Consolidated Report for R&D Projects
[March 2011 to Feb 2014]
(Extended to Sept. 2014)

Project Title

Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venoms and complete characterization of their major toxins

DBT Sanction Order No. & Date: No. BT/43/NE/TBP/2010, 14/03/2011

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Section-A : Project Details

A1. **Project Title:** Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venoms and complete characterization of their major toxins

A2. **DBT Sanction Order No. & Date:** No. BT/43/NE/TBP/2010, 14/03/2011

A3. **Name of Principal Investigator (NE):**

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Name of Co-PI/Co-Investigator:

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University of Madras, Guindy Campus,
Chennai - 600 025, Tamil Nadu, INDIA

Dr. B. S. Vishwanath
Department of Studies in Biochemistry
University of Mysore, Manasagangotri,
Mysore – 570 006.

A6. **Total Cost:** **Rs. 104.9 lakhs**
Tezpur University: Rs. 69.84 lakhs
University of Mysore: Rs. 24.28 lakhs
University of Madras: Rs. 10.78 lakhs

A7. **Duration:** Three years (14 March 2011 to 13 March 2014, Extended to Sept. 2014)

A8. **Approved Objectives of the Project:**

1. Determination of venom composition (*Tezpur University and Mysore University*)
2. Isolation and purification of major toxins (*Tezpur University and Mysore University*)
3. Biochemical and pharmacological characterization of the toxin (*Tezpur University and Mysore University*)
4. Determination of amino acid sequences of toxins (*Tezpur University and Mysore University*)
5. Determination of three dimensional structures (*Madras University*)

A9. **Specific Recommendations made by the Task Force (if any): None**

1. **The training undertaken by the NER PI and the recruited manpower at the Collaborating Institution/s. Details of personnel trained**

a. Visit of PI from Tezpur University to collaborating Institute

1. Dr. R. Doley, PI visited University of Mysore, Department of studies in Biochemistry for discussion on project and to training on animal experimentation. During 12-13 June 2011.
2. Dr. R. Doley, PI visited University of Mysore, Department of studies in Biochemistry for discussion on final project report. During 27 Dec 2014 to 05 Jan. 2015

b. Visit of Research Person from Tezpur University

1. Mr. Diganta Das (JRF, DBT project) was sent to University of Mysore, Department of studies in Biochemistry under Prof. B.S. Vishwanath for training in animal handling, experimentation and haemostasis studies from 20th January, 2013 to 28th of February, 2013.
2. Mr. Diganta Das (JRF, DBT project) was sent to University of Mysore, Department of studies in Biochemistry under Prof. B.S. Vishwanath for training in animal handling, experimentation and haemostasis studies from 8th September, 2013 to 10th of November, 2013

2. The details of visits of the Collaborating institutes PI and personnel's to NER

a. Visit of PI from Collaborating institute

1. Prof. B. S. Vishwanath from University of Mysore, visited Tezpur University for the planning and proper execution of the project work at its different stages. During 8-9 March 2012

2. Prof. Velumurugan from Madras University, Chennai visited Tezpur University for the planning and proper execution of the project work at its different stages. 9-10 April 2012

b. Visit of Research Person from Collaborating institute

1. Dhanusa Yesudhas, Project assistant (DBT project) of Prof. Velumurugan from Madras University had visited Tezpur University for protein purification work from 10th of November, 2012 to 16th of February, 2013 for protein purification.

2. Mr. V. Vishwanath, Project assistant (DBT project) of Prof. Velumurugan from Madras University had visited Tezpur University for purification and haemostasis studies from 17th of April, 2013 to 4th of May, 2013.

3. Mr. Vilas Hiremath, PhD. Student of Prof. B.S. Vishwanath from University of Mysore had visited Tezpur University for purification and biological studies from 4th August 2013 to 9th August 2013.

3. Details of Publications & Patents, if any:

1. Diganta Das, Nanjaraj Urs, Vilas Hiremath, Bannikuppe Sannanaik Vishwanath and **Robin Doley** (2013). Biochemical and Biological Characterization of *Naja kaouthia* venom from North-East India and its neutralization by polyvalent antivenom. *J Venom Res*, 4, 31-38.

2. Maitreyee Sharma, Neeharika Gogoi, B. L. Dhananjaya, Jaideep C. Menon, and **Robin Doley**. (2013). Geographical variation of Indian Russell's viper venom and neutralization of its coagulopathy by polyvalent antivenom. *J. Toxicol. Toxin. Rev.* Vol. 33, No. 1-2: 7–15.

3. Maitreyee Sharma, Diganta Das, Iyer Janaki Krishnamurthy, R. Manjunatha Kini and Robin Doley. Unveiling the complexities of Daboia russelii venom, a medically important snake of India, by tandem mass spectrometry. **Toxicon (Accepted)**

4. Maitreyee Sharma, Iyer Janaki Krishnamurthy, R. Manjunatha Kini and Robin Doley. Ruvitoxin, a FX and FXa binding PLA_2 enzyme of *Daboia russelii* venom. (**Under Preparation**)

4. Poster presentations in National Seminar/Conference

1. Sharma, M. and **Doley, R. Indian Russell's viper venom analysis using proteomics tool and neutralization of its coagulopathy by polyvalent antivenom.** The 4th Annual Conference & International Colloquium; Venoms, Toxins and Human health- Revisiting the Translational Approach. Organized by Department of Clinical and Experimental Pharmacology, Calcutta School of Tropical Medicine, Kolkata, India, November 20-22, 2014.

2. Sharma, M. and **Doley, R. Unveiling the venom composition of Indian Daboia russelli by Tandem Mass spectrometry.** National seminar on "Recent Advances in Biotechnological Research in North East India: Challenges and Prospects". Organized by Department of Molecular Biology and Biotechnology, Tezpur University, November 27-29, 2014.

3. Sharma, M, Menon, J.C and **Doley, R. Compositional analysis of two Russell's viper venom of South India.** 2nd National Conference on Snakebite Management and Annual conference of Toxinological Society of India. Organized by Department of Studies in Biochemistry, University of Mysore and Karnataka Open University. December 10-12 2012.

4. Das, D and **Doley, R. Purification and partial characterization of an anticoagulant protein from Indian monocled cobra *(Naja kaouthia)* of North East origin.** 2nd National Conference on Snakebite Management and Annual conference of Toxinological Society of India. Organized by Department of Studies in Biochemistry, University of Mysore and Karnataka Open University. December 10-12 2012.

5. Das, D, Saikia, D, Kalita, R.D., Mukherjee. A. K. and **Doley, R. Green medicine for snakebite** in "1ST NATIONAL CONFERENCE ON ANIMAL, MICROBIAL, PLANT TOXINS

& SNAKEBITE MANAGEMENT (AMPTOX 2010) "Biotoxins in Health & Diseases" Kolkata, December 2010.

5. Oral presentations

1. Das, D and **Doley, R. Neurotoxic three finger toxin (3FTx) from *Naja kaouthia* with anticoagulant activity: its biochemical and biological characterization.** National seminar on "Recent advances in Biotechnological Research in North East India: Challenges and Prospects". Organized by Department of Molecular Biology and Biotechnology, Tezpur University, November 27-29, 2014.

6. Conference and Seminars attended by PI

1. The 4th Annual Conference & International Colloquium; **Venoms, Toxins and Human health- Revisiting the Translational Approach.** Organized by Department of Clinical and Experimental Pharmacology, Calcutta School of Tropical Medicine, Kolkata, India, November 20-22, 2014.

2. National seminar on **"Recent Advances in Biotechnological Research in North East India: Challenges and Prospects".** Organized by Department of Molecular Biology and Biotechnology, Tezpur University, November 27-29, 2014.

3. National Conference on **Snakebite Management and Annual conference of Toxinological Society of India**. Organized by Department of Studies in Biochemistry, University of Mysore and Karnataka Open University. December 10-12 2012.

4. Participated in workshop of **"Thrombosis and Haemostasis: Discovery and development of Tools and Therapeutics""** jointly by International Society on Thrombosis and Haemostasis, Dept. of Studies in Biochemistry, Mysore University and KSOU (Karnataka State Open University, Mysore) December 2012.

Objectives of project to be fulfilled by Tezpur University

1. Determination of venom composition of *Daboia russelii*
2. Isolation and purification of major toxins from the venom of *Daboia russelii*
3. Determination of amino acid sequences of toxins isolated from the venom of *Daboia russelii*
4. Biochemical and pharmacological characterization of the toxin isolated from the venom of *Daboia russelii*

Objective 1: Determination of venom composition of *Daboia russelii*

1.1 Materials

a) Chemicals and reagents: All reagents used were of analytical grade from Sigma-Aldrich, (USA).

b) Collection of snake venom, preparation and storage: Venom of "Big Four" (*Naja naja, Daboia russelli, Bungarus caeruleus* and *Echis carinatus)* were procured from Irula Snake Catcher Industrial Co-operative Society limited Ind 1969 Vadanemmeli Village, District Kancheepuram, Tamil Nadu, India. Lyophilized venoms are stored in -20 °C until further use.

1.2 Methods

a) Determination of protein content: Total protein content of crude venom/fractions was determined according to Lowry's method using BSA as standard (LOWRY et al., 1951). Protein estimation was also done on Nano Drop 2000 spectrophotometer (Thermo Scientific, USA).

b) SDS-PAGE: Reducing and non-reducing SDS-PAGE was performed according to the method of (Laemmli, 1970). The gel were stained with 0.25% coomassie brilliant blue in methanol:water:acetic acid (40:50:10) and subsequently destained in the same solution to visualize the protein bands.

c) Gel filtration: 50 µg of crude *Daboia russelii* venom was subjected to superdex 75 column of 120ml volume (GE Healthcare, USA) gel filtration column. Fractionation was carried out in AKTA purifier system (Amersham Biosciences, Uppsala, Sweden). The crude venom was dissolved in 20 mM TrisCl pH-7.4 and eluted with the same buffer at a flow rate of 1 ml/min. Elution of the venom components were monitored at 215 and 280 nm and 0.8 ml fractions were collected. The fractions containing the peaks were pooled together and the protein content was determined by the method of Lowry (Lowry et al., 1951).

d) ESI-LC MS/MS:

For ESI-LC-MS/MS analysis of gel filtration peaks, proteolysis of the fractions were carried out using trypsin with Protease Max surfactant (Promega, Madison, WI, USA) according to the manufacturer's instructions. Each gel filtration fraction (~50 µg) was dissolved in 50 µl of MilliQ water. To this 41.5 µl of 50 mM of ammonium bicarbonate, 2 µl of 1% Protease Max and 1 µl of 0.5

M DTT were added. The reaction mixtures were incubated at 56°C for 20 min. Then 2.7 µl of 0.55 M IAA was added and incubated in dark for 15 min. Finally 1 µl of 1% Protease Max and 1.8 µl of Trypsin (1 µg/µl in 50 mM acetic acid) were added and the reaction mixtures were incubated at 37°C for 3 h. To stop the reaction 0.5 µl of 100% TFA was added and incubated at room temperature for 5 min. The reaction mixtures were centrifuged at 12,000 rpm for 10 min.

