor of thrombin and factor Xa. The peptide holds good promise for future application as a cardiovascular drug for prevention of unwanted thrombosis.

Details of Publications & Patents, if any:

***Indian Patents*:**

1. Indian Patent on **"SYNTHETIC ANTICOAGULANT PEPTIDES DERIVED FROM *NAJA NAJA* SNAKE VENOM"**; Provisional Patent Application no: **201831010001**; filing date: 19.03.2018.
2. Indian Patent on **"TOXIN EPITOPE-BASED DETECTION OF SPECIES-SPECIFIC SNAKE ENVENOMATION"**; Provisional Patent Application no: **201831010002**; filing date: 19.03.2018.

Research publications in peer-reviewed international journals and book chapters from project:

1. **Mukherjee, A. K.**, Dutta, S., Mackessy, S. P. (2014) A new C-type lectin (RVsnaclec) purified from venom of *Daboia russelii russelii* shows anticoagulant activity via inhibition of FXa and concentration-dependent differential response to platelets in a Ca^{2+}-independent manner. ***Thrombosis Research*** 134, 1150-1156.
2. Thakur, R, Chattopadhyay D., Ghosh S. S., **Mukherjee, A. K.** (2015) Elucidation of procoagulant mechanism and pathophysiological significance of a new prothrombin activating metalloprotease purified from *Daboia russelii russelii* venom. ***Toxicon*** 100, 1-12.
3. Saikia D., **Mukherjee A.K.** (2015) Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A2 Enzymes. In: Gopalakrishnakone P., Inagaki H., Mukherjee A., Rahmy T., Vogel CW. (eds) ***Snake Venoms. Toxinology***. Springer, Dordrecht, 1 – 14.
4. Dutta, S., Gogoi, D., **Mukherjee, A. K.** (2015). Anticoagulant mechanism and platelet deaggregation property of a non-cytotoxic, acidic phospholipase A_2 purified from Indian cobra (*Naja naja*) venom: Inhibition of anticoagulant activity by low molecular weight heparin. ***Biochimie*** *110*, 93-106.
5. Thakur R, and **Mukherjee, A. K.** (2016) Pathophysiological significance and therapeutic implications of Russell's viper proteins and peptides effecting blood coagulation. Invited article submitted in ***Snake Venoms and Envenomation: Modern Trends and Future Prospects*** (Utkin, Y. editor), Nova Science Publishers, INC. USA, 93-114.
6. **Mukherjee, A. K.**, Dutta, S., Kalita, B., Jha, D. K., Deb, P., Mackessy, S. P. (2016). Structural and functional characterization of complex formation between two Kunitz-type serine protease inhibitors from Russell's Viper venom. ***Biochimie*** *128*, 138-147.
7. Thakur, R., **Mukherjee, A. K.** (2017). Pathophysiological significance and therapeutic applications of snake venom protease inhibitors. ***Toxicon*** 131, 37-47.

Paper presented in National and International conferences:

1. **Mukherjee, A. K.** Therapeutic application of anticoagulant proteins purified from snake venom for the treatment of cardiovascular disorders: Prospects and challenges. ***83rd Annual Conference of Society of Biological Chemists of India***, KIIT University, Bhubaneswar 17-21 December, 2014.
2. Dutta, S., **Mukherjee, A. K.** Characterization of biochemical and pharmacological properties of a noncytotoxic, acidic phospholipase A_2 purified from Indian cobra (*Naja naja*) venom. ***83rd Annual Conference of Society of Biological Chemists of India***, KIIT University, Bhubaneswar 17-21 December, 2014.
3. **Mukherjee, A.K.**, Dutta, S., Kalita, B., Mackessy, S. P. Potential biomedical application of Kunitz-type protease inhibitors from *Daboia russelii russelii* venom. ***18th World Congress of International Society on Toxinology***, Oxford, UK from 25-30 September, 2015.

4. **Mukherjee, A.K.**, Dutta, S., Kalita, B., Jha, D.K., Deb, P., Mackessy, S.P. Characterization of Rusvikunin complex isolated from *Daboia russelii russelii* venom to comprehend its potent biological activity, ***National Seminar on Snake Venom Research and Snake Bite Therapy: National and International Perspectives (SnakSymp 2016)***, held at Tezpur University, 22-24 November, 2016.
5. Das, A., Ramani, S., Dutta, S., Gogoi, D., Bora, B., **Mukherjee, A.K.** Cardiovascular drug development from snake venom: Fact or fiction?, ***National Seminar on Snake Venom Research and Snake Bite Therapy: National and International Perspectives (SnakSymp 2016)***, held at Tezpur University, 22-24 November, 2016.
6. Dutta, S., Kalita, B., **Mukherjee, A.K.** Protein and peptide-based anticoagulant cardiovascular drug development from snake venom: Phospholipase A_2 is an example. ***3rd International Conference on Translational Research: Application in Human Health and Agriculture.*** Organized by Indian Society of Translational Research, Amity University, Kolkata, 22-25 September, 2017.

