ANNEX-I: Sections B1-B7

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

DBT TWINING PROJECT

Assessment of thrombolytic potential and anticancer activity of fibrinolytic enzymes purified from Russell's viper venom and bacterial isolated from NE India

FINAL PROJECT REPORT

(1st April, 2011 to 18th September, 2014)

Submitted By

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DEPARTMENT OF BIOTECHNOLOGY, NEW DELHI FUNDED RESEARCH PROJECT

INFORMATION SHEET

Title of the project	Assessment of thrombolytic potential and anticancer activity of fibrinolytic enzymes purified from Russell's viper venom and bacteria isolated from NE-India.
DBT sanction no. and date	BT/38/NE/TBP/2010, 18.3.2011
Name of the PI, designation and address where the project is implemented	**(a) From North-East India:** Dr. A. K. Mukherjee, Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784 028 E-mail: akm@tezu.ernet.in **(b) Collaborative Institute:** Dr. Dulal Panda, Professor, Department of Bioscience and Bioengineering, Indian Institute of Technology Bombay, Mumbai-400 076, E-mail: panda@iitb.ac.in
Category of the project	R & D under DBT-twinning programme
Duration of project	3 years (due to non release of fund in time, project was extended up to 18.9.2014)
Effective date of starting the project	1st April, 2011
Total budget sanctioned	Total: Rs 160.04 lakh (a) Tezpur University: Rs 120.86 lakh (b) IIT Bombay: Rs 39.18 lakh
Project report No.	Third and Final
Duration of report	1st April, 2011 to 18th September, 2014
Objectives of the project	1. Purification and biochemical characterization of fibrinolytic enzymes from protease secreting bacteria isolated from environment and fermented food-samples of North-East India and from Russell's viper venom. 2. To study the biophysical properties of the fibrinolytic enzymes and their derivatives 3. Characterization of pharmacological properties and cytotoxicity of purified fibrinolytic enzymes and their derivatives 4. Assessment of *in vitro* thrombolytic activity, anticancer activity, gastrointestinal absorption and toxicity of the fibrinolytic enzymes on mammalian cell lines.

A. K. Mukherjee
(PI/Coordinator, NE India)
Dated: 14/8/2015

D. Panda
(PI, Collaborative Institute)
Dated:

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DETAIL TECHNICAL REPORT OF THE PROJECT

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period

1. **Objectives sanctioned Vs Objectives Achieved: at a Glance**

No	Objective	Achieved or not (Y/N)
1	Purification and biochemical characterization of fibrinolytic enzymes from protease secreting bacteria isolated from environment and fermented food-samples of North-East India and from Russell's viper venom	Yes
2	To study the biophysical properties of the fibrinolytic enzymes and their derivatives	Yes
3	Characterization of pharmacological properties and cytotoxicity of purified fibrinolytic enzymes and their derivatives	Yes
4	Assessment of *in vitro* thrombolytic activity, anticancer activity, gastrointestinal absorption and toxicity of the fibrinolytic enzymes on mammalian cell lines.	Yes

Brief Outline of Achievements in 1st Year (2011-2012)

1. Procurement of equipment, chemicals, appointment of Project Staff
2. Isolation, purification and biochemical characterization of a direct-acting fibrinolytic enzyme from *Bacillus* sp. strain AS-S20-I, isolated from a soil sample from Assam, northeast India [Work done at Tezpur University]
3. Screening, and taxonomic identification of fibrinolytic enzyme producing bacteria from fermented food samples of north-east India: Isolation and partial purification of a fibrinolytic enzyme. [Work done at Tezpur University]
4. Statistical optimization of fibrinolytic enzyme production by potential bacterial strains
5. Isolation and purification of an anticoagulant peptide from Russell's viper venom showing anticancer activity. [Work done jointly with IIT, Mumbai and Tezpur University].

Brief Outline of Achievements in 2nd Year (2012-2013)

The work done in second year is the continuation of the work done in the second year to achieve the target of the second year. In brief, we fulfill the following targets.

1. Biophysical and pharmacological characterization and cytotoxicity assay of a direct-acting fibrinolytic enzyme (Bafibrinase) from *Bacillus* sp. strain AS-S20-I, isolated from a soil sample from Assam, northeast India.
2. Purification, biochemical and pharmacological characterization of fibrinolytic enzymes purified from (a) *Bacillus cereus* strain FF01
3. Biochemical characterization and *in vitro* cytotoxicity of an anticoagulant peptide (Ruviprase) purified from Russell's viper venom.

Brief Outline of Achievements in 3rd Year (2013-2014)

1. Purification, biochemical and pharmacological characterization of a fibrinolytic enzymes from *Bacillus brevis* strain FF01
2. Assessment of in vitro thrombolytic activity and anticoagulant activity of purified protease and comparison of these properties with commercial drugs
3. Elucidation of anticancer mechanism of Ruviprase in MCF-7 cells
4. Purification and characterization of a non-toxic protease showing in vivo anticoagulant activity from Russell's Viper (*Daboia russelii russelii*) venom.

Summary of Major Findings

1. **Purification and characterization of a fibrin(ogen)olytic serine protease (Bacethrombase) showing antiplatelet and antithrombotic activity from *Bacillus cereus* strain FF01**

Fibrin(ogen)olytic enzymes offer great promise for the treatment of thrombosis associated disorders. An extracellular fibrin(ogen)olytic protease-producing bacterial strain (FF01) was isolated from a rice beer alcohol producing starter culture of North East India, Assam. By polyphasic approach (phenotypic characterization, biochemical properties and ribotyping) the stain FF01 was identified as *Bacillus cereus* strain FF01. Initially, Plackett-Burman design was applied to screen the significant factors influencing the yield of fibrinolytic enzyme (measured in terms of fibrinolytic / caseinolytic ratio) in submerged fermentation after 24 h of incubation at pH 8.0 and 37°C. Further, response surface methodology (RSM) associated central composite design showed optimum enzyme production after 120 h of incubation with 0.0057% (w/v) of casein and 0.0318%(w/v) of ammonium sulfate at pH 8.0 of the MSM. This resulted in 5.24 fold enhancement of fibrin(ogen)olytic enzyme production from this bacterium with a corresponding increase in F/C value of culture supernatant from 85 (from initial, unoptimized conditions) to 446 (production under RSM optimized conditions).T

An extracellular fibrin(ogen)olytic serine protease (named Bacethrombase) was then purified from *Bacillus cereus* strain FF01by hydrophobic interaction chromatography of 40% ammonium sulphate preceipitated fraction followed by gel-filtration of active fraction. The molecular mass of the Bacethrombase was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Matrix Assisted Laser Desorption/Ionization-Time-of-Flight–Mass Spectroscopy (MALDI-ToF-MS) analyses at 39.5 kDa (Fig. 1A) and 38450.51 Da, respectively.

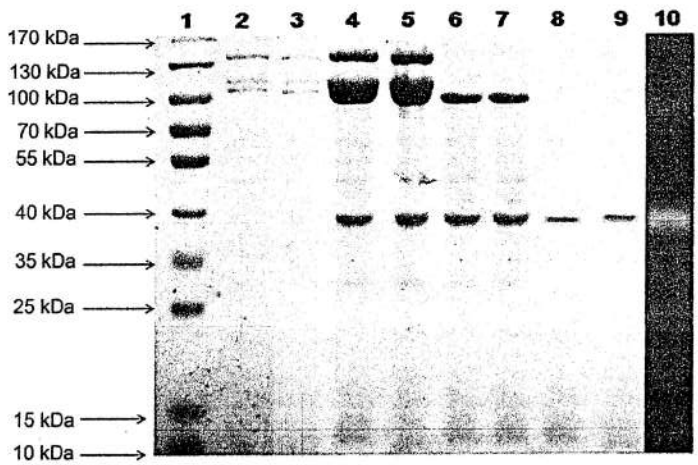

Fig. 1A. Determination of purity and molecular mass of Bacethrombase by 12.5% SDs-PAGE analysis.

The peptide mass fingerprinting and analyses of the composition of the amino acids revealed the similarity of the Bacethrombase to the bacterial serine proteases. The secondary structure of the

Bacethrombase was composed of 14% helix, 6.6% beta-sheet, and 79.4% random coil. Bacethrombase was found to contain 48% sialic acid and it preferentially degraded the Aα-chain of fibrinogen (Fig. 1B), as well as fibrin (Fig 1C).