The tryptic digests of individual samples were loaded onto Accela LCQ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) for MS/MS analysis. Each sample (~80 µl) was injected into a Hypersil Gold C_{18} column (50 x 2.1 mm, 1.9 µm, Thermo Scientific, Waltham, MA, USA) pre-equilibrated with 0.1% formic acid. Elution was carried out at a flow rate of 200 µl/ min with a linear gradient of 40%-80% acetonitrile in 0.1% formic acid. The eluent from liquid chromatography (LC) column was directly fed to the mass spectrometer. Ion polarity of the system was set to positive ionization mode. Spectra were obtained in MS/MS mode and MS/MS scan range was set from 500 to 2000 m/z. Oxidation of methionine residues and *S*-carbamidomethylation of cysteine residues were set as modification. The MS/MS spectra were analyzed by the software Proteome Discoverer 3.1 using Sequest program. Based on sequence similarity, the peptide fragments were assigned to the proteins in the NCBI database. Analysis and identification of the proteins and peptides were validated by the parameters like protein score (~2-105), coverage (~8-86) (calculated by the Sequest program) and the presence of at least one unique peptides. Peptides with lower score and coverage were manually validated by NCBI BLASTp search. The relative abundance of the protein families were calculated considering all the protein families as 100%.

1.3 Results and Discussion

Snake venom is a cocktail of proteins and polypeptides responsible for the pathophysiological manifestation in prey/victims. Hence, elucidation of venom composition helps in deciphering its pharmacological profile. Such an understanding clarifies the complexity of the venom proteome and helps in the identification of proteins in the venom including the scarcely abundant proteins. This in turn may provide strategies to design specific and effective antivenoms to combat clinical challenges of snake envenomation.

Further, comparative analysis of the proteome profiles along with the experimental evaluation will help in correlating the probable mechanism of action of the venom proteins on various physiological

systems of the prey or the victim. Such comprehensive information about the complete venom composition will supplement the existing knowledge of characterization of some of the toxins isolated from *Daboia russelii* venom of Indian origin.

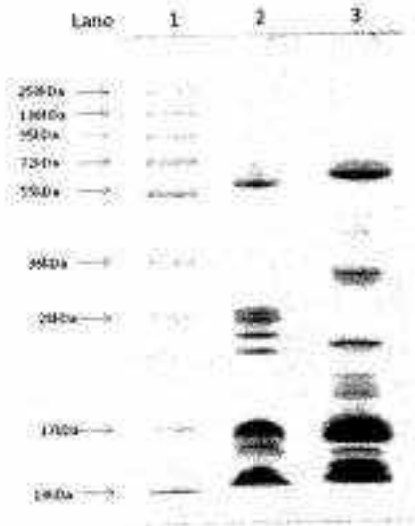

Figure 1: SDS-PAGE (12.5%) of crude Russell's viper venom (30 µg). Lane 1: Molecular weight marker; lane 2: Non-reduced *Daboia russelli* venom; lane 3: Reduced *Daboia russelli* venom.

In the present study, the complexity of *Daboia russelii* venom of Indian origin was analysed using biochemical and proteomic techniques. The abundance of low and high molecular weight proteins in the crude venom was confirmed by SDS-PAGE (Figure 1). The prominent protein bands at 95, 55 and 14 kDa might be the presence of LAAO, SVMP and PLA_2 enzymes in the venom. To evaluate the venom complexity, the crude venom was resolved by gel filtration chromatography into 9 distinct protein peaks (Figure 2).

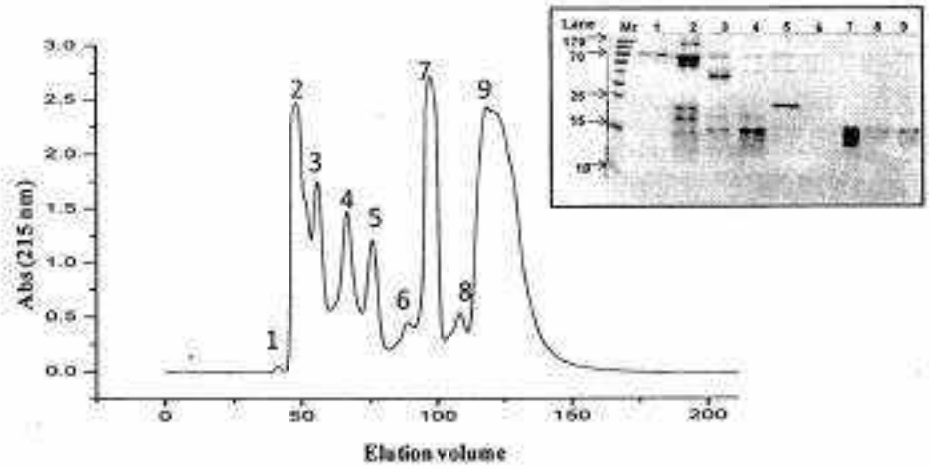

Figure 2: Elution profile of *Daboia russelli* on superdex 75 column. 9 peaks were eluted and are named as Rv-GF1 to 9. **Inset:** SDS-PAGE of Gel filtration fractions under reduced condition. Lane 1-10: Mol. Wt. marker;Rv-GF-1, Rv-GF-2; Rv-GF-3; Rv-GF-4; Rv-GF-5; Rv-GF-6; Rv-GF-7; Rv-GF-8 and Rv-GF-9.

Individual peaks were subjected to tryptic digestion and ESI-LC-MS/MS. A thorough analysis of the peptide fragments provided a comprehensive overview of different proteins present in the crude venom (Table 1). Based on the sequence homology search, 66 different proteins were identified which belong to 12 distinct snake venom protein families (Figure 3).

PLA_2 enzymes were found to be the most abundant enzymatic family of this venom (Figure 3). The members of this protein family are reported to exert wide range of pharmacological effects including myotoxicity, neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathic, haemorrhage, edema, tissue damage and convulsion (Kini, 2003). In viperid venom, PLA_2 enzymes have been reported to exist either as monomers or as complex like vipoxin, and viperotoxin F (for a review, see (Doley and Kini, 2009)). Vipoxin from the venom of *Vipera ammodytes meridionalis* is a heterodimeric complex with a PLA_2-like natural inhibitor (Chain A) and a highly toxic basic enzymatically-active PLA_2 (Chain B) (Mancheva et al., 1987). In this proteome, five peptide fragments including three unique peptides similar to chain A of vipoxin were observed (Accession 1408314, Table 1). One of the peptide fragments homologous to this acidic subunit with the substitution of His 48 to Gln 48 at the active site was also found. However, no peptides homologous to toxic basic PLA_2 (Chain B) was found in the proteome. Although this protein lacks enzymatic

activity, but for pharmacological manifestation in victims/prey it might function through non-enzymatic mechanism. Similarly, RV-4 (enzymatically active) and RV-7 (non-toxic acidic subunit) are the two subunits of viperotoxin F, the major toxic component of *Vipera russelii formosensis* (Wang et al., 1992). Peptide sequence similar to RV-7 subunit (Accession. No. 400714) from *D. siamensis* was observed while the active form, RV-4 was absent. The absence of Chain B of vipoxin and RV-4 subunit of viperotoxin F indicates the absence of vipoxin and vipertoxin F in *Daboia russelli* venom from South India, although the functionally active forms of both vipoxin and vipertoxin F are reported in *Daboia russelli siamensis* from Myanmar (Risch et al., 2009). Similarly, daboiatoxin, a major PLA_2 toxin in *Daboia russelii siamensis* from Myanmar which shows myotoxic, neurotoxic and cytotoxic activity (Maung et al., 1995), was not identified in our venom sample. These differences might be due to the difference in the geographical origin of *Daboia russelii*. It would be interesting to analyze and validate whether the inactive forms of PLA_2 might interact with some other PLA_2 components of the venom from South India and impose distinct synergistic functional characteristics. VRV-PL-VIIIa, a basic PLA_2 enzyme reported as the major toxin in *Daboia russelii* venom were found in this venom (Gowda et al., 1994). VRV-PL-VIIIa exhibits diverse pharmacological effects like neurotoxicity, myonecrosis, *in-vitro* tissue damaging activity and anticoagulant effect (Kasturi and Gowda, 1989). In the present analysis we have observed six peptide fragments of this toxin in gel filtration peak 5, 6 and 8. Peptide fragments homologous to ammodytin I1 (C) (Accession No. 50874332, Table 1) a presynaptic neurotoxic PLA_2 from the venom of *Vipera berus berus* was observed (Jan et al., 2007).

Table 1: List of various snake venom protein families as identified by ESI-LC MS/MS in the gel filtration fractions of crude *Daboia russelii* venom. The number of similar peptides identified from different snake venom families are shown.

Sl. No.	MS/MS derived Sequence	No. of peptides	Peptide ion m/z	MH+ [Da]	z	Coverage & Score	Accession No.	Protein	Homology with protein from	Protein family
Gel filtration Peak 1										
1.	ARNEcDVPEHcTGQSAEcPR	2	792.19	2373.57	3	37.00 & 103.27	300079900	Factor X activator heavy chain	*Daboia russellii russellii*	SVMPs
	ASDLMTRKSHDNALLFTDMR	1	774.82	2321.47	3					
	cILYPPLR	1	1032.53	1031.53	1					
	KSHDNALLFTDMR	1	517.07	1548.22	3					
	LKPGAEcGNGLccYQcK	1	1008.63	2015.26	2					
	NEcDVPEHcTGQSAEcPR	1	1074.22	2146.45	2					
	NPCNmHYScmDQHKGMVDPGTKcEDGK	1	1057.98	3170.93	3					
	SVGIVQVQGNR	1	579.67	1157.35	2					
2.	ARDEcDVPEHcTGQSAEcPR	1	792.19	2373.57	3	49.41 & 97.67	162329887	Chain A, of Russell's Viper Venom Metalloproteinase	*Daboia siamensis*	SVMPs
	DEcDVPEHcTGQSAEcPR	1	1074.58	2147.16	2					
	GYcYNGDcPIMR	1	1506.45	1505.45	1					
	KSHDNALLFTDMR	1	517.07	1548.22	3					
	LKPGAEcGNGLccYQcK	1	1008.63	2015.26	2					
	LVSTSAQFNK	1	548.58	1095.16	2					
	NPCNmHYScmDQHKGMVDPGTKcEDGK	1	1057.98	3170.93	3					
3.	KGSHLVSLHSR	1	611.46	1220.91	2	59.35 & 22.04	251205	Coagulation factor X activating enzyme light chain, RVV-X-light chain	*Daboia russelii*	SVMPs
	SMTcNFIAPVVcK	1	764.63	1527.27	2					
	VLDcPSGWLSYEQHcYK	1	1072.16	2142.32	2					
4.	VEHQVKKHLEDVFSNR	1	656.21	1965.64	3	8.10 & 2.35	521759683	Carnosine dipeptidase 1 (metallopeptidase M20 family)	*Crotalus horridus*	SVMPs
	FSIRQVPHmDLSK	1	788.07	1574.14	2					
	KVFNQDPDMIR	1	682.62	1363.23	2					
5.	DcQNPCCDAATCKLTPGAEcGNGLCCEKCK	2	1064.96	3191.89	3	25.98 & 15.15	73621852	Coagulation factor X-activating enzyme heavy chain	*Macrovipera lebetina*	SVMPs
	ENGRKIPcAPQDIK	1	813.92	1625.85	2					
	GSYYGYcR	1	513.99	1025.98	2					
	KNcIcNDSScIMSAVLSSQPSK	1	1244.35	2486.70	2					
	LFSNcSNHDYRR	1	1570.43	1569.43	1					
	QcISLFGSRATVAEDSCFQENQK	1	874.01	2619.04	3					
	TAVIMAHELGHNLGMYHDR	1	722.97	2165.90	3					
6.	DEcDVTEHcTGQSAEcPR	1	1076.02	2150.04	2	22.83 & 9.68	83523646	Group III snake venom metalloproteinase	*Echis ocellatus*	SVMPs
	IPcAPQDVK	1	1028.36	1027.36	1					
	NQcISLFGSR	1	1182.43	1181.43	1					
	VFSSCSYDDYRMYLAK	2	975.07	1948.14	2					