Consolidated Utilization Certificate

(For the project tenure 25th November, 2014 - 31st March, 2018)

1	Title of the Project/Scheme	Discovering novel drug formulations from non-toxic, potent anticoagulant components of snake venom for the prevention or treatment of cardiovascular disease (CVD)
2	Name of the Organization	Department of Molecular Biology & Biotechnology, Tezpur University.
3	Principal Investigator	Prof. Ashis K. Mukherjee
4	Deptt. of Biotechnology sanction order No. & date of sanctioning the project	BT/HRD/NBA/34/01/2012-13 dated November 25, 2014
5	Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given	NA
6	Amount received from DBT during the financial year (2014-2018)	Rs. 14,60,709.00
7	Other receipts/interest earned, if any, on the DBT grants:	Rs. 4,509.00
8	Total amount that was available for Expenditure for the project:	Rs. 14,65,218.00
9	Actual expenditure incurred during the project tenure 2014-2018 :	Rs. 14,62,262.00
10	Unspent balance refunded, if any *(Please give details of cheque No. etc.):*	Rs. 2956.00 (DD No. 454269 ; dated 18-07-18)
11	Balance amount available at the end of the project :	Rs. 2,956.00
12	Amount allowed to be carried forward to the next financial year vide letter No. & date	NA

Joint Registrar
Tezpur University
25/06/18

1. Certified that the amount of ₹ 14,62,262.00 (Rupees fourteen lakhs sixty two thousand two hundred sixty two only) mentioned against col. 9 has been utilized on the project/scheme for the purpose for which it was sanctioned and that the balance of ₹ 2,956.00 (Rupees two thousand nine hundred fifty six only) remaining unutilized at the end of the year has been surrendered to Govt. (vide No. 454269 dated 18-07-2018)/will be adjusted towards the grants-in-aid payable during the next year 2015-2016.
2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Orders for chemicals were placed after T&PC approval of price.
2. Stock entry of chemical, consumable & contingency items etc.
3. Appointment of JRF following the DBT guidelines after floating the advertisement.
4.
5.

(Signature of Principal Investigator)

(Signature of Finance officer)

25/06/18

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular
Biology & Biotechnology
Tezpur University (Central)
Tezpur- 784028, Assam

(Signature of Head of the Institute)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

Title of the Project : Discovering novel drug formulations from non-toxic, potent anticoagulant components of snake venom for the prevention or treatment of cardiovascular disease (CVD)

1. **Sanctioned Project Cost:** Rs. 15.00 Lakhs (Rupees Fifteen lakhs only)
2. **Revised cost, if any** : Nil
3. **Duration of the project:** Three Years (2015-2018)
4. **Sanction Order No. & Date:** Sanction order no. BT/HRD/NBA/34/01/2012-13 dated 25-11-2014
5. **Date of commencement of Project:** 25th November 2014
6. **Extension, if any:** Nil
7. **Date of completion of project:** 31st March 2018

❖ **Details of grant, expenditure and balance**

Sl. No.	Heads	Total sanctioned (Rs.)	Year wise releases made (in Rs.)					Year wise expenditure incurred (in Rs.)					
			1st yr (25.11.14-31.3.15)	2nd yr (01.04.15-31.03.16)	3rd yr (01.04.16-31.03.17)	4th yr (01.04.17-31.03.2018)	Total (Rs.)	1st yr (25.11.14-31.3.15)	2nd yr (01.04.15-31.03.16)	3rd yr (01.04.16-31.03.17)	4th yr (01.04.17-31.03.2018)	Total (Rs.)	Balance (Rs.)
(i)	Manpower	1500000.00	500000.00	0.00	500000.00	460709.00	1460709.00	0.00	54000.00	52529.00	86333.00	192862.00	Rs. 2,956.00
(ii)	Consumable and Analysis charge							0.00	286034.00	327355.00	302253.00	915642.00	
(iii)	Travel - Domestic							0.00	0.00	49820.00	38276.00	88096.00	
(iv)	Contingency							0.00	159932.00	14704.00	70676.00	245312.00	
(v)	Equipment								0.00	20350.00	0.00	20350.00	
(vi)	Maintenance							0.00	0.00	0.00	0.00	0.00	
(vii)	Overheads							0.00	0.00	0.00	0.00	0.00	
(viii)	Interest earned		0.00	2686.00	1329.00	494.00	4509.00	0.00	0.00	0.00	0.00	0.00	
Totals		**1500000.00**	**500000.00**	**2686.00**	**501329.00**	**461203.00**	**1465218.00**	**0.00**	**499966.00**	**464758.00**	**497538.00**	**1462262.00**	

(Signature of Principal Investigator)
Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular
Biology & Biotechnology
Tezpur University (Central)
Tezpur- 784028, Assam

(Signature of Head of the Institute)
Registrar
Tezpur University

(Signature of Finance officer)
Joint Registrar
Tezpur University