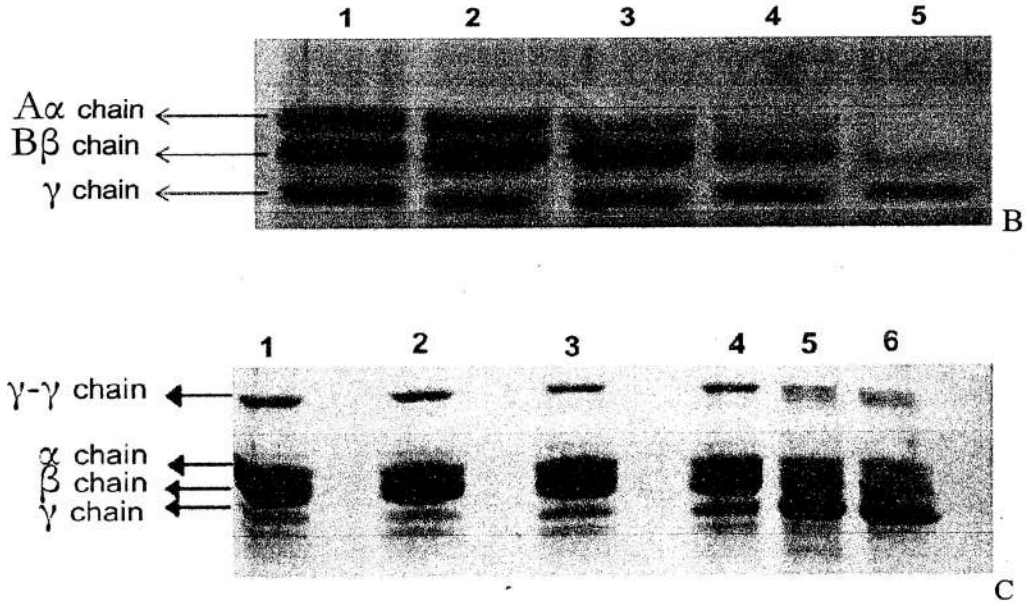

Fig. 1B. Time-dependent fibrin degradation by Bacethrombase (1.0 μM) at 37 °C, pH 7.4. The fibrin degradation products were separated by 12.5% SDS-PAGE under reduced conditions. (a) Lane 1, control fibrin (without Bacethrombase treatment); lanes 2 to 6, fibrin degradation by Bacethrombase after 30, 60, 90, 120 and 180 min of incubation, respectively at 37 °C, pH 7.4.

Fig. 1C. Time-dependent fibrin degradation by Bacethrombase (1.0 μM) at 37 °C, pH 7.4. The fibrin degradation products were separated by 12.5% SDS-PAGE under reduced conditions. (a) Lane 1, control fibrin (without Bacethrombase treatment); lanes 2 to 6, fibrin degradation by Bacethrombase after 30, 60, 90, 120 and 180 min of incubation, respectively at 37 °C, pH 7.4.

The anticoagulant potency of the Bacethrombase was comparable with that of warfarin and heparin (Fig. 1D), and was corroborated by its fibrinogenolytic activity rather than the inhibition of thrombin, prothrombin or FXa. Bacethrombase demonstrated antiplatelet activity, and dose-dependently inhibited the ADP-induced platelet aggregation. Bacethrombase (10 mg/kg) did not show toxicity after i.v. administration in Wistar rats; however, it revealed an *in vivo* anticoagulant effect and significantly inhibited the carrageenan-induced *in vivo* thrombus formation in rats.

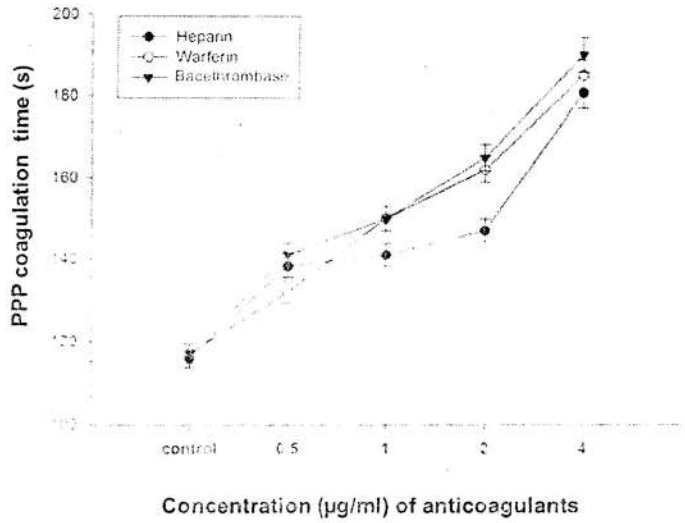

Fig. 1D. A comparison of the dose-dependent anticoagulant activity among Bacethrombase, warfarin, heparin, and plasmin under identical experimental conditions (37 °C, pH 7.4). Data represent mean ± SD of triplicate determinations.

2. **Characterization, mechanism of anticoagulant action, and antithrombotic activity of a fibrinolytic serine protease (Brevithrombolase) purified from *Brevibacillus brevis* strain FF02B**

In this study, biochemical and pharmacological characterization of Brevithrombolase, a fibrinolytic serine protease purified from *Brevibacillus brevis* strain FF02B has been reported. An assessment of its thrombolytic potency has also been made.The strain was isolated from fermented food sample of India and pure culture was obtained by serial dilution technique. By ribotyping (16s rDNA sequencing), sequencing of ISR (16s-23s region), and phenotypic characterization, the strain FF02B was identified as *Brevibacillus brevis* strain FF02B.

By using multidimensional chromatographic technique, an extracellular fibrinolytic serine protease was purified from this strain. This enzyme was named Brevithrombolase. The molecular mass of this monomeric protease (Brevithrombolase) was determined as ~55kDa (Fig. 2A), and 56043 Da (Fig 2B), respectively, by SDS-PAGE and MALDI-TOF-MS. In the analytical studies, the N-terminal sequence of Brevithrombolase was found to be blocked; however, the peptide mass fingerprinting and amino acid composition analyses demonstrated the similarity of Brevithrombolase with endopeptidases in possessing serine in their catalytic triad. This finding was confirmed by the observation that the serine protease inhibitors decrease the catalytic (fibrinolytic) activity of Brevithrombolase.

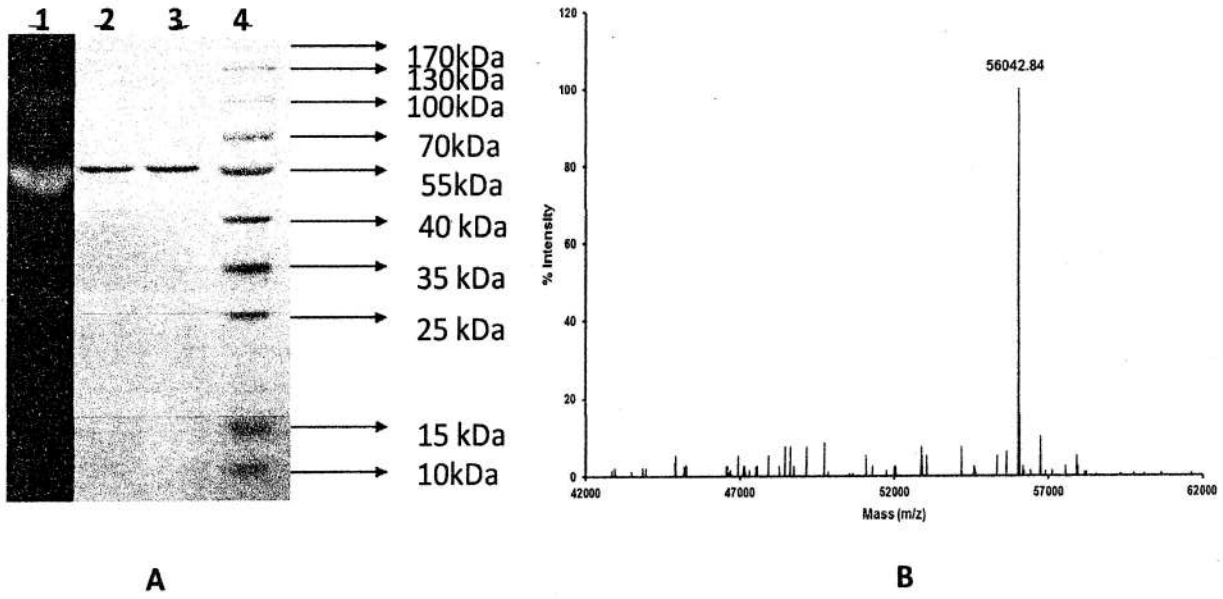

Fig. 2. Determination of purity and molecular mass of Brevithrombolase by- (A) 12.5% SDS-PAGE, and (B) MALDI-ToF-MS.

The secondary structure of Brevithrombolase was composed of 30.6% alpha helix and 69.4% random coil. Brevithrombolase showed the *Km* and *Vmax* values towards the chromogenic substrate for plasmin at 0.39 mM and 14.3 µmol/min, respectively. Brevithrombolase demonstrated optimum fibrinolytic activity at pH 7.4 and 37°C, and showed marginal hydrolytic activity towards globulin, casein and fibrinogen. The anticoagulant potency of Brevithrombolase was comparable to the low molecular mass heparin/antithrombin-III and warfarin (Fig. 2C).