7.	DLQTFcYPSIIQK EGWYANLGPMR HIVIVGAGMSGLSAAYVLAGAGHK IFFAGEYTANAHGWIDSTIK KDLQTFcYPSIIQK LNEFVQETENGWYFIK SAGQLYQESLGK VTVTYQTTQK	1 1 1 1 2 1 1 1	807.62 648.09 1140.60 1121.94 871.42 1009.67 1281.45 585.54	1613.24 1294.18 2279.20 2241.89 1740.85 2017.33 1280.45 1169.08	2 2 2 2 2 2 1 2	54.56 & 98.63	395406796	L-amino-acid oxidase	*Daboia russelii russelii*	LAAO
8.	KFWEDDGIQGGK RFDEIVGGMDQLPTSMYR VYFAGEYTANAHGWIDSTIK	1 1 1	691.10 1058.70 748.54	1380.19 2115.41 2242.63	2 2 3	32.74 & 43.72	347602327	L-amino-acid oxidase	*Echis acellatus*	LAAO
9.	EYLIK HDDIFAYEK SAGQLYEESLR	1 1 1	666.37 1138.40 627.03	665.37 1137.40 1252.06	1 1 2	29.31 & 28.94	538259837	L-amino acid oxidase	*Protobothrops flavoviridis*	LAAO
10.	HDDIFAYEK LNEFSQENDNAWYFIK SAGQLYEESLGK RFDEIVDGmDK	1 1 1 1	1138.40 673.78 641.86 671.09	1137.40 2018.35 1281.72 1340.19	1 3 2 2	22.49 & 27.10	10120762	Chain H, of L-amino acid oxidase	*Calloselasma rhodostoma (Malayan pit viper)*	LAAO
11..	cTERQAcCQDYEDTcVLPTQSWScSK DFYTFDSEGIVR FGPVSGEIIMALQMADR GKNEVTSFENIEVYNLMcDLLK NPFYNPSPAK	1 1 1 1 1	1072.02 725.31 918.72 873.30 568.78	3213.07 1448.62 1835.44 2616.90 1135.55	3 2 2 3 2	38.66 & 66.52	586829527	Phosphodiesterase	*Macrovipera lebetina*	Phosphodiesterase
12.	NLHNcVNLILLADHGMEEIScDR TFLPIFVNPVN VNLMVDQQWMAVR VRDVELLTGLNFYSGLK	2 1 1 1	908.92 1261.53 795.90 642.64	2723.77 1260.53 1589.80 1924.92	3 1 2 3	33.73 & 35.20	538259853	Phosphodiesterase	*Protobothrops flavoviridis*	Phosphodiesterase
13.	TSTHIAPLSLPSSPPSVGSVcR WDKDIMLIK DIMLIK	1 1 1	1126.31 1162.40 733.41	2250.62 1161.40 732.41	2 1 1	18.22 & 22.01	380875417	Serine protease VLSP-3	*Macrovipera lebetina*	SVSPs
15.	FFcLSNK TSTYIAPLSLPSSPPR WDKDIMLIK	1 1 1	916.36 844.95 1162.40	915.36 1687.90 1161.40	1 2 1	20.70 & 26.99	311223824	Serine beta-fibrinogenase-like protein precursor	*Daboia siamensis*	SVSPs
16.	TLcAGILQGGIDTcK DIMLIK MVLIR	1 1 1	1608.43 733.41 633.37	1607.43 732.41 632.37	1 1 1	10.12 & 22.05	380875421	Beta-fibrinogenase	*Macrovipera lebetina*	SVSPs
17.	KcFGLEK GTGYRSWFNLNCEEPYPFVCKVPPNC YFcYR	1 1 1	882.36 1007.77 809.34	881.36 3020.32 808.34	1 3 1	24.05 & 5.64	73621141	Coagulation factor X-activating enzyme light chain 2	*Macrovipera lebetina (Levantine viper)*	SVMPs
18.	AWNEGTNcFVFK GSHLLSLHNIAEADFVLK	1 1	737.54 655.99	1473.09 1964.99	2 3	57.43 &	73620113	Snaclec 5	*Daboia siamensis*	Snaclec

	NHWSHMDcSSTHNFVcK QDcLSDWSFYEGYcYK	1 1	716.78 1061.43	2147.35 2120.85	3 2	22.60				
19.	TWFNLScGDDYPFVcK NcFGLEK AFDEPKR NVIER SGDAEK	1 1 1 1 1	670.70 868.35 863.49 631.40 607.20	2009.11 867.35 862.49 630.40 606.20	3 1 1 1 1	26.28 & 12.33	218526485	Snaclec A14	*Macrovipera lebetina (Levantine viper)*	Snaclec
20.	WSDGVNLDYK QDcLSDWSFYEGYcYK VFNEK	1 1 1	599.444 1061.43 637.35	1196.89 2120.85 636.35	2 2 1	20.67 & 11.74	300490464	Dabocetin beta subunit	*Daboia russellii russellii*	Snaclec
21.	FcMEHPNNGHLVSVESmEEAEFVAK TWEAAER FcmEHPNNGHLVSVESmEEAEFVAK MWFNR YLFVcKVPPEc	1 1 1 1 1	969.87 863.39 975.75 754.41 706.68	2906.62 862.39 2924.27 753.41 1411.37	3 1 3 1 2	33.54 & 10.99	300490458	Factor X activator light chain 2	*Daboia siamensis*	SVMPs
22.	IKDKEQEcSSEWSDGSSVSYDNLGK cEEPYPFVcK ESGYRMWFNHK mWFNHKCEEPYPFVcKVPPEC	1 1 1 1	950.67 665.26 728.11 886.62	2849.01 1328.53 1454.22 2656.86	3 2 2 3	41.77 & 9.12	300079896	Factor X activator light chain 2	*Daboia russellii russelli (India)*	SVMPs
23.	LRQGAQCAEGLCCDQcRFmK MHMEAGEEcDCGSPGNPCCDAATCKLR MHMEAGEECDCGSPGNPcCDAATCK	1 1 1	778.84 962.66 872.90	2333.52 2884.98 2615.71	3 3 3	50.56 & 4.81	50365991	Adinbitor	*Gloydius brevicaudus*	Disintegrin
24.	cTGQDcYGGVAR YLGYLNVIFDDK AEVNK EVVKFMNSLR	1 1 1 1	673.05 730.89 561.21 612.72	1344.11 1459.77 560.21 1223.43	2 2 1 2	7.41 & 6.54	338855300	Ecto-5' nucleotidase	*Crotalus adamanteus*	Nucleotidase
25.	FHEcNLGNLIcDAVIYNNLR DIPEDQVVK AQVNK	1 1 1	812.79 522.43 561.29	2435.38 1042.86 560.29	3 2 1	8.33 & 3.31	586829529	5'-nucleotidase, partial	*Macrovipera lebetina*	Nucleotidase
26.	AcSGENGEQATSQNNSGDNER QLPCmHEFHFHCIDRWLSENSTcPICR RHPSITLDLQVRR	1 1 1	742.74 1126.14 796.09	2225.21 3375.43 1590.18	3 3 2	9.62 & 2.35	387016758	Hypothetical protein LOC100554767	*Crotalus adamanteus*	Hypothetical protein
27.	YmLYSLLDcGEESEQc CCFVHDCCYGRVNGCDPK MRILWIVAVCLIGAEGHLSQFGDMINK VAAIcFGENmNTYDK KTGIFGImSYIYYGcYCGWGGK	1 2 1 1 1	1007.56 674.08 1005.95 583.98 847.50	2013.12 2019.23 3014.87 1748.94 2539.50	2 3 3 3 3	71.01 & 2.13	50874332	Ammodytin I1(C) variant	*Vipera berus berus (common viper))*	PLA_2
Gel filtration Peak 2										

28.	DLQTFcYPSIIQK FDEIVGGMDQLPTSMYR HIVIVGAGMSGLSAAYVLAGAGHK IFFAGEYTANAHGWIDSTIK KFWEDDGIQGGK LNEFVQETENGWYFIK NPLEEcFREDDYEEFLEIAK SAGQLYQESLGK VTVLEASERPGGR VTVTYQTTQK YPVKPSEAGK	1 1 1 1 1 1 1 1 1 1 1	807.58 980.70 760.87 748.27 691.16 1009.81 849.86 641.69 686.47 585.06 539.15	1613.17 1959.39 2279.62 2241.82 1380.31 2017.61 2546.59 1281.38 1370.94 1168.12 1076.29	2 2 3 3 2 2 3 2 2 2 2	56.94 & 78.87	395406796	L-amino-acid oxidase	*Daboia russellii russellii*	LAAO
29.	FDEIVGGMDKLPTSMYR HDDIFAYEK SAGQLYEESLGK	2 1 1	654.31 570.14 642.22	1959.93 1138.27 1282.44	3 2 2	20.24 & 41.31	75570145	L-amino-acid oxidase	*Gloydius blomhoffii (mamushi)*	LAAO
30.	EGWYANLGPMR FDEIVGGMDQLPTSMYR VTVLEASER	1 2 1	647.94 980.70 503.10	1293.87 1959.39 1004.20	2 2 2	19.21 & 34.56	347602330	L-amino-acid oxidase	*Vipera ammodytes ammodytes (western sand viper)*	LAAO
31.	EGWYANLGPMR HDDIFAYEK SAGQLYEESLR	1 1 1	647.94 570.14 627.73	1293.87 1138.27 1253.47	2 2 2	21.39 & 30.57	538259837	L-amino acid oxidase	*Protobothrops flavoviridis*	LAAO
32.	FDFFWIGLR QDcLSDWSFYEGYcYK SSEEMDFVIR TTDNQWLR TWEDAEK WSDGVNLDYK	1 1 1 1 1 1	601.58 1061.73 607.18 518.03 880.36 599.40	1201.16 2121.47 1212.37 1034.07 879.36 1196.81	2 2 2 2 1 2	54.00 & 49.10	300490464	Dabocetin beta subunit	*Daboia russellii russellii*	Snaclec
33.	GFDcPFGWSSYEGYcYK AWSGKSYcLVSK mNWEDAESFcR TTNNEWLSMDCSR	1 1 1 1	708.79 693.46 731.62 779.25	2123.38 1384.91 1461.24 1556.50	3 2 2 2	59.20 & 2.48	3023231	Alboaggregin-A subunit beta	*Trimeresurus albolabris (white-lipped tree viper)*	Snaclec
34.	NPFIcK TWEDAEK YHAWIGLR	1 1 1	779.33 880.36 508.94	778.33 879.36 1015.88	1 1 2	27.92 & 24.74	123899657	Dabocetin subunit alpha	*Daboia siamensis*	Snaclec
35.	FGSVWIGLNDPWHNcNWEWSDNAR GSHLASIHSSEEEAFVSK IFWFNRGcEKFVSFVcK RPYcTVMVLKPDR	1 1 1 1	987.90 958.72 742.46 817.95	2960.70 1915.44 2224.39 1633.91	3 2 3 2	48.00 & 19.28	300490484	P31 beta subunit	*Daboia siamensis*	Snaclec