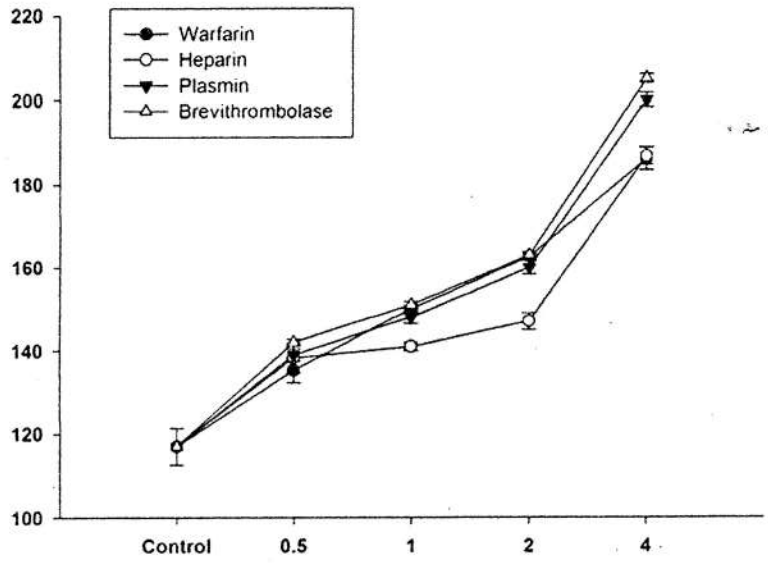

Fig. 2C. A comparison of the dose-dependent anticoagulant activity among Brevithrombolase, warfarin, heparin and plasmin under identical experimental conditions. Data represent mean ± SD of triplicate determinations.

However, among the three enzymes—Brevithrombolase, plasmin and streptokinase—the fibrinolytic activity and *in vitro* thrombolytic potency of Brevithrombolase was found to be superior. The RP-HPLC and SDS-PAGE analyses suggested a similar pattern of fibrin degradation by Brevithrombolase and plasmin, indicating that former enzyme is a plasmin-like fibrinolytic serine protease. Brevithrombolase did not show *in vitro* cytotoxicity on HT29 and HeLa cells or hemolytic activity. Brevithrombolase did not inhibit factor Xa, and its mechanism of anticoagulant action was associated with the enzymatic cleavage of thrombin (Fig. 2D).

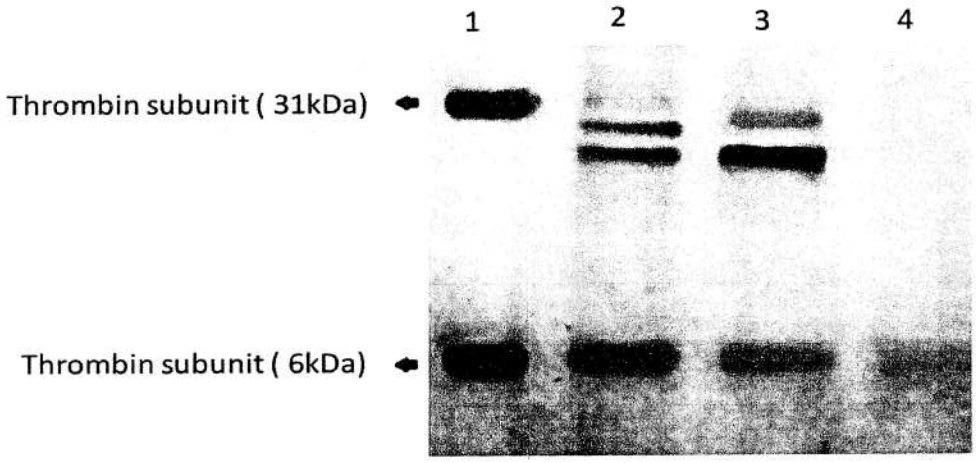

Fig. 2D. Kinetics of thrombin degradation by Brevithrombolase at 37°C.The thrombin degraded products were analyzed by 15% SDS-PAGE under reduced conditions. Lane1, control thrombin; lanes 2-4,thrombin treated with Brevithrombolase (7.0μM) for 30, 60 and 120 min, respectively.

At a dose of 10 mg/ kg, which is almost 20 and 5- to 10-fold higher dose than the therapeutic dose of streptokinas and plasmin, respectively, Brevithrombolase did not exhibit lethality or toxicity on Wistar strain albino rats. Taken together, the biochemical and pharmacological properties, thrombolytic potency and strong anticoagulant activity of Brevithrombolase by hydrolysis of thrombin may lead us to anticipate that it is a promising candidate for the peptide-based lead molecule for the treatment and/or prevention of cardiovascular disorders.

3. **Purification and characterization of a non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease (Bafibrinase) showing *in vivo* anticoagulant activity and thrombolytic potency from *Bacillus* sp. strain AS- S20-I**

Our previous study has shown that *Bacillus* sp. strain AS-S20-I is capable of secreting significant amount of fibrinolytic enzyme in culture medium. In this project, a non-toxic, direct-acting fibrinolytic serine protease (Bafibrinase) demonstrating thrombolytic and anticoagulant properties was purified from *Bacillus* sp. strain AS-S20-I. Bafibrinase was monomeric, with a molecular mass of 32.3 kDa (Fig. 3A). The peptide mass fingerprinting of Bafibrinase revealed only 8.3% sequence coverage, suggesting it was

a novel fibrinolytic enzyme. However, two of the tryptic digested *de novo* peptide sequences of Bafibrinase demonstrated good similarity with endopeptidases possessing serine in their catalytic triad. Further, catalytic activity of Bafibrinase was inhibited by serine protease inhibitor reinforcing this is a subtilisin-like serine protease.

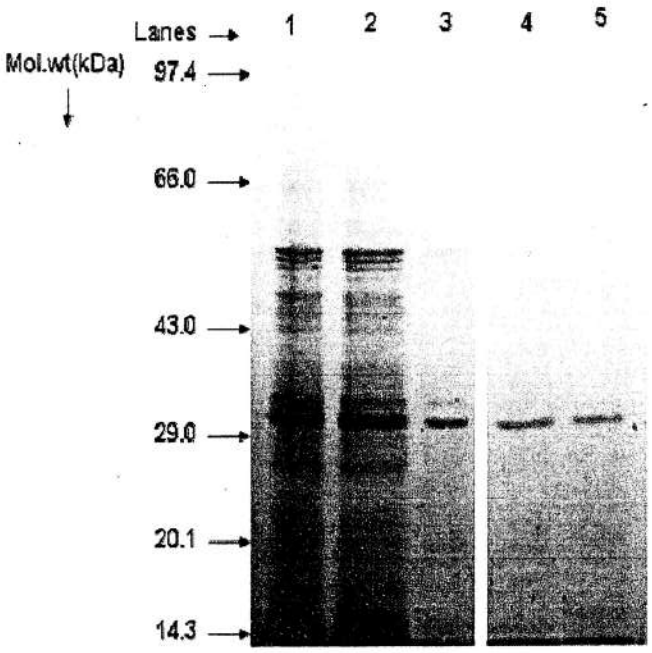

Fig. 3A. SDS-PAGE (12.5%) of RP-HPLC fraction from *Bacillus* sp. AS-S20-I. Lane 1 –crude protease under reduced conditions (50 µg); Lane 2: crude protease under non-reduced conditions (50 µg), Lane 3: acetone precipitated fraction (10.0 µg); Lane 4: RP-HPLC fraction under reduced conditions (8.0 µg); Lane 5: RP-HPLC fraction under non-reduced conditions (8.0 µg).

The apparent Km and Vmax values of Bafibrinase towards fibrin were determined as 0.24 µM and 2.8 µmol/min, respectively. It showed a Km value of 0.139 mM towards a chromogenic substrate for plasmin (D-Val-Leu-Lys-p-Nitroanilide dihydrochloride) and optimum activity at physiological conditions (37 °C and pH 7.4). Based on the cleavage pattern of fibrin and fibrinogen, Bafibrinase may be classified as an α, β-fibrinogenase (Fig. 3B). Bafibrinase could not degrade collagen and was non-cytotoxic to HT29 cells or mammalian erythrocytes. Further, Bafibrinase at a dose of 2mg/ kg was devoid of toxicity as well as hemorrhagic activity on BALB/c mouse model, supporting its suitability for the development of a better and safer thrombolytic drug. Bafibrinase was also superior to human plasmin in degrading *in vitro* thrombus. The *in vivo* anticoagulant nature of Bafibrinase is being explored for the treatment and prevention of thrombosis and other cardiovascular diseases.

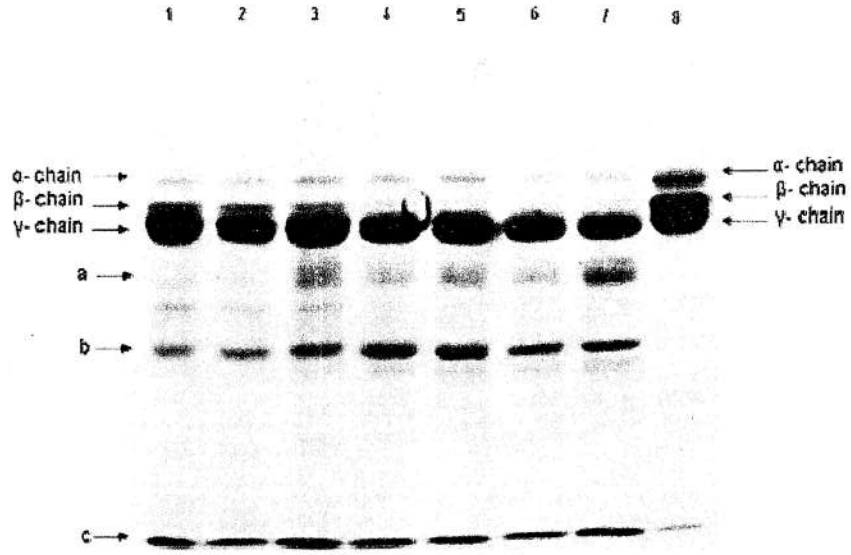

Fig 3B. Fibrinogen degradation by Bafibrinase from *Bacillus* sp. strain AS-S20-I. The fibrinogen degradation products were separated by 12.5% SDS-PAGE. Lanes 1-7: fibrinogen degradation pattern by 5.0 µg of Bafibrinase after 30, 60, 120, 240, 360, 480 and 720 min of incubation, respectively at 37°C; Lane 8: control (fibrinogen without Bafibrinase).

In conclusion, The fibrinolytic protease Bafibrinase isolated from *Bacillus* sp. strain AS- S20-I is a novel plasmin-like, direct-acting fibrinolytic enzyme with high specificity toward fibrin and lower fibrinogen-degrading activity. Thrombolytic drugs in current use, such as t-PA, SK and u-PA, promote effects such as rapid dissolution of thrombi by activating the natural fibrinolytic system and generating plasmin from plasminogen. However, Bafibrinase acts directly on fibrin, thereby reducing the undesirable side effects associated with currently available thrombolytic therapy. Moreover, the much greater specificity of Bafibrinase for fibrin, as compared to plasmin, as well as the lack of both *in vitro* and *in vivo* toxicity, offers an attractive potential alternative to the treatment of thrombosis-associated diseases.

4. **Purification and characterization of Ruviprase-a dual inhibitor of thrombin and FXa from *Daboia russelii russelii* venom**

Compounds showing dual inhibition of thrombin and factor Xa (FXa) are the subject of great interest owing to their broader specificity for effective anticoagulation therapy against cardiovascular disorders. This is the first report on the functional characterization and assessment of therapeutic potential of a 4,423.6 Da inhibitory peptide (Ruviprase) purified from *Daboia russelii russelii* venom. The secondary structure of Ruviprase is composed of α-helices (61.9 %) and random coils (38.1%). The partial N-terminal sequence (E^1-V^2-X^3-W^4-W^5-W^6-A^7-Q^8-L^9-S^{10}) of Ruviprase demonstrated significant similarity (80.0 %) with an internal sequence of apoptosis-stimulating protein reported from the venom of

Ophiophagus hannah and *Python bivittatus;* albeit Ruviprase did not show sequence similarity with existing thrombin/FXa inhibitors, suggesting its uniqueness. Ruviprase demonstrated a potent *in vitro* anticoagulant property (Fig. 4A) and inhibited both thrombin (Fig. 4B) and FXa following slow binding kinetics.

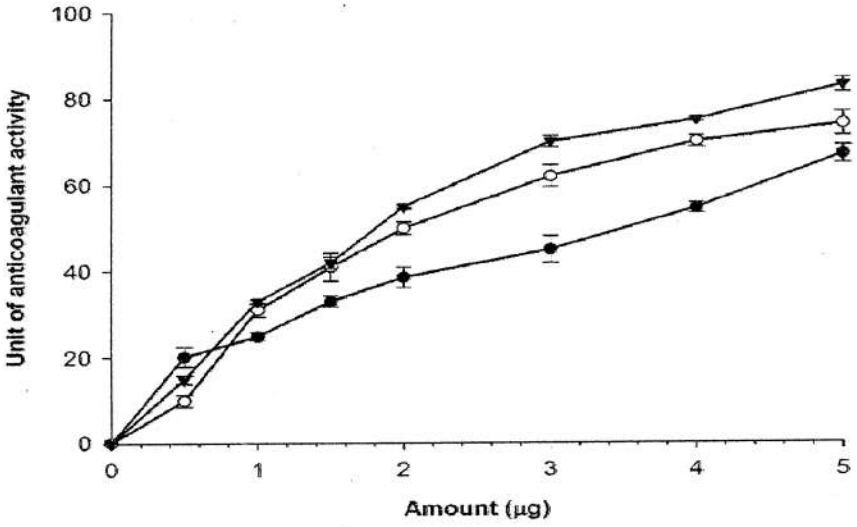

Fig. 4A. Comparison of dose dependent plasma recalcification activity of Ruviprase (▼) with warfarin (○) and Heparin (●).

Ruviprase inhibited thrombin by binding to its active site via an uncompetitive mechanism with a *Ki* value and dissociation constant (K_D) of 0.42 µM and 0.46 µM, respectively (Fig. 4C).

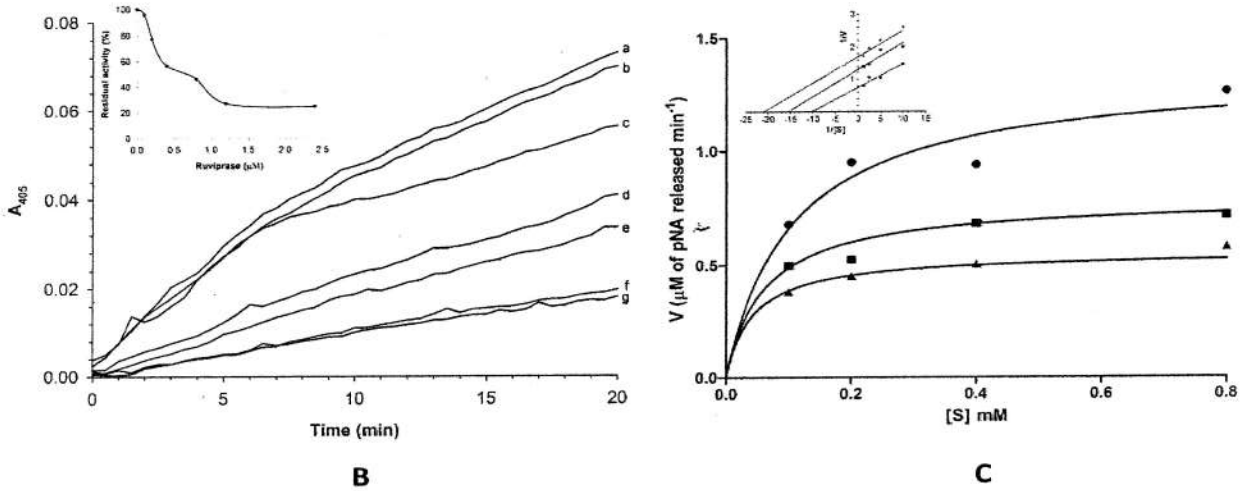

Fig. 4B. Inhibition of the amidolytic activity of thrombin by Ruviprase. Thrombin (0.03 NIH/ml) was preincubated with (a) 0 µM; (b) 0.1 µM; (c) 0.2 µM;(d) 0.4 µM; (e) 0.8 µM; (f) 1.2 µM; or (g) 2.4 µM Ruviprase for 30 min at 37°C. Chromogenic substrate for thrombin (T1637, 0.2 mM) was added and hydrolysis of the substrate was monitored for 30 min at 405 nm at 30 s intervals. Inset: Dose response curve of inhibition of the amidolytic activity of thrombin by Ruviprase. Residual activity of thrombin was calculated by considering the absorbance after 20 min of substrate hydrolysis in the absence of inhibitor

as 100 %. **(C)** Michaelis-Menten plots and Lineweaver-Burk plots (inset) showing inhibition of thrombin activity towards T1637 by (●) 0 μM; (■) 0.6 μM; or (▲) 1.2 μM Ruviprase. The plots are the means of 3 independent measurements.

Ruviprase demonstrated mixed inhibition (*Ki* = 0.16 μM) of FXa towards its physiological substrate prothrombin. Ruviprase at a dose of 2.0 mg/kg was non-toxic and showed potent *in vivo* anticoagulant activity after 6 h of intraperitoneal treatment in mice. The potent *in vitro* and *in vivo* anticoagulant effects of Ruviprase suggest the pharmacological significance of Ruviprase. This new basic knowledge about Ruviprase permits us to foresee the development of it as a therapeutic agent to dissolve blood clots (thrombus). Future studies on the structural aspects of Ruviprase designed to enable us to understand its binding mechanisms as well as its therapeutic potential in terms of its inhibitory effects on thrombin and FXa are warranted.