36.	AWNEGTNcFVFK GSHLLSLHNIAEADFVLK QDcLSDWSFYEGYcYK TWEDAEK	1 1 1 1	737.53 655.93 1061.73 880.36	1473.07 1964.79 2121.47 879.36	2 3 2 1	56.08 & 16.42	73620113	Snaclec 5	*Daboia siamensis*	Snaclec
37.	DGIYVWIGLR FcTEQANSGHLVSIK WDYVNcAEHYR	1 1 1	596.97 846.62 757.51	1191.94 1691.25 1513.03	2 2 2	34.18 & 15.14	300490478	P31 alpha subunit	*Daboia russellii limitis*	Snaclec
38.	cEEPYPFVcK EEFRKcFVLQK FcMEHPNNGHLVSIESMEEAEFVAK VPPEc	1 1 1 1	665.33 742.59 969.75 602.28	1328.65 1483.18 2906.26 601.28	2 2 3 1	32.28 & 13.27	300079896	Factor X activator light chain 2	*Daboia russellii russellii*	SVMPs
39.	TWFNLScGDDYPFVcK AFDEPK TWFNLScGDDYPFVcKFPPRc FPPR FIKNCFGLEKESDYR	1 1 1 1 2	1005.84 707.36 890.02 516.45 889.45	2009.68 706.36 2667.05 515.45 2665.34	2 1 3 1 3	17.09 & 8.76	300490470	P68 alpha subunit	*Daboia siamensis*	Snaclec
40.	KGSHLVSLHSR VFTEEmNWADAEKFCTEQKK MEWSDR	1 1 1	611.72 817.79 824.36	1221.45 2450.36 823.36	2 3 1	24.66 & 8.59	73620112	Snaclec 4	*Daboia siamensis*	Snaclec
41.	DIMLIK FFcLSNK IMGWGAITSPNETFPGVTHcANINILPYSVcR TSTYIAPLSLPSSPPR	1 1 1 1	733.42 916.41 1193.23 563.60	732.42 915.41 3576.69 1687.80	1 1 3 3	32.03 15.47	311223824	Serine beta-fibrinogenase-like protein precursor	*Daboia siamensis*	SVSPs
42.	DIMLIR NMEIYLGVHSK NMEIYLGVHSKK	1 1 1	761.49 646.35 710.12	760.49 1290.71 1418.24	1 2 2	23.61 & 11.51	90116798	Thrombin-like enzyme elegaxobin-1	*Protobothrops elegans*	SVSPs
43.	TSTHIAPLSLPSSPPSVGSVcR DIMLIK SSELVIGGDECNINEHR	1 1 1	751.25 733.42 937.24	2250.75 732.42 1872.48	3 1 2	17.44 & 8.67	380875417	Serine protease VLSP-3	*Macrovipera lebetina (Levantine viper)*	SVSPs
44.	SIIAGNTAATcPP FGAHSQKVLNEDEQIRNPK NNEVLDKDImLIK	1 1 1	637.65 737.10 521.01	1273.29 2210.10 1560.04	2 3 3	17.44 & 7.51	13959617	Snake venom serine protease 1	*Trimeresurus gramineus (Indian green tree viper)*	SVSPs
45.	FHcSGTLLNEEWVLTAAHCDmENmQJYLGVHDK DIMLIR KAYGGLPEK TLCAGVLQGGIDTCLADSGGPLicNGQFQGIVAWGR	1 1 1 1	1298.61 761.49 963.46 1216.93	3892.83 760.49 962.46 3647.79	3 1 1 3	32.31 & 7.35	381141431	Serine protease VLSP-1	*Macrovipera lebetina (Levantine viper)*	SVSPs
46.	VLDcPSGWLSYEQHcYK KGSHLVSLHSR MEWSDR	1 1 1	1072.35 611.72 824.36	2142.70 1221.45 823.36	2 2 1	27.64 & 9.44	251205	RVV-X-light chain	*Daboia russelii*	SVMPs

47.	DQLQQNGQPcQNNR DScFQENLK NEcDVPEHcTGQSAEcPR NQcISLFGSR	1 1 1 1	851.18 571.74 1074.16 592.07	1700.36 1141.47 2146.32 1182.14	2 2 2 2	24.39 & 45.24	300079900	Factor X activator heavy chain	*Daboia russellii russellii*	SVMPs
48.	EAVHSYAIYGcYcGWGGQGR AVCECDRAAAICLGENVNTYDK CCFAQDCcYGR	1 1 1	764.95 787.11 664.10	2291.86 2358.34 1326.20	3 3 2	43.44 & 7.36	1408314	Phospholipase A2 acidic subunit	*Vipera aspis (aspic viper)*	PLA_2
50.	KDDENDKDIMLIR SSELVIGGDECNINEHR	1 1	536.08 937.24	1605.23 1872.48	3 2	11.63 & 4.69	406609998	Hypothetical protein	*Gloydius blomhoffii*	SVSPs
Gel Filtration Peak 3										
52.	AAAIcLGENVNTYDK CcFAQDCcYGR EAVHSYAIYGcYcGWGGQGR NLFQFGDmILQK NYEYYSISHcTEESEQc	1 2 1 1 1	820.68 691.917 764.781 735.999 1100.68	1639.36 1381.83 2291.34 1469.10 2199.37	2 2 3 2 2	80.33 & 31.58	1408314	Phospholipase A2 acidic subunit	*Vipera aspis*	PLA_2
53.	AAAIcLGQNVNTYDK EVVHSYAIYGcYcGWGGQGR NYEYYSISHcTEESEQc	1 1 1	547.12 774.15 1100.68	1638.36 2319.46 2199.37	3 3 2	69.57 & 22.98	400714	Acidic phospholipase A2 RV-7	*Daboia siamensis*	PLA_2
54.	ccFVHDccYGKLWSCSPK GTWCEKQIcEcDKAAAIcFR NGAIVcARGTWCEKQIcECDK	1 1 2	770.266 816.331 1221.11	2307.80 2445.10 2440.23	3 3 2	33.33 & 11.15	123907686	Basic phospholipase A2 Tpu-G6D49	*Trimeresurus puniceus*	PLA_2
55.	ccFVHDCCYGNLPDcNPK LAIPSYSSYGCYcGWGGKGTPK VNGAIVcEKGTScENR YmLYPDFLCKGELRC	1 1 1 1	734.72 784.90 898.40 934.10	2201.15 2351.71 1794.81 1866.20	3 3 2 2	68.61 & 5.97	408407675	Basic phospholipase A2 DsM-S1	*Daboia siamensis*	PLA_2
56.	CCLVHDCcYTRVGDCSPK EAAICLGENVNTYDK VGDCSPKmTLYSYRFENGDIICDNK	1 1 1	687.16 547.70 962.35	2058.47 1640.11 2884.05	3 3 3	46.67 & 4.99	40889259	Chain A, Acidic Phospholipase A2	*Indian Saw-Scaled Viper*	PLA_2
57.	FDFFWIGLR QDcLSDWSFYEGYcYK SSEEMDFVIR TTDNQWLR WSDGVNLDYK	1 1 1 1 1	601.52 1061.63 607.56 517.85 599.75	1201.04 2121.26 1213.12 1033.70 1197.50	2 2 2 2 2	51.33 & 26.92	300490464	Dabocetin beta subunit	*Daboia russellii russellii*	Snaclec
58.	NPFIcK YHAWIGLR YHEWITLPcGDKNPFIcK	1 1 1	779.36 508.96 760.33	778.36 1015.91 2277.10	1 2 3	38.31 & 18.80	123899657	Dabocetin subunit alpha	*Daboia siamensis*	Snaclec

59.	FDYK FGSVWIGLNDPWHNcNWEWSDNAR GSHLASIHSSEEEAFVSK IFWFNRGcEK	1 1 1 1	573.402 987.923 958.684 679.656	572.40247 2960.76999 1915.36894 1357.31120	1 3 2 2	37.33 & 15.35	300490484	P31 beta subunit	*Daboia siamensis*	Snaclec
60.	EDDYEEFLEIAKNGLK EGWYANLGPmRVPEK LNEFVQETENGWYFIK SAGQLYQESLGK	1 1 1 1	638.52 588.35 1009.83 641.43	1912.56 1762.05 2017.67 1280.87	3 3 2 2	26.19 24.15	395406796	L-amino-acid oxidase	*Daboia russelii russellii*	LAAO
62.	DEcDVPEHcTGQSAEcPR DQLQQNGKPcQNNR NPcNMHYScMDQHK YKPKCIFNPPLR	2 1 1 1	716.74 850.92 608.54 738.67	2147.22 1699.84 1822.63 1475.35	3 2 3 2	20.84 & 6.97	162329887	Chain A, Russell's Viper Venom Metalloproteinase	*Daboia siamensis*	SVMPs
63.	TLcAGILEGGKDSCR TLCAGILEGGK	1 1	791.37 531.40	1580.73 1060.80	2 2	6.44 & 4.06	82117246	Full=Beta-fibrinogenase brevinase	*Gloydius blomhoffii (mamushi)*	SVSPs
64.	SLLQQDSCQDAGmQSKcSASCFCQNK SVDFDSESPR VLEGIKcGENIYMSPNPMK YTNcKSLLQQDSCQDAGMQSKcSASCFCQNK	1 1 1 1	961.837 570.742 1090.99 1178.63	2882.51 1139.48 2179.97 3532.88	3 2 2 3	41.82 & 5.05	190195337	Cysteine-rich secretory protein Da-CRPa	*Deinagkistrodon acutus*	CRISP
65.	cSGccTDESMK FMEHTAcEcRPR HTADIQJMR MEVMKFmEHTACEcRPR WKQGEPEGPK	1 2 1 1 1	668.472 798.092 543.665 724.447 579.19	1334.94 1594.18 1085.33 2170.34 1156.38	2 2 2 3 2	56.94 & 32.54	327478537	Snake venom vascular endothelial growth factor toxin	*Daboia russelii russellii*	VEGF
66.	QQGEVISFLTVYER QENHcEPCSERR CSCKFTDSRcK	1 1 1	835.685 773.384 1335.28	1669.37 1544.77 1334.28	2 2 1	19.27 & 6.28	327488518	Vascular endothelial growth factor A	*Vipera ammodytes ammodytes (western sand viper)*	VEGF
Gel filtration Peak 4										
67.	cGENIYMSPYPMK cILNHSPYNSR RPEIQNEIVDLHNSLR RSVTPTASNMLK WTAIIHEWHK	2 1 1 1 1	796.09 1361.59 967.78 653.69 661.46	1590.18 1360.59 1933.56 1305.38 1320.92	2 1 2 2 2	53.56 & 69.14	190195321	Cysteine-rich secretory protein Dr-CRPK	*Daboia russelii*	CRISP
68.	KPEIQNEIVDLHNSLR SLVQQAGcEDKQIQSDcSAICFCQNKII SVDFDSESPRKPEIQNEIVDLHNSLR	1 1 1	953.80 1062.84 1009.40	1905.59 3185.53 3025.20	2 3 3	61.09 & 28.64	190195307	cysteine-rich secretory protein Ch-CRPKa	*Crotalus horridus*	CRISP
69.	KPEIQNKIVDLHNFLR mEWYPEAAANAER SGPPCGDCPSACDNGLCTNPCTK	1 2 1	655.69 777.78 747.93	1964.08 1553.55 2240.80	3 2 3	54.30 & 24.21	190195319	Cysteine-rich secretory protein Cv-CRP	*Crotalus viridis* (western rattlesnake)	CRISP

70.	DFKYGVGAVPSNAATGHYTQVVWYK mEWYPEAAANAER SYRGGcAAAYCPSSK GGcAAAYcPSSK	1 2 1 1	925.08 777.78 790.05 615.52	2772.24 1553.55 1578.10 1229.03	3 2 2 2	28.96 & 12.38	1778013	Prepro-cysteine-rich venom protein	*Protobothrops mucrosquamatus*	CRISP
71.	cILDHSPYNSR mEWYPEAAANAER SVDFDSESPR	1 2 1	1363.45 777.78 570.71	1362.45 1553.55 1139.43	1 2 2	46.15 & 21.81	190195323	Cysteine-rich secretory protein Dr-CRPB	*Daboia russelii*	CRISP
72.	IVDLHNSLR mEWYPEAAANAER YTNcNSLVQKSGCQDTWmQSNcPAICFCQNK	1 2 1	534.61 777.78 1216.06	1067.23 1553.55 3645.19	2 2 3	22.08 & 11.43	190195329	Cysteine-rich secretory protein Pg-CRP	*Cerrophidion godmani*	CRISP
73	AAAIcLGENVNTYDK EAVHSYAIYGcYcGWGGQGR NLFQFGDmILQK NYEYYSISHcTEESEQc	1 1 1 1	820.60 764.78 735.66 1100.56	1639.21 2291.33 1469.31 2199.13	2 3 2 2	86.07 & 23.82	1408314	Phospholipase A2 acidic subunit	*Vipera aspis (aspic viper)*	PLA_2
74.	AAAIcLGQNVNTYDK NLFQFGEMILQK NYEYYSISHcTEESEQc	1 1 1	820.14 735.35 1100.56	1638.29 1468.71 2199.13	2 2 2	69.67 & 18.33	37927199	Chain G, of Rv4RV7 complex	*Daboia siamensis*	PLA_2
75.	VNGAIVcEQGTScENR ccFVHDCCYGNLPDcNPK LAVPFYSSYGcYcGWGGK RVNGAIVcEQGTScENR	1 1 1 1	898.08 734.95 691.81 651.18	1794.16 2201.86 2072.43 1950.54	2 3 3 3	58.68 & 14.85	298351762	Basic phospholipase A2	*Daboia russellii russellii*	PLA_2
77.	NLFQFAEMIVK GKPQDATDRccFVHDccYEKVK	1 1	670.94 925.11	1339.88 2772.33	2 3	27.27 & 6.27	3914259	Basic phospholipase A2 RVV-VD	*Daboia russellii russellii*	PLA_2
78.	SPIFSYGDYGcYcGWGGK VAAIcFGENVNTYDKK CcFVHDcCYGR	1 1 1	692.49 915.12 710.63	2074.47 1828.24 1419.27	3 2 2	37.50 & 2.39	129506	Acidic phospholipase A2	*Cerastes cerastes*	PLA_2
79.	ccFVHDccYGKATGCDPKK	1	769.94	2306.81	3	13.67 & 2.39	403399517	Acidic phospholipase A2 Tgc-E6	*Trimeresurus gracilis*	PLA_2
80.	ccFVHDccYGKLAK DATDRCcFVHDCCYGK GTWCEEQICEcDRVAAECLR	1 1 1	910.15 631.73 791.42	1818.30 1892.18 2371.26	2 3 3	57.38 & 2.01	223635543	Basic phospholipase A2 A	*Crotalus durissus ruruima*	PLA_2
81.	YHEWITLPcGDKNPFIcK cFGLNK TWEDAEK	1 1 1	760.66 739.34 879.34	2278.98 738.34 878.34	3 1 1	37.01 & 17.16	123899657	Dabocetin subunit alpha	*Daboia siamensis*	Snaclec
82.	TWEDAEWFCTK cFGLK	1 1	708.56 625.37	1415.13 624.37	2 1	10.13 & 6.14	82129809	Crotocetin-1	*Crotalus durissus terrificus*(tropical rattlesnake)	Snaclec

83.	TTDNQWLR KTWEDAEK VFNEK	1 1 1	517.88 504.39 637.31	1033.76 1006.79 636.31	2 2 1	48.00 & 11.53	300490464	Dabocetin beta subunit	*Daboia russellii russellii*	Snaclec
84.	GNVVTVMVDVNLNNNVYK INTAcVcVISR HWNSYcTTTDTFVR mSMLcYTLIIAFLIGIRAAPK THEALKTSRNTDQHYPAPNK	1 1 1 1 1	997.28 647.37 597.15 800.46 770.36	1992.56 1292.74 1788.45 2398.37 2308.09	2 2 3 3 3	41.98 & 10.75	335892642	Venom nerve growth factor 2	*Daboia russelii*	VNGF
86.	SAGQLYQESLGK LNEFVQETENGWYFIK HIVIVGAGMSGLSAAYVLAGAGHK EGWYANLGPMR VTVLEASERPGGR	1 1 1 1 1	641.50 1009.73 760.66 648.10 686.64	1280.10 2017.47 2278.98 1294.21 1371.29	2 2 3 2 2	23.41 & 9.39	395406796	L-amino-acid oxidase	*Daboia russellii russellii*	LAAO
87.	c5GccTDESMK cTPVGKHTADIQIMRMNPR HTADIQIMR QGEPEGPKEPR	2 2 1 1	668.50 1113.64 543.66 613.25	1335.00 2225.29 1085.32 1224.51	2 2 2 2	43.75 & 8.07	327478537	Snake venom vascular endothelial growth factor toxin	*Daboia russellii russellii*	VEGF
88.	ATVAEDAcFQFNRLGSDYGYcRK DECDmADLCNGQSDEcPK DENKGmVEPGTKcENGK NGHPcQNNNGYcYNGK	2 2 1 1	910.39 682.86 955.70 949.62	2728.17 2045.58 1909.40 1897.25	3 3 2 2	36.27 & 7.34	297593790	Metalloproteinase	*Echis carinatus sochureki*	SVMPs
89.	ARNECDVPEHCTGQSAECPR cEDGKVcNNK DQLQQNGQPcQNNR GMVDPGTK LFSNcSIHDYQRYLTR	1 1 1 2 1	735.19 612.52 851.12 805.37 691.81	2202.56 1223.05 1700.23 804.37 2072.43	3 2 2 1 3	22.94 & 5.53	300079900	Factor X activator heavy chain	*Daboia russellii russellii*	SVMPs
Gel filtration Peak 5										
90.	LAIPSYSSYGcYcGWGGK MILEETGK VNGAIVcEKGTScENR YMLYPDFLcK	1 1 1 2	676.48 921.38 898.045 675.90	2026.43 920.38 1794.09 1349.80	3 1 2 2	76.86 & 45.27	24638087	Basic phospholipase A2 VRV-PL-VIIIa	*Daboia russellii russellii*	PLA_2
91.	LAVPFYSSYGcYcGWGGK RVNGAIVcEQGTScENR SLLEFGMMILEETGK	1 1 1	1037.08 976.28 850.14	2072.16 1950.57 1698.29	2 2 2	66.94 & 36.61	298351762	Basic phospholipase A2 3	*Daboia russellii russellii*	PLA_2
93.	AAAIcLGQNVNTYDK TATYSYSFENGDIVcGDNDLcLR mRTLWIVAVCLIGVEGNLFQFGEMILEK EVVHSYAIYGcYcGWGGQGRAQDATDR	1 1 1 1	820.14 1336.56 1076.51 1026.42	1638.27 2671.12 3226.53 3076.25	2 2 3 3	67.39 & 5.24	400714	Acidic phospholipase A_2 RV-7 (Viperotoxin non-toxic acidic component)	*Daboia siamensis*	PLA_2

94.	IPcAPEDVK IQNDADSSASISACNGLKGHFK LYcFDNLPEHK MPQcILIKPSR	1 1 1 1	514.81 755.25 718.95 672.77	1027.63 2262.76 1435.91 1343.55	2 3 2 2	16.29 & 10.04	123896981	Zinc metalloproteinase-disintegrin-like Eoc1	*Echis ocellatus*	SVMP
95.	EKFFcLSSK FFcLSSK FYcAGTLINQEWVLTAAR NVPNEDQQIRVPK TLCAGILQGGIDSCK	1 1 1 1 1	1146.44 889.316 1057.87 769.85 739.94	1145.44 888.31 2113.75 1537.70 1477.87	1 1 2 2 2	21.15 & 7.21	13959655	Venom serine proteinase-like protein 2	*Macrovipera lebetina*	SVSP
96.	WDKDIMLIK TLcAGVLEGGIDScK VPAEKVFCVSSKTYTR	1 1 1	582.21 790.62 606.29	1162.43 1579.24 1815.87	2 2 3	16.88 & 6.22	297593764	serine protease	*Echis carinatus sochureki*	SVSP
99.	RPEIQNEIVDLHNSLR cPAScFcHNEII	1 2	967.74 754.89	1933.48 1507.77	2 2	11.72 & 4.86	190195321	Cysteine-rich secretory protein Dr-CRPK	*Daboia russelii*	CRISP
Gel filtration peak 6										
100.	KYmLYPDFLcK LAIPSYSSYGcYcGWGGK QNLNTYSK VNGAIVcEK SLLEFGKMILEETGK VNGAIVcEKGTScENR	3 3 1 1 1 2	748.16 676.18 968.43 991.39 848.59 898.15	1494.32 2025.53 967.43 990.39 1695.18 1794.30	2 3 1 1 2 2	82.64 & 72.67	24638087	Basic phospholipase A_2 VRV-PL-VIIIa	*Daboia russellii russellii*	PLA_2
101.	IcECDKAAAICFRR IYmLYPDFLcK LAVPFYSSYGcYcGWGGK SLLEFGmMILEETGK VNGAIVcEQGTScENR	1 3 1 1 2	829.30 740.44 1037.08 858.14 898.15	1656.61 1478.89 2072.16 1714.28 1794.30	2 2 2 2 2	81.82 & 45.98	298351762	Basic phospholipase A_2 3	*Daboia russellii russellii*	PLA_2
103.	CcFVHDccYGKVNGCNPK EVCECDKAAAIcFRDNK NVAGR	1 1 1	721.11 987.01 516.37	2160.33 1972.02 515.37	3 2 1	32.79 & 6.21	3914268	Acidic phospholipase A2	*Trimeresurus gramineus*	PLA_2
104.	FPNGLDKDIMLIR RPVTYSTHIAPVSLPSR WcEPLYPWVPADSR	1 1 1	767.17 941.69 889.10	1532.34 1881.39 1776.19	2 2 2	47.03 & 26.89	134129	Factor V activator RVV-V alpha	*Daboia siamensis*	SVSP
105.	cKEFIYGGcHGNANKFPSR cRQTcGASAKGRPT EFIYGGcHGNANK FcYLPADPGEcLAHMR	1 1 1 1	748.64 775.39 734.58 646.89	2242.91 1548.79 1467.16 1937.69	3 2 2 3	65.56 & 18.18	159883540	Trypsin Inhibitor-5 precursor	*Daboia siamensis*	KSPI