5. Elucidation of mechanism of anticancer activity of Ruviprase against MCF-7 cell

Ruviprase, a 4.4 kDa anticoagulant peptide isolated from Russell's Viper (*Daboia russelii russelii*) venom demonstrated antiproliferative activity against EMT6/AR1, U-87MG, HeLa and MCF-7 cancer cells with an IC_{50} value of 23.0, 8.8, 5.8 and 4.0 $ng\mu l^{-1}$, respectively.

Rationale behind inhibition of MCF-7 cells by RVV protein was further examined for apoptotic activity by propedium iodide/annexin V staining. RVV protein treatment caused apoptotic bodies with definite loss in cell membrane integrity. Further binding of annexin V and propedium iodide to MCF-7 cell membrane and nucleus respectively suggested that the anticancer protein under study inhibits MCF-7 proliferation through apoptotic pathway (Fig. 5A).

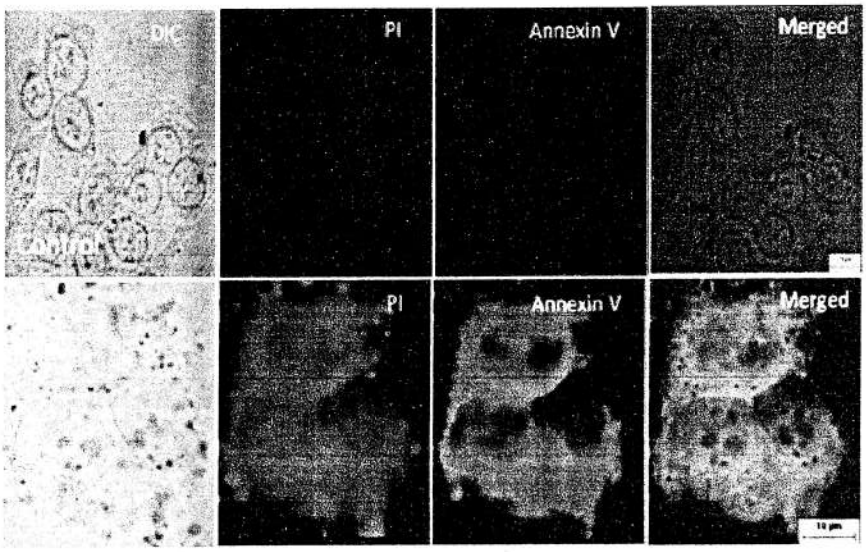

Fig. 5A. Detection of Ruviprase-induced apoptosis in MCF-7 cells. The MCF-7 (0.5×10^5 cells ml^{-1}) cells were treated with Ruviprase (2 IC_{50} value) for 24 h were subjected to annexin V/propidium iodide (PI) staining and observed under an Eclipse TE 2000U microscope (Nikon, Tokyo, Japan) at 40X magnification.

Our further study also showed that Ruviprase induces apoptosis in MCF-7 cells through p53 and p21 pathways. Activation of p53 produces downstream signal that leads to p21 expression and its nuclear accumulation (Fig. 5B). Furthermore, p53 is also involved in inhibiting cell cycle progression from the G2 phase to mitosis (the G2/M checkpoint), thereby making it a suitable target for most anticancer drugs.

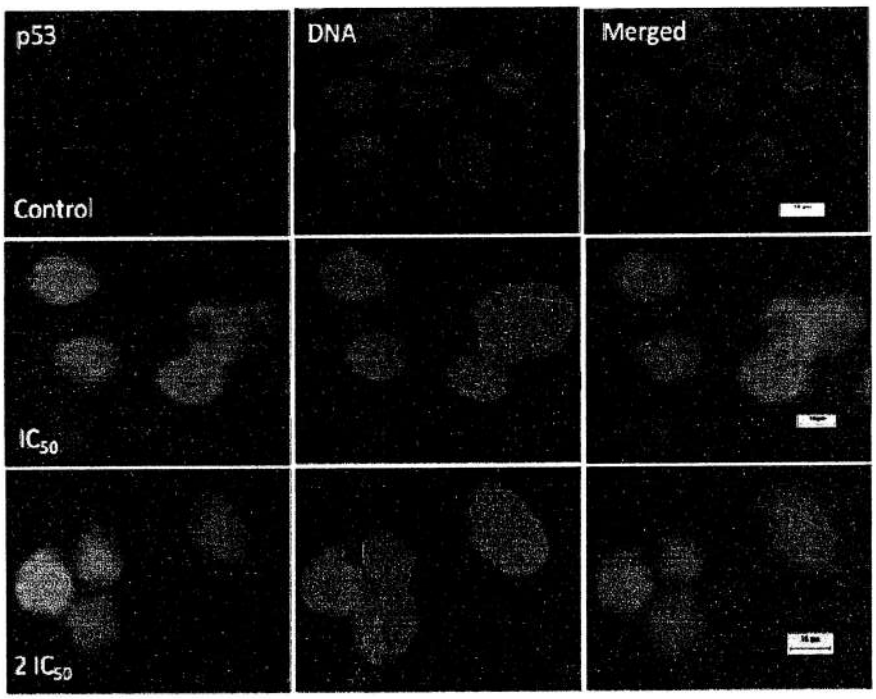

Fig 5B. Immunofluorescence microscopic analysis of Ruviprase-treated MCF-7 cells for assessment of nuclear accumulation of p53 and p21. Ruviprase treated MCF-7 cells (0.5×10^5 cells ml^{-1}) were incubated with mouse monoclonal anti-p53/p21 antibody for 2 h at room temperature (~23 °C). Cells were washed and incubated with Alexa 568-labeled anti-mouse IgG antibody at room temperature (~23 °C) for 2 h.

However at high Ruviprase concentration (1.5 µg/100 µl) loss of DNA integrity was observed Furthermore, Ruviprase altered the mitochondrial transmembrane potential and induced ROS generation in MCF-7 cells suggesting Ruviprase-mediated apoptosis induction in MCF-7 cell involved intrinsic (mitochondrial) pathway. In summary, Ruviprase leads to p53-mediated cell-cycle arrest in addition to the decrease in mitochondrial transmembrane potential and ROS generation that ultimately leads to apoptosis in MCF-7 cells. Unlike Ruviprase, none of the snake venom anticancer proteins have been shown to possess anticoagulant activity which might have additional advantage for the Ruviprase to prevent cancer progression.

6. Purification and characterization of fibrinogenolytic serine protease isoenzymes showing in vivo anticoagulant activity from *Daboia russelii rus*selii venom

Four new RVV protease isoenzymes of molecular mass 32901.044 Da, 333631.179 Da, 333571.472 Da, and 34594.776 Da, were characterized in this study (Fig. 6A). These proteins are named RV-FVP$_\alpha$, RV-FVP$_\beta$, RV-FVP$_\gamma$ and RV-FVP$_\delta$, respectively.

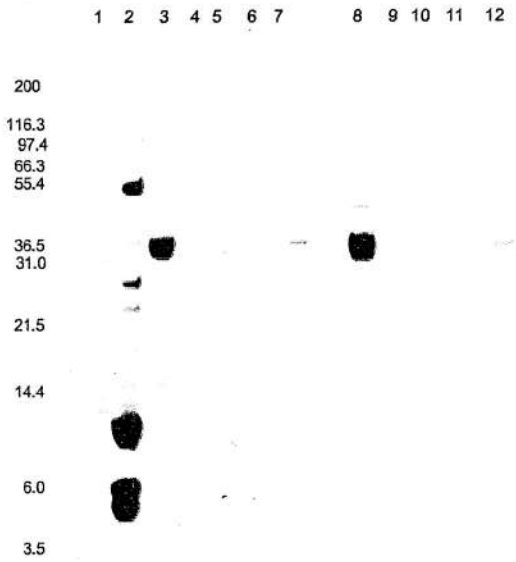

Fig. 6A. Determination of purity and molecular mass of fibrinogenolytic serine protease isoenzymes from D. russelii russelii venom by SDS-PAGE analysis.

The first 10 N-terminal residues of these serine protease isoenzymes showed significant sequence homology with N-terminal sequences of snake venom thrombin-like and factor V-activating serine proteases, which was reconfirmed by peptide mass fingerprinting analysis. These proteases were found to be different from previously reported factor V activators isolated from snake venoms. These proteases showed significantly different fibrinogenolytic, BAEE-esterase and plasma clotting activities but no fibrinolytic, TAME-esterase or amidolytic activity against the chromogenic substrate for trypsin, thrombin, plasmin and factor Xa. Their *Km* and *Vmax* values towards fibrinogen were determined in the range of 6.6 to 10.5 μM and 111.0 to 125.5 units/mg protein, respectively. On the basis of fibrinogen degradation pattern, they may be classified as A/B serine proteases isolated from snake venom (Fig. 6B).