106.	FcHLPVDSGlcR DQcRHTcGGK	1 1	731.39 609.77	1460.78 1217.54	2 2	26.19 & 2.25	123913154	Kunitz protease inhibitor 4	*Daboia russellii russellii*	KSPI
108.	HANFPILSANIRPK ASGNPILLNK ETPVLSNPGPYLEFRDEVEELQNHANK	1 1 1	527.25 514.50 1042.91	1578.80 1026.99 3125.74	3 2 3	9.86 & 8.65	538259847	5' nucleotidase, partial	*Protobothrops flavoviridis*	Nucleotidase
Gel filtration Peak 7										
109.	IYmLYPDFLcK LAVPFYSSYGcYcGWGGK RVNGAIVcEQGTScENR SLLEFGmMILEETGK	2 1 2 2	740.38 1037.13 651.08 857.89	1478.76 2072.26 1950.26 1713.78	2 2 3 2	76.86 & 50.08	298351762	Basic phospholipase A_2	*Daboia russellii russellii*	PLA_2
Gel Filtration Peak 8										
112	SLLEFGmmILEETGK LAVPFYSSYGcYcGWGGK IYmLYPDFLcK RVNGAIVcEQGTScENR	3 2 2 2	866.11 1037.36 740.28 976.225	1730.22 2072.72 1478.56 1950.45	2 2 2 2	86.78 & 104.89	298351762	Basic Phospholipase A_2	*Daboia russellii russellii*	PLA_2
113	VNGAIVcEKGTScENR LAIPSYSSYGcYcGWGGK KYMLYPDFLcK	2 1 3	1795.25 1014.34 740.28	1794.25 2026.68 1478.56	1 2 2	82.64 & 59.37	24638087	Basic phospholipase A2 VRV-PL-VIIIa	*Daboia russellii russellii*	PLA_2
114	FAIIAYSNYGcYcGWGGK ccFVHDccYGR	1 1	696.8767 767.965	2087.63 1533.93	3 2	21.01 & 3.70	13936543	Phospholipase A_2	*Echiscoloratus*	PLA_2
115	SPIFSYGDYGcYcGWGGK ccFVHDccYGR mIFKMTGK	2 1 1	692.2833 767.965 973.32	2073.85 1533.93 972.32	3 2 1	30.83 & 2.33	129506	Acidic phospholipase A_2	*Cerastes cerastes*	PLA_2
116	TSVSSHYcTGR CTTGPCCRQcK LKPAGTTcWR	1 1 1	628.6 629.085 596.145	1255.20 1256.17 1190.29	2 2 2	74.42 & 8.67	123916448	Disintegrin CV short precursor	*Ceraste svipera*	Disintegrin
117	FcYLPADPGEcMAYIR SFYYDSESK cRQTcRAPR	1 1 1	982.81 563.925 603.185	1963.62 1125.85 1204.37	2 2 2	37.78 & 4.57	159883524	Trypsin inhibitor-4 precursor	*Daboia siamensis*	KSPI
118.	LTPGSQCAEGLCcDQCKFIK GAVQPKNPccDAATcK	1 1	734.86 889.52	2201.59 1777.04	3 2	32.43 & 6.49	48428042	Piscivostatin alpha	*Agkistrodon piscivorus piscivorus* (eastern cottonmouth)	Disintegrin

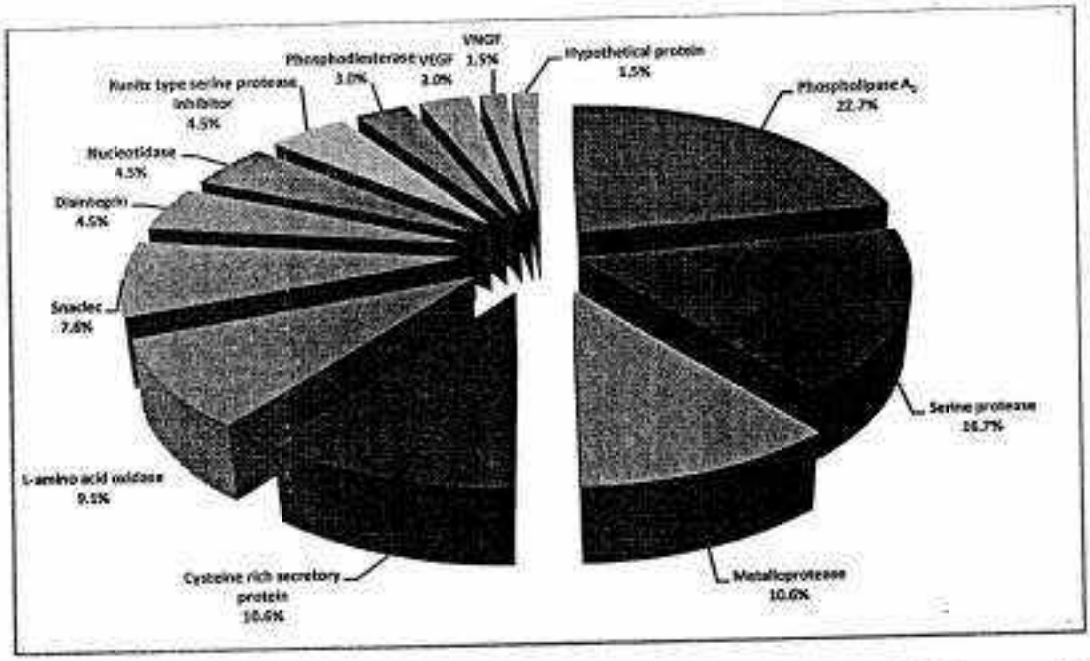

Figure 3: Relative abundance of snake venom protein families in *Daboia russelii* venom. Peptide sequences were obtained by ESI-LC MS/MS analysis. Protein families were assigned based on the sequence as identified by the Proteome discoverer 3.1 with the sequest program pre-loaded into the system.

Based on the N-terminal amino acid residues, the PLA_2 enzymes of *D. russelii* were classified into N-type (asparagine at N-terminus) and S-type (serine at N-terminus) (Tsai et al., 1996). The N-type venom is reported to be present in *D. r. siamensis*, *D. r. russelii* and *D. r. formosensis* while the S-type is found in *D. r. pulchella* (Tsai et al., 1996). However, in this venom, both the S-type and N-type PLA_2 enzymes were observed along with peptide fragments homologous to PLA_2 with (13936543, 50874332 and 223635543) H at the N-terminal. This suggests the absence of any specific taxonomic pattern for classification of Indian *Daboia russelii* venom based on the N-terminal amino acid residue of PLA_2 enzymes. The observed anticoagulant, PLA_2 activity (48.89±3.85 µmol/min/ml/mg) and edema could be attributed to the presence of large number of PLA_2 isoenzymes.

SVSPs are the second most abundant proteins in this venom proteome. The abundance of these proteins correlates with the proteolytic and fibrinogenolytic activities (data not shown) of the crude venom. Homolog of elegaxobin (Accession No. 90116798) from *Protobothrops elegans* (Oyama and Takahashi, 2000), RVV-Vα from *D. siamensis* were observed. RVV-V converts coagulation factor V to Va by cleaving at the Arg^{1545}-Ser^{1546} bond of factor V (Nakayama et al., 2011), which is an important co-factor in the prothrombinase complex. Apart from these, three isoforms of β-fibrinogenase similar to proteins reported from *D. siamensis*, *Macrovipera lebetina* and *Gloydius blomhoffii* were observed in the present study (Table 1).

SVMPs are the third most abundant protein family in this proteome. Based on the difference in the domain structure, the SVMPs can be categorized into three broad classes, namely PI, PII, (a, b, c, d) PIII (a, b, c and d) (Fox and Serrano, 2005; Fox and Serrano, 2008; Fox and Serrano, 2009). During envenomation SVMPs cause both local and systemic injuries which include symptoms like myonecrosis, haemorrhage, edema formation and blistering (Fox and Serrano, 2010). Viperidae venoms are rich source of these proteins; these venoms induce the most profound damaging effects to the basement membrane of the blood vessel capillaries leading to excess bleeding and distortion of the skeletal muscles (Fox and Serrano, 2010). Six isoforms of heavy chain and light chain of RVV-X (coagulation factor X activating enzyme) were observed (Table 1) suggesting the presence of this protein complex in the crude venom might be Responsible for the observed procoagulant activity. RVV-X belongs to the PIIId family of metalloprotease with a heavy chain and two light chains (Takeya et al., 1992) connected to each other via an inter-chain disulphide bond between Cys79 of chain A and Cys77 of chain B while the light chain is connected to the heavy chain via

another disulphide bond between Cys133 of chain A and Cys339 of heavy chain. It converts coagulation factor X to Xa by cleaving at the Arg^{51}-Ile^{52} of FX leading to severe coagulopathy in victim/prey (Fujikawa et al., 1972).

Presence of large number of these SVMPs and SVSPs in the venom could be responsible for the observed procoagulant and proteolytic activities. These enzymes together may be responsible for consumptive coagulopathy in the victim. However, the thrombus formed by SV-TLE is readily dissolved by the plasmin due to absence of proper cross-linked fibrins leading to excess blood loss at the site of injury (Phillips et al., 2010).

LAAO (57-68 kDa, monomeric form) are homodimeric high molecular weight proteins (Tan and Fung, 2010). These enzymes cause oxidation of hydrophobic L-amino acids releasing α–keto acid, ammonia and H_2O_2 (Chen et al., 2012; Du and Clemetson, 2002) The liberated H_2O_2 is causes various pathologies like edema formation, ADP or collagen induced platelet aggregation inhibition or activation, apoptosis, antibacterial effect, antiparasitic, anticoagulant, haemolytic and haemorrhagic effect (Chen et al., 2012; Suhr and Kim, 1996; Du and Clemetson, 2002). Six isoforms of LAAO identified were found to be similar with protein sequence previously reported from *Echis ocellatus* (Accession No. 347602327), *Calloselasma rhodostoma* (Accession No. 10120762), *D. r. russelli* (Accession No. 395406796), *Protobothrops flavoviridis* (Accession No. 538259837), *Gloydius blomhoffii* (Accession No. 75570145), and *V. a. ammodytes* (Accession No. 347602330) (Table 1). Most of these proteins are reported to inhibit ADP and collagen induced platelet aggregation (Chen et al., 2012; Samel et al., 2006). As such it could be suggested that the LAAO together with SVSPs, SVMPs and PLA_2 might exert severe coagulopathy in the prey or victim.

PDEs are high molecular weight (> 90 kDa) (Dhananjaya et al., 2010) proteins reported to act as endonucleases on both double and single stranded RNA and DNA releasing 5' mononucleotides (Dhananjaya et al., 2010). In this proteome, analogous sequence of PDEs reported previously from *Macrovipera lebetina* (Accession No. 586829527) and *Protobothrops flavoviridis* (Accession No. 538259853) (Aird et al., 2013) were identified (Table 1). Pharmacologically, PDEs inhibit platelet aggregation, decrease the mean arterial pressure and hinder locomotion (Russell et al., 1963).

Nucleotidases belong to the metallophosphatase superfamily of proteins (Aird et al., 2013). Presence of homologous peptides suggest their presence in the venom and they might be involved in the

release of adenosine (purines) which cause hypotension and paralysis leading to prey immobilization and digestion (Aird, 2002; Aird, 2005).