These proteases contain ~42% to 44% of N-linked carbohydrates by mass whereas partially deglycosylated enzymes showed significantly less catalytic activity as compared to native enzymes. *In vitro* these protease isoenzymes induce blood coagulation through factor V activation, whereas *in vivo* they provoke dose-dependent defibrinogenation (Fig. 6C) and anticoagulant activity in the mouse model (Fig 6D). At a dose of 5 mg/kg, none of these protease isoenzymes were found to be lethal in mice, suggesting therapeutic application of these anticoagulant peptides for the prevention of thrombosis.

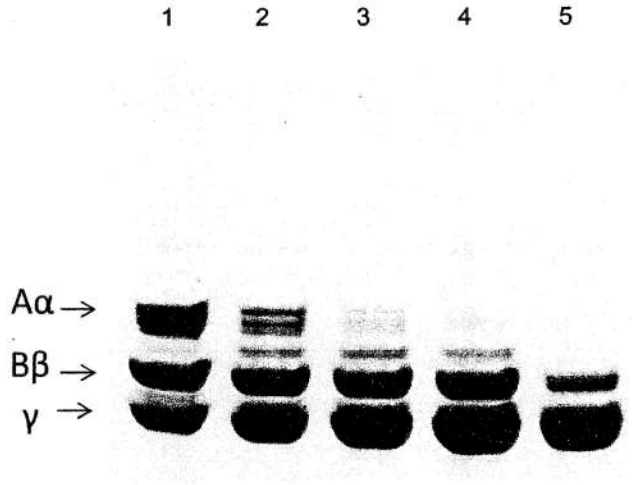

Fig. 6B. The dose-dependent fibrinogenolytic activity of RV-FVP$_\alpha$ was analyzed by 12.5% SDS-PAGE under reduced conditions. Lane 1, control fibrinogen; lanes 2 to 5, fibrinogen was incubated with RV-FVP$_\alpha$ at a concentration of 250 nM, 500 nM, 1000 nM, and 2000 nM, respectively for 5 h at 37 °C

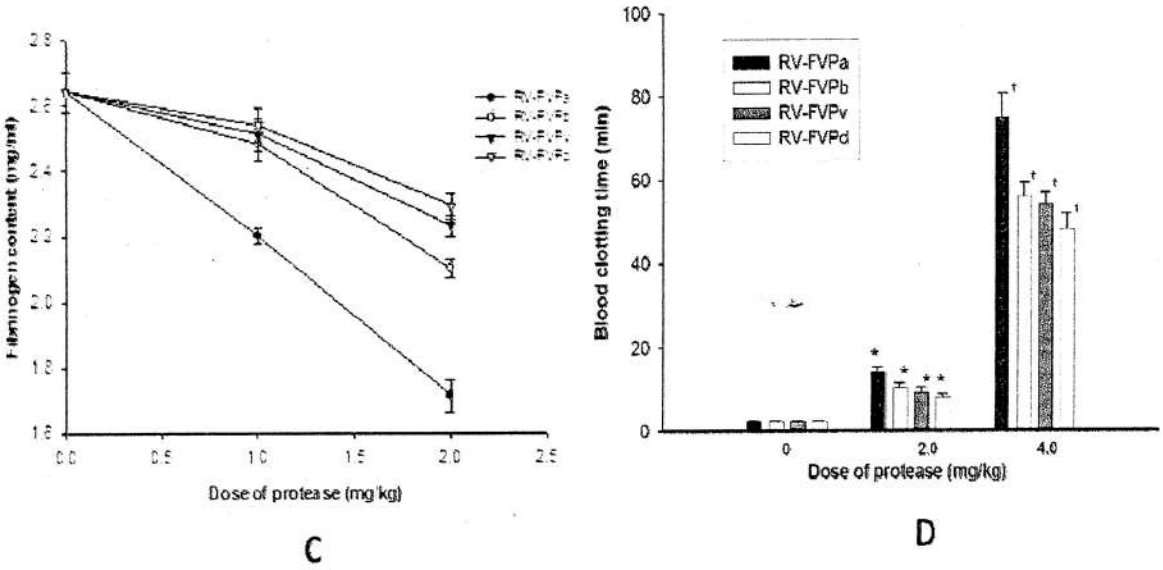

Fig. 6. Dose-dependent *in vivo* defibrinogenating activity and *in vitro* blood clotting activity of RVV protease isoenzymes in NSA strain mouse model. The figure (A) shows *in vivo* defibrinogenating activity and figure (B) shows *in vitro* clotting blood of control and protease-treated (2 and 4 mg/kg dose) mice after 6 h i.p. injection. The values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control, *$p<0.01$; $^{\dagger}p<0.001$.

B3. Connectivity of the partnering institutes (Institute wise achievements to be given separately for each objective).

Tezpur University: Isolation and taxonomic identification of fibrinolytic enzyme producing bacteria; purification, biochemical and pharmacological characterization of fibrinolytic enzyme from bacteria and Russell's viper (*Daboia russelii russelii*) venom.

IIT Bombay: N-terminal sequencing of proteins, cell cytotoxicity study, anticancer activity assay of Ruviprase

B4. Details of New Leads Obtained, if any:

1. Purification of direct-acting fibrinolytic enzymes from bacteria and snake venom which surpass the *in vitro* thrombolytic potential as well as anticoagulant activity of commercially available drugs such as plasmin and streptokinase, and warfarin and heparin, respectively. These enzymes are promising candidates for their therapeutic application for the prevention and/or treatment of thrombosis.
2. Purification of an anticoagulant, anticancer peptide from Russell's viper venom which at a very low dose shows apoptotic activity against MCF-7 breast cancer cells. This molecule may be developed as peptide based therapeutic agent for the treatment of cancer. In fact, this is the first report on purification and characterization of a smallest peptide from snake venom showing dual inhibition against thrombin and FXa.

B5. Details of Publications & Patents, if any from the Project Work:

1. Mukherjee, A.K., Rai, S.K.,Thakur, R., Chattopadhyay, P, Kar, S. (2012) Bafibrinase: A non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease from *Bacillus* sp. strain AS- S20-I exhibits *in vivo* anticoagulant activity and thrombolytic potency. ***Biochimie*** 94, 1300-1308 (Published by Elsevier Science).
2. Mukherjee, A. K. (2014) The pro-coagulant fibrinogenolytic serine protease isoenzymes from *Daboia russelii russelii* venom coagulate the blood through factor V activation: Role of glycosylation on enzymatic activity. ***PLoS One*** 9(2): e86823. doi:10.1371/journal.pone.0086823
3. Thakur, R., Kumar A., Bose B., Panda D., Saikia D, Chattopadhyay P, Mukherjee A. K. (2014) A new peptide (Ruviprase) purified from the venom of *Daboia russelii russelii* shows potent anticoagulant activity via non-enzymatic inhibition of thrombin and factor Xa. ***Biochimie*** 105, 149-158.
4. Majumdar, S., Sarmah, B., Gogoi, D., Banerjee, S., Ghosh, S. S., Banerjee, S., Chattopadhyay, P., Mukherjee, A. K. (2014) Characterization, mechanism of anticoagulant action, and assessment of therapeutic potential of a fibrinolytic serine protease (Brevithrombolase) purified from *Brevibacillus brevis* strain FF02B. ***Biochimie*** 103, 50-60
5. Majumdar, S., Goswami, S., Keppen, C., Rai, S. K., Mukherjee, A. K. (2015) Statistical optimization for improved production of fibrin(ogen)olytic enzyme by *Bacillus cereus* strain FF01 and its *in vitro* thrombolytic potential assessment. ***Biocatalysis and Agricultural Biotechnology*** 4, 191-198

6. Majumdar, S., Dutta, S., Das, T., Chattopadhyay, P., Mukherjee, A. K. (2015) Antiplatelet and antithrombotic activity of a fibrin(ogen)olytic protease from *Bacillus cereus* strain FF01. ***International Journal of Biological Macromolecules*** 79,477-489

Revised manuscript communicated:

1. Thakur, R., Kini, S., Panda, D., Mukherjee, A. K. (2015) Ruviprase, a non-toxic, anticoagulant peptide purified from *Daboia russelii russelii* venom induces apoptosis in MCF-7 breast cancer cell via p53 - mediated pathway.

B6. The training undertaken by the NER PI and the recruited manpower at the Collaborating Institution/s (Details of personnel trained, duration of training and training undertaken).

1. The PI of the NE India (Prof. A. K. Mukherjee) has undergone a training/advance research on "Proteomics strategy to analyze snake venom proteomes for the discovery of novel antithrombotic and anticancer drugs" at University of Northern Colorado, Greeley, USA from September, 2011 to September, 2012 under DBT-Crest award. At UNC, USA, Prof. A. K. Mukherjee also pursued a substantial part of this project.

B7. The details of visits of the Collaborating institutes PI and personnel's to NER (Purpose and duration of visits).

The PI of the collaborating Institute Prof. Dulal Panda visited Tezpur University in July, 2011 for 4 days for research related discussion with PI of the NE Institute (Tezpur University).

The PI of NE Institute (Tezpur University) Prof. A. K. Mukherjee visited IIT Bombay, Mumbai for 3 days in the month of December, 2013.

Dated: 14/8/2015

(A. K. Mukherjee)

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

Appendix-B

Utilization Certificate

(For the financial year ending 18th September, 2014)

(a) Tezpur university, Assam (122.36 Lakhs)

(b) IIT BOMBAY (39.18 Lakhs)

1	Title of the Project/Scheme:	Assessment of thrombolytic potential and anticancer activity of fibrinolytic enzymes purified from Russell's viper venom and bacteria isolated from NE-India.
2	Name of the Organization:	Department of Molecular Biology & Biotechnology, Tezpur University.
3	Principal Investigator:	Prof. Ashis K. Mukherjee
4	Dept. of Biotechnology sanction order No. & date of sanctioning the project:	Sanction order no.- BT/38/NE/TBP/2010 Dated- 18-03-2011
5	Amount brought forward from the previous financial year 2012-2013 quoting DBT letter No. & date in which the authority to carry forward the said amount was given:	₹ 167.00
6	Amount received from DBT during the financial year (2013-2014):	₹ 5,53,000.00
7	Other receipts/interest earned, if any, on the DBT grants:	₹ 2,78,476.00
8	Total amount that was available for Expenditure during the financial year:	₹ 8,31,643.00
9	Actual expenditure (excluding commitments) incurred during the financial year 2013-2014 (statement of expenditure is enclosed)	₹ 5,18, 554.00
10	Unspent balance refunded, if any *(Please give details of cheque No. etc.)*:	Not applicable
11	Balance amount available at the end of the financial year 2013-2014 (excluding commitments expenditure):	₹ 3,13,089.00
12	Amount allowed to be carried forward to the next financial year vide letter No. & date:	NA

1. Certified that the amount of ₹ 5, 18,554.00 (Rupees five lakh eighteen thousand five hundred and fifty four only) mentioned against col. 6 has been utilized on the project/scheme for the purpose for which it was sanctioned and that the balance of ₹ 3, 13,089.00 (Rupees three lakh thirteen thousand eighty nine only) remaining unutilized at the end of the year has been surrendered to Govt. (vide DD no. _____ dated _____)
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. Equipments were procured following Central govt. purchase rules
2. Chemicals were purchased from T&PC approved vendors and approval rules
3. Travel grant was utilized for the purpose for which it was granted
4. Contingency grant was also utilized following the rule
5. Items purchased from equipment, chemical/consumable, contingency and travel head were entered in stock register

20/7/2015

(PROJECT INVESTIGATOR)

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

31-7-15

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

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

Appendix-C

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the period from 1st April, 2014 to 18th September, 2014.

Items	Heads	Unspent balance carried forward from previous year (2012-2013) (₹)	Grant received from DBT during the year (2013-2014) (₹)	Other receipts / Interest earned if any, on the DBT grant (₹)	Total (2+3+4) (₹)	Expenditure (excluding commitments) incurred during the year (2013-2014) (₹)	Balance (5-6) (₹)	Remarks
1		2	3	4	5	6	7	8
1. Non - recurring								
(i)	Equipments	0.00	0.00	0.00	0.00	0.00	0.00	
2. Recurring								
(i)	Human resource	9,381.00	1,80,000.00	0.00	1,89,381.00	1,45,200.00	44,181.00	
(ii)	Consumable	-12999.00	3,00,000.00	0.00	2,87,001.00	3,00,000.00	-12,999.00	
(iii)	Travel	1,995.00	24,000.00	0.00	25,995.00	23,903.00	2,092.00	
(iv)	Contingency	2.00	25,000.00	0.00	25,002.00	24,971.00	31.00	
(v)	Overhead	1,788.00	24,000.00	0.00	25,788.00	24,480.00	1,308.00	
Totals		**167.00**	**5,53,000.00**	**2,78,476.00**	**8,31,643.00**	**5,18,554.00**	**3,13,089.00**	

20/7/2015

(PROJECT INVESTIGATOR)

Professor A.K.Mukherjee,Ph.D.
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21-7-15

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

Statement of Expenditure of the Utilisation Certificate

Showing grants received the Department of Biotechnology and the expenditure incurred during the entire project period from 1st April, 2011 to 18th September, 2014.

Items	Heads	Grant received from DBT during the year 2011-2012 (₹)	Grant received from DBT during the year 2012-2013 (₹)	Grant received from DBT during the year 2013-2014 (₹)	Total grant received (2011-2015) (₹)	Expenditure during the year 2011-2012 (₹)	Expenditure during the year 2012-2013 (₹)	Expenditure during the year 2013-2014 (₹)	Expenditure during the year 2014-2015 (₹)	Total expenditure (2011-2015) (₹)	Balance (₹)
1	2	3	4	5	6	7	8	9	10	11	12
1. Non - recurring											
(i)	Equipments	89,39,000.00	0.00	0.00	89,39,000.00	7,40,798.00	80,66,945.00	0.00	0.00	88,07,743.00	1,31,257.00*
2. Recurring											
(i)	Human resource	3,07,000.00	3,07,000.00	1,80,000.00	7,94,000.00	1,90,507.00	1,80,000.00	2,34,112.00	1,45,200.00	7,49,819.00	44,181.00
(ii)	Consumable	6,00,000.00	3,48,000.00	3,00,000.00	12,48,000.00	5,50,358.00	61,320.00	4,80,578.00	3,00,000.00	13,92,256.00	-1,44,256.00
(iii)	Travel	50,000.00	37,000.00	24,000.00	1,11,000.00	36,511.00	5,422.00	43,072.00	23,903.00	1,08,908.00	2,092.00
(iv)	Contingency	50,000.00	50,000.00	25,000.00	1,25,000.00	46,633.00	3,320.00	50,045.00	24,971.00	1,24,969.00	31.00
(v)	Overheads	2,00,000.00	0.00	24,000.00	2,24,000.00	1,04,000.00	88,612.00	5,600.00	24,480.00	2,22,692.00	1,308.00
Total interest earned in the project					2,78,476.00						2,78,476.00
Totals		**1,01,46,000.000**	**7,42,000.00**	**5,53,000.00**	**1,17,19,476.00**	**16,68,807.00**	**84,05,619.00**	**8,13,407.00**	**5,18,554.00**	**1,14,06,387.00**	**3,13,089.00**

*The amount of ₹ 1,31,257.00 as unspent balance from equipment head in the year 2011-2012 was adjusted with amount of ₹ 3,48,000.00 in consumable head of fund received in the year of 2012-2013.Therefore, total amount of (1,31,257.00 + 3,48,000.00) ₹ 4,79,257.00 was considered as a part of consumable head for the expenditure. (Vide letter no. BCIL/NER-BPMC/2013-911, dated 20/08/2013)

24/7/2015

(PROJECT INVESTIGATOR)

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(HEAD OF THE INSTITUTE)

31-7-15

(FINANCE OFFICER)

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. Title of the Project : Assessment of thrombolytic potential and anticancer activity of fibrinolytic enzymes purified from Russell's viper venom and bacteria isolated from NE-India.

2. Sanctioned Project Cost : Rs. 122.36 Lakh (Rupees One Core twenty two lakh and thirty six thousand only)

3. Revised cost, if any : Nil

4. Duration of the project : Three Years (2011-2014)

5. Sanction Order No. & Date : Sanction order no. BT/38/NE/TBP/2010, Dated - 18-03-2011

6. Date of commencement of Project : 18-3-2011

7. Extension, if any : 6 months (19th March, 2014 to 18th September, 2014)

8. Date of completion of project : 18th September, 2014

Details of grant, expenditure and balance

Sl. No.	Heads	Sanctioned cost (Rs.)	Year wise releases made				Year wise expenditure incurred					
			1st yr (Rs.)	2nd yr (Rs.)	3rd yr (Rs.)	Total (Rs.)	1st yr (Rs.)	2nd yr (Rs.)	3rd yr (Rs.)	4th yr (Rs.)	Total (Rs.)	Balance (Rs.)
A. Non -recurring												
(i)	Equipments	89, 39,000.00	89,39,000.00	0.00	0.00	89,39,000.00	7,40,798.00	80,66,945.00	0.00	0.00	88,07,743.00	1,31,257.00
B. Recurring												
(i)	Human resource	9, 47000.00	3,07,000.00	3,07,000.00	1,80,000.00	7,94,000.00	1,90,507.00	1,80,000.00	2,34,112.00	1,45,200.00	7,49,819.00	44,181.00
(ii)	Consumable	1,600,000.00	6,00,000.00	3,48,000.00	3,00,000.00	12,48,000.00	5,50,358.00	61,320.00	4,80,578.00	3,00,000.00	13,92,256.00	-1,44,256.00
(iii)	Travel	3,00,000.00	50,000.00	37,000.00	24,000.00	1,11,000.00	36,511.00	5,422.00	43,072.00	23,903.00	1,08,908.00	2,092.00
(iv)	Contingency	1,50,000.00	50,000.00	50,000.00	25,000.00	1,25,000.00	46,633.00	3,320.00	50,045.00	24,971.00	1,24,969.00	31.00
(v)	Overheads	3,00,000.00	2,00,000.00	0.00	24,000.00	2,24,000.00	1,04,000.00	88,612.00	5,600.00	24,480.00	2,22,692.00	1,308.00
Total interest earned in the project						2,78,476.00						2,78,476.00
Totals		**1,22,36,000.00**	**1,01,46,000.000**	**7,42,000.00**	**5,53,000.00**	**1,17,19,476.00**	**16,68,807.00**	**84,05,619.00**	**8,13,407.00**	**5,18,554.00**	**1,14,06,387.00**	**3,13,089.00**