CRISP are the largest group of non-enzymatic proteins (10.6%) identified in *D. russelii* venom. Several peptides similar to Da-CRPa Dr-CRPK, Ch-CRPKa, Cv-CRP, Dr-CRPB, Pg-CRP and a prepro CRISP from crotalinae and viperinae subfamily were identified (Table 1). They are reported to inhibit cyclic nucleotide gated ion channels (Brown et al., 1999), potassium activated smooth muscle contraction (Osipov et al., 2005) and vascular smooth muscle contraction (Ito et al., 2007). The presence of CRISP in the venom might cause visual, olfactory and locomotory impairment of the victim thus playing a crucial role in prey immobilization and subjugation.

Snaclecs are the second most abundant non-enzymatic component of the crude venom. Peptide sequence homologous to α and β subunits of dabocetin documented earlier from subspecies of *D. russelii* were identified (Table 1). α dabocetin is reported to bind to glycoprotein Ib of platelet to induce inhibitory effect on ristocetin-induced platelet aggregation (Zhong et al., 2006). Similarly peptide sequences analogous to α and β subunits of P31 (Accession No. 300490484) and α subunit of P68 (Accession No. 300490470), crotocetin-1 (Accession No. 82129809) and snaclec A14 (Accession No. 218526485) were also observed. Moreover, β subunit of alboaggregin-A (Accession No. 3023231) reported earlier from *Trimeresurus albolabris* is a strong activator of platelet, binding via GPIb α and GP VI of platelets was also identified in this proteome (Asazuma et al., 2001; Andrews et al., 1996; Dormann et al., 2001). The abundance of snaclecs in this proteome might be the cause of enhanced coagulopathic disorders along with SVMPS, SVSPs and PLA_2 enzymes in the envenomed victims.

KSPI are the low molecular weight proteins having 50-60 amino acid residues with a conserved Kunitz motif typical to bovine pancreatic trypsin inhibitor (BPTI) (Mourao and Schwartz, 2013; Morjen et al., 2014). Upon envenomation, KSPI may inhibit diverse pharmacological effects on the victim like fibrinolysis (Qiu et al., 2013), trypsin and chymotrypsin (Guo et al., 2013), haemorrhage and anticoagulation (Earl et al., 2012) as well as potassium and calcium ion channel (Schweitz et al., 1994; Stotz et al., 2000). We have identified three isoforms of KSPI having sequence similarity with trypsin inhibitor precursor - 4 and 5 reported earlier from *D. siamensis* (Guo et al., 2013) and KPI- 4 from *D. r. russelii* (Table 1).

Disintegrin are the polypeptides rich in cysteine residues liberated by the proteolytic cleavage of multidomains of metalloproteases in the venom (Calvete et al., 2005). They are found in the viperidae family and are known to cause inhibition of integrin receptors (Calvete et al., 2005). Peptide sequence analogous to adinbitor (Accession No. 50365991) from *Gloydius brevicaudus* which is reported to inhibit angiogenesis and platelet aggregation and piscivostatin α (Accession No. 48428042) isolated from *Agkistrodon p. piscivorus* (Okuda and Morita, 2001) were identified. Both these toxins have RGD as the integrin inhibitory motif. Sequences similar to disintegrin CV with RTS integrin inhibitory motif studied previously from *Cerastes vipera* were also observed in the crude venom (Sanz et al., 2006). Disintegrin CV is reported to inhibit cell adhesion and migration via collagen I and II, anti angiogenic and interact with α1 and β1 of integrin (Sanz et al., 2006). The integrin receptor inhibitory loop with RGD motif is antagonist to β1and β3 ($\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$) integrins while the inhibitory loop with RTS is specific to α1β1 (Calvete, 2005; Calvete et al., 2010).

Peptide sequence similar to VNGF-2 reported earlier from *Daboia russelii* of Taiwan was observed. VNGFs are reported to cause apoptosis, vascular permeability and wound healing (Li et al., 1980; Otten et al., 1984). Peptide sequences identified in the proteome were similar to Vegf toxin and Vegf-A described earlier from snakes of viperinae family (Yamazaki et al., 2003; Yamazaki et al., 2003; Yamazaki et al., 2009). These proteins are reported to cause nitric oxide induced hypotension, angiogenesis, capillary permeability, cell proliferation, migration and anti-apoptosis (Yamazaki et al., 2009).

Apart from these well known protein families, peptide sequences similar to a hypothetical protein was also observed in the venom proteome. This protein was reported earlier from the transcriptome of *Crotalus adamanteus* (Accession No. 387016758) (Rokyta et al., 2012). Details of its structure and function of this protein is under investigation.

Objective 2: Isolation and purification of major toxins from the venom of *Daboia russelii*

2.1 Materials

a) Chemicals and reagents: All reagents used were of analytical grade from Sigma-Aldrich, (USA).
b) Columns: Gel filtration Superdex 75 column and ion exchange (Hiprep CM FF 16/10) columns from GE Healthcare (USA) and RP HPLC columns (Phenomenex, USA) were used.

2.2 Methods

a) Gel filtration: Fractionation of crude venom was done as described in section 1.2 c.

b) Ion-exchange chromatography: Ion-exchange chromatography was performed on a cation exchanger column on Hiprep CM FF 16/10. Fractionation was carried out in AKTA purifier system (Amersham Biosciences, Uppsala, Sweden). The fractionation was carried at a flow rate of 2.5 ml/min and eluted by a linear gradient of 50mM Tris, pH 7.4 containing 0.8M NaCl. Elution of the protein was monitored at 215 and 280 nm.

c) Reversed Phase HPLC

RP-HPLC was performed using C18 column (5µ, 4.6x250 mm, 300Å) (Phenomenex, USA). The column was pre-equilibrated with milli Q in 0.1% TFA and fractionated using 80% ACN containing 0.1%TFA on a HPLC system (Waters, USA). Elution of the protein peaks were monitored at 215 and 280 nm. Peaks were manually collected and SDS-PAGE was done to check the purity of the preparation.

d) Determination of molecular mass:

Molecular mass of the purified protein was determined by electrospray ionization mass spectrometry (ESI-MS) using an LC Q fleet Ion Trap, Thermo Scientific (USA) mass spectrometry system. Ion spray voltage was set at 4.4KV. Nitrogen was used as a curtain gas at a flow rate of 0.6 l/hr and compressed air was used as a nebulizer. The sample was infused by flow injection at flow rate of 50µl/min. Solvent used was 50% acetonitrile in 0.1% formic acid at a flow rate of 200µl/min. Promas for Xcaliber was used to analyze and decipher the raw mass data.

g) Secondary structure determination

The secondary structural conformation of the native purified protein was studied by circular dichroism study on Jasco spectropolarimeter J-810. About 200µl of 0.4mg/ml of purified protein dissolved in Milli Q water was loaded on quartz cuvette (0.1cm path length). The far UV scan for the spectra was done from 260-190nm at a speed of 50nm/min.

Temperature dependent scan: About 200µl of 0.4mg/ml of purified protein dissolved in Milli Q water was scanned from 25^0C to 100^0C to determine its limit of thermal stability. Milli Q water was used as blank for the experiment.

pH dependent scan: For the pH dependent stability about the same concentration of the purified protein was reconstituted in phosphate buffer saline (PBS) at pH 3.0, pH 7.4 and pH 12.0. PBS buffer with the respective pH were used as the blank for each condition.

2.3 Results and Discussion

A major protein named as Ruvitoxin was purified by successive steps of chromatography. Peak Rv-GF-7 (Figure 2) from gel filtration chromatography was found to be the major protein present as it covers 31% of the total crude venom of *Daboia russelii*. Peak Rv-GF-7 was subjected to ion exchange chromatography on Hiprep CM FF 16/10 on an AKTA purifier system (Amersham Biosciences, Uppsala, Sweden). Fraction revealed one major peak (II) and two minor peaks (I) (Figure 4A). Under reduced condition Rv-GF-7 CM II which is the major peak migrated as single band. Under non-reduced condition the band migrated as smear and the size of was twice as the reduced condition which clearly indicates Rv-GF-7 CM II is a covalent dimmer and constituted 24.5% of the total protein in the crude venom (Figure 4B). Rv-GF-7 CM II was further subjected to RP-HPLC for desalting and to remove if it contains any contaminating protein (Figure 5). The RP-HPLC profile showed single symmetrical peak and silver staining of the peak after SDS-PAGE showed the protein was purified to homogeneity. This purified protein is one of the major proteins of the crude venom and was named as "Ruvitoxin". Molecular weight of the protein was evaluated using ESI-MS and it was found to be 13597.62 Da which is in the range of PLA_2. Secondary structure analysis of Ruvitoxin by CD reveals the presence of α-helix: 42.72% and β-sheet: 12.36% which is typical for secretary PLA_2. The secondary structure found to be stable at pH 3 and 7.4 however at pH 12 it lost its secondary structure (Figure 7). The protein was found to be heat stable as structure did not distorted up to 70 °C (Figure 8). The biochemical, biophysical and biological

characterization of this protein was carried out to understand its structure. The role of this major protein in venom toxicity was also evaluated.

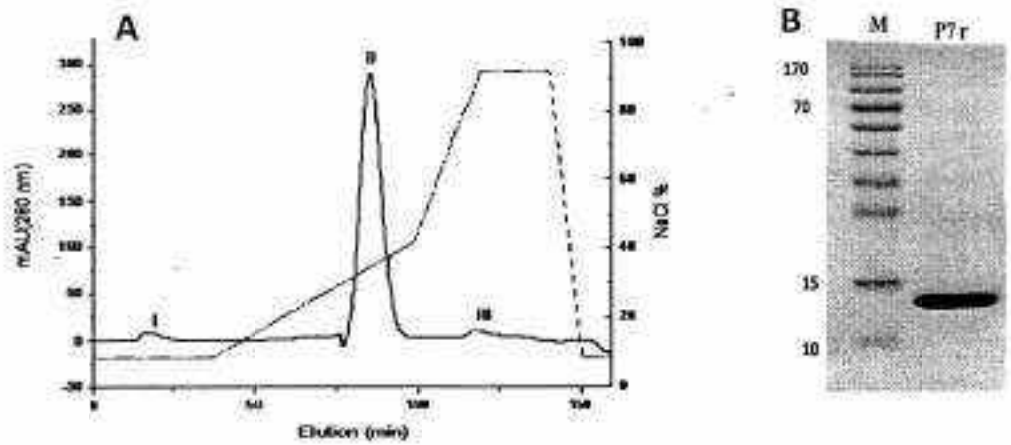

Figure 4: A. Cation exchange profile of Rv-GF-7, B. SDS -PAGE profile of Rv-GF-7 under reduced (r) condition.