20/7/2015

(PROJECT INVETIGATOR)

Professor A.K.Mukherjee,Ph.D.
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(HEAD OF THE INSTITUTE)

31-7-15

(FINANCE OFFICER)

Appendix-A

Details of Assets acquired wholly or substantially out of Govt. grants Register to be maintained by Grantee Institution

Name of the Sanctioning Authority : Department of Biotechnology Government of India, Ministry of Science and technology, (NER Division)

1.	**Sl. No.**	: 358,359
2.	**Name of the Grantee Institution**	: Department of MBBT, Tezpur University
3.	**No. & Date of sanction order**	: No.- BT/38/NE/TBP/2010, Dated - 18-03-2011
4.	**Amount of the sanctioned grant**	: Rs 122.36 Lakh (Rupees One crore twenty two lakh and thirty six thousand (To Tezpur University)
5.	**Brief purpose of the grant**	: To work on Assessment of thrombolytic potential and anticancer activity of fibrinolytic enzyme purified from Russell's viper venom and bacteria
6.	**Whether any condition regarding the right of ownership of Govt. in the Property or other assets acquired out of the grant was incorporated in the grant-in-aid sanction order.**	: Nil
*7.	**Particulars of assets actually credited or acquired.**	: Enclosed below
8.	**Value of the assets as on 10.02.2014**	: ₹ 88,07,743.00
9.	**Purpose for which utilized at present**	: To work for DBT twining project for NE and partially for other's research activity
10.	**Encumbered or not**	: Not
11.	**Reasons, if encumbered**	: NA
12.	**Disposed of or not**	: Not
13.	**Reasons and authority, if any, for Disposal**	: NA
14.	**Amount realized on disposal**	: NA
15.	**Remarks**	: Nil

20/7/2015

(PROJECT INVESTIGATOR)

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31-7-15

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

Section-C: Details of Grant Utilization

C1. Equipment Acquired or Placed Order with Actual Cost:

Sl. No.	Name of Equipments	Equipment Acquired	Placed of Order (₹) / USD / EURO	Actual Cost (₹)
1	Micropipette set of various capacities	Received & installed	Rs. 123385.90	1,23,385.90
2	Digital water bath	Received & Installed	Rs. 359580.00	3,59,580.00
3	Dancing Shaker	Received & Installed	Rs. 212245.00	21,224.50
4	Refrigerator with Freezer (- 4 °C)	Received & Installed	Rs. 29490.00	29,490.00
5	pH meter	Received & Installed	Rs. 34617.50	34,617.50
6	Digital electronic balance	Received & Installed	Rs. 828350	82,835.00
7a	UPLC System with FLR detector	Received & Installed	USD 67000.0	33,97,557.00
7b	UPLC accessories	Received & Installed	Rs. 3.29,925.00	3,29,925.00
8	AKTA Purifier Protein System	Received & Installed	USD 63000.00	31,95989.00
9	Refrigerated table top centrifuge	Received & Installed	EURO 6337.00	4,60,197.00
10	UV-Vis spectrophotometer	Received & Installed	EURO 9556.00	6,83,277.00
11	Accessories of UV-Vis spectrophotometer	Received & Installed	Rs. 89665.00	89,665.00
			TOTAL	₹ 88, 07,743.00

(Total expenditure: Eighty eight lakh seven thousand seven hundred forty three forty three only)

31-7-15

20/7/2015

(Signature of Project investigator)

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

Section-C: Details of Grant Utilization # (1st April, 2014 – 18th September, 2014)

C1. Equipment Acquired or Placed Order with Actual Cost: NA

No equipment was purchased from 1/4/2014 to 18/9/2014

20/7/2015

(Signature of Project investigator)

31-7-15

(Signature of Finance officer)

[illegible].Ph.D.
[illegible]
Biology & Biotechnology
Tezpur University (Central)
Tezpur-784028, Assam

(Signature of Head of the institute)

Annexure A

C2. Manpower Staffing and Expenditure Details (In the financial wise manner) 1st April, 2014 – 18th September, 2014

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY (₹)	TOTAL SALARY RELEASE 2013-2014 (₹)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR 2011-2012 (₹)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR 2012-2013 (₹)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR 2013-2014 (₹)	TOTAL SALARY PAID DURING PROJECT PERIOD (₹)
Sourav Majumdar	JRF*	08.06.2013	-NA-	12000@pm	11,6000.00	1,17,200.00	1,08,000.00	2,49,867.00	4,75,067.00
	SRF	08.06.2013	-NA-	14000@pm		-NA-	-NA-		
Pankoj Borah	Project Assistant	14.06.2011	18.08.2011	8000@pm	Nil	16,507.00	Nil	Nil	16,507.00
Pranjal Baruah	Project Assistant	29.08.2011	29.03.2013	8000@pm	Nil	56,800.00	72,000.00	23,484.00	1,52,284.00
Bhargab Kalita	Project Assistant	12.08.2013	-NA-	8000@pm	64,000.00	Nil	Nil	1,05,961.00	1,05,961.00
				TOTAL	**1,80,000.00**	**1,90,507.00**	**1,80,000.00**	**3,79,312.00**	**7,49,819.00**

*JRF up to 7.06.2013

20/7/2015

(Signature of Project investigator)

Professor A.K. Mukherjee, Ph.D.
Department of Molecular
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Tezpur University (Central)
Tezpur-784028, Assam

31-7-15

(Signature of Finance officer)

(Signature of Head of the institute)

Annexure B

Manpower Expenditure Details (In financial year wise manner): 1st April, 2014 - 18th September, 2014

SANCTIONED POSTS	NUMBER	SCALE OF PAY	ANNUAL OUTLAY	OUTLAY FOR THE ENTIRE PERIOD	REVISED SCALE, IF ANY	REVISED ANNUAL OUTLAY	REVISED PROJECT OUTLAY	ACTUAL RELASES BY DBT	ACTUAL EXPENDITURE	BALANCE
SRF	1	14000/-pm	168000	659000	NA	NA	NA	116000	92400	23600
Project Assistant	1	8000/-pm	96000	288000	NA	NA	NA	64000	52800	11200

20/7/2015

(Signature of Principal Investigator)

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

31-7-15

(Signature of Accounts Officer)

(Signature of Head of the institute)

Tezpur University

Due- Drawn Statement (1st April, 2014 - 18th September, 2014)

Name of the Project Staff	Month and Year	Due	Drawn	Difference
Sourav Majumdar	March and 2014	14000/-	14000/-	
	April and 2014	14000/-	14000/-	
	May and 2014	14000/-	14000/-	
	June and 2014	14000/-	14000/-	
	July and 2014	14000/-	14000/-	
	August and 2014	14000/-	14000/-	
	September and 2014 (up to 18/9/2014)	8400/-	8400/-	
Name of the Project Staff	**Month and Year**	**Due**		**Difference**
Bhargab Kalita	March and 2014	8000/-	8000/-	
	April and 2014	8000/-	8000/-	
	May and 2014	8000/-	8000/-	
	June and 2014	8000/-	8000/-	
	July and 2014	8000/-	8000/-	
	August and 2014	8000/-	8000/-	
	September and 2014 (up to 18/9/2014)	4800/-	4800/-	

20/7/2015

(Signature of Principal Investigator)

Professor A.K Mukherjee, Ph.D.
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Tezpur University (Central)
Tezpur-784028, Assam

31-7-15

(Signature of Accounts Officer)

(Signature of Head of the institute)

C3. Details of Recurring Expenditure: (1st April, 2014 – 18th September, 2014)

Sl. NO.	HEAD	AMOUNT SANCTIONED (₹)	TOTAL EXPENDITURE (₹)	BALANCE AMOUNT (₹)
1	Manpower	1,80,000.00	1,45,200.00	44,181.00
2	Consumable	3,00,000.00	3,00,000.00	0.00
3	Travel	24,000.00	23,903.00	97.00
4	Contingency	25,000.00	24,971.00	29.00
5	Overhead Charges	24,000.00	24,480.00	-480.00
	TOTAL	**5,53,000.00**	**5,18,554.00**	**43,827.00**

C4. Financial Requirements for the Next Year with Justifications

Not applicable

28/7/2015

(Signature of Principal Investigator)

31-7-15

(Signature of Accounts Officer)

Professor A.K. Mukherjee, Ph.D.
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Tezpur-784028, Assam

(SIGNATURE OF THE REGISTRAR)