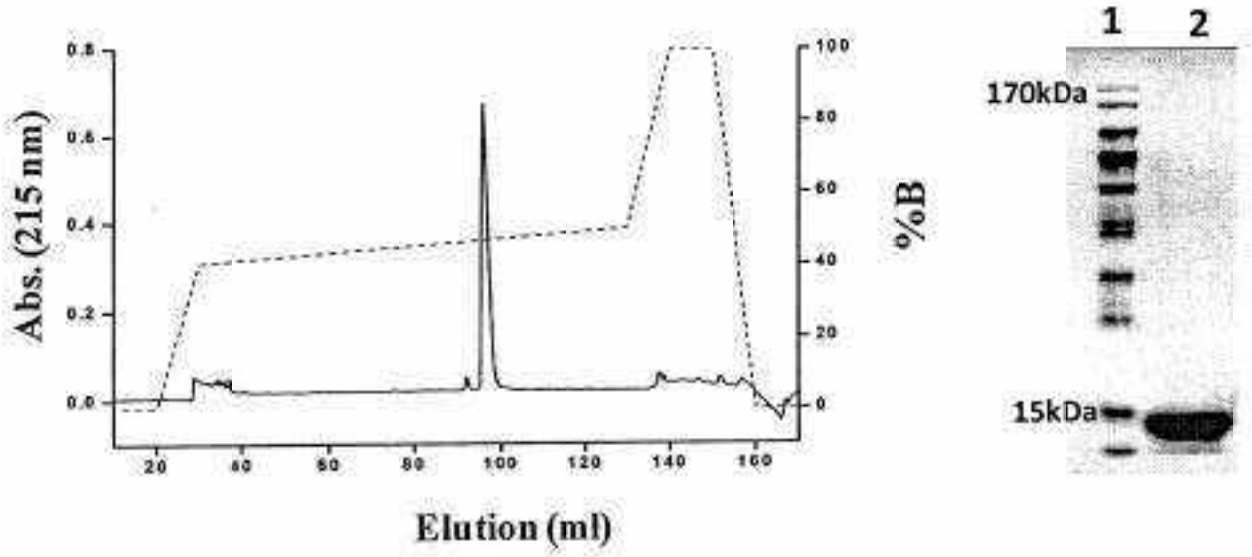

Figure 5: RP-HPLC profile of Rv-GF-7 CM II on C18 column. Inset: SDS -PAGE profile of Rv-GF-7 CM II after silver staining.

Table 2: Summary of purification of Ruvitoxin.

Purification	Sample	Total protein (mg)	% protein in crude venom	Recalcification time (1µg) (NCT= 115.68 sec)	Specific activity PLA_2 (µmol/min/ml/mg)
Un-fractionated	Crude venom	88.0	100	35.0	20.05
Gel filtration	Rv-GF-7	28.0	31.8	No clot	47.0
Ion-exchange	Rv-GF-7-CM-II	21.6	24.5	No clot	50.0
RP-HPLC	Ruvitoxin	15.02	21.03	No clot	65.12

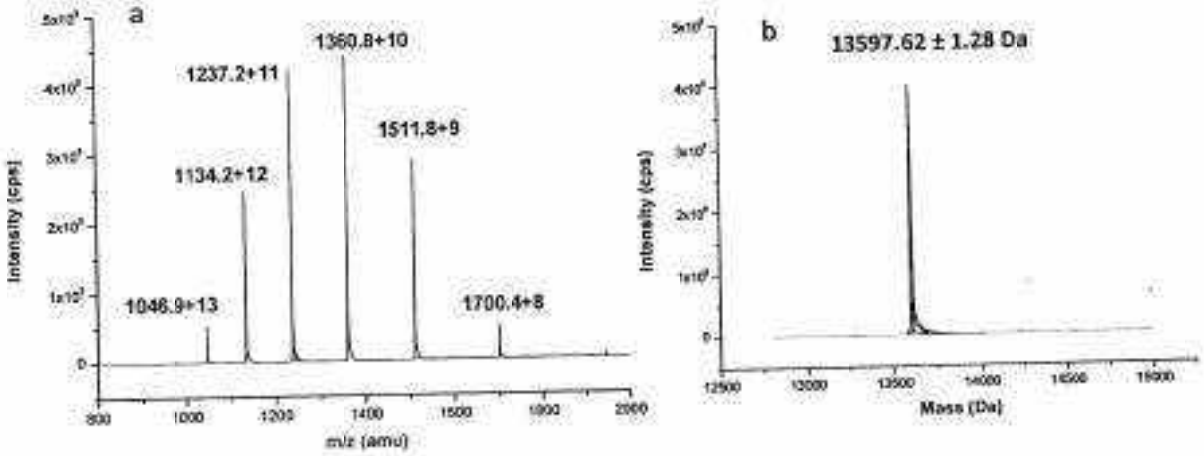

Figure 6: Molecular mass determination of Ruvitoxin by ESI/MS: a) The spectrum shows a series of multiply charged ions, corresponding to a single, homogenous peptide with a molecular weight of 13597.62 Da. **b)** Reconstructed mass spectrum of the protein, CPS = counts/s; Da = Dalton.

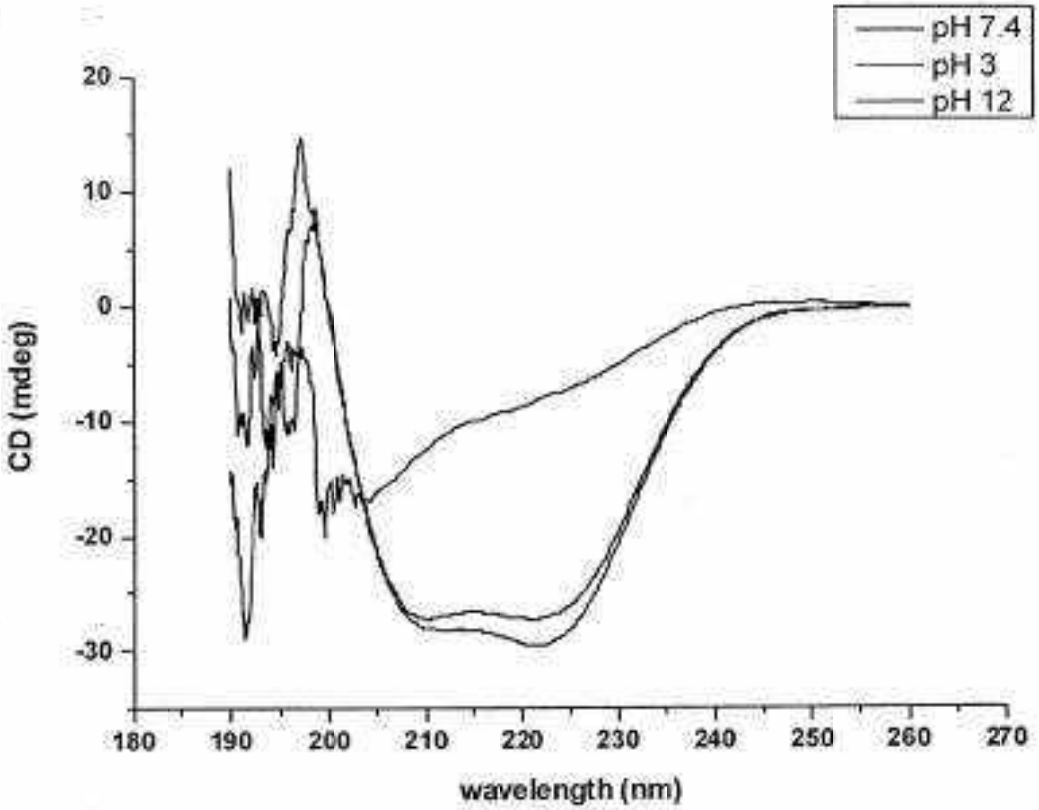

Figure 7: CD analysis of Ruvitoxin for determination of the secondary structure. Secondary structure was determined at different pH to check the stability of Ruvitoxin.

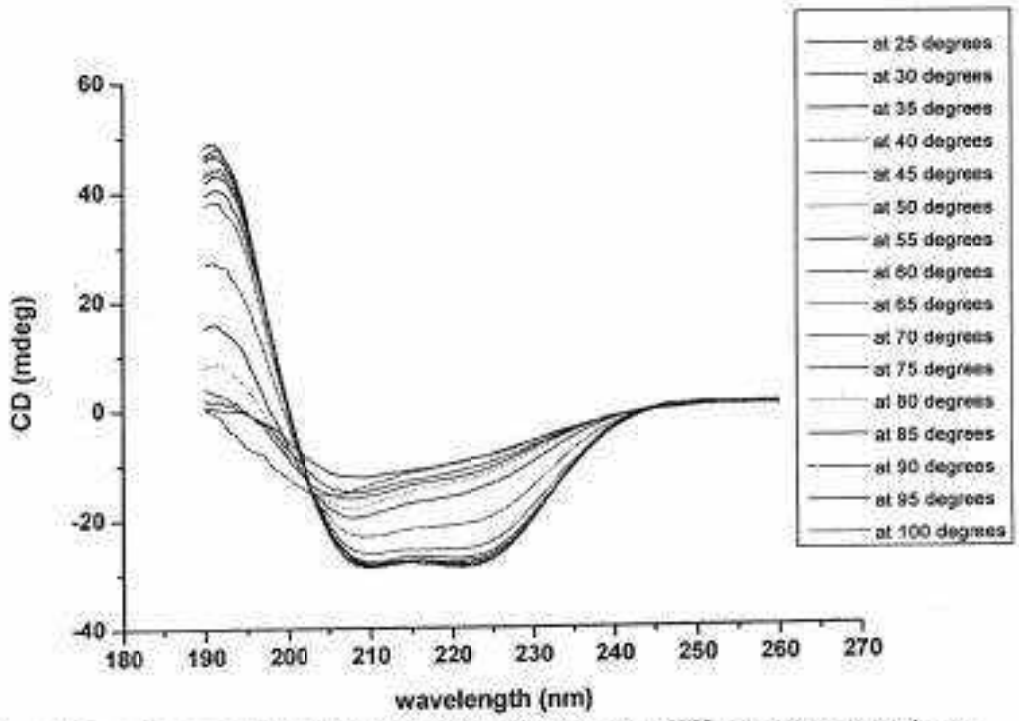

Figure 8: Analysis of secondary structure of Ruvitoxin at different temperature.

Objective 3: Determination of amino acid sequences of toxins isolated from the venom of *Daboia russelii*

4.1 Materials and Methods

a) N-terminal sequencing of the purified protein

N-terminal sequencing was determined by automated Edman degradation process using PPSQ 31 (Shimadzu, USA) with phenlythiohydantion (PTH) derivative analyzer. Briefly, Lyophilized protein was resuspended in 100µl of denaturant solution (6.0 M guanidinium hydrochloride, 0.13M Tris, 1mM EDTA, pH-8.0) containing 0.07M β-mercaptoethanol and incubated at 37°C for 2hrs. Subsequently blocking of sulfhydral groups was done by adding 4-vinylpyridine and incubated at room temperature. The protein was desalted by RP HPLC after 2hr and subjected to N-terminal sequencing. About 89µg (6545.56 picomoles) of purified protein was dried on PVDF membrane and loaded onto the sequencer. Sequencing was done for 50 cycles or till the signals were readable.

b) ESI LC-MS/MS

The protein was reduced with dithiothreitol (DTT) and reduced group was blocked by alkylation using Iodoacetamide (IAA). Further, the protein was digested with trypsin and loaded on LCQ Fleet Ion Trap, Thermo Scientific (USA) and LC-MS/MS was performed as described in section 1.2d.

4.2 Results and Discussion

The first 30 amino acid residues of Ruvitoxin were obtained by N-terminal amino acid sequencing (Figure 9). Serine is first amino acid residue in the protein and PLA_2 enzymes with S, N and H are well reported in this family of protein (Tsai et al., 1996). To obtain the complete sequence of the protein, it was subjected to proteolytic cleavage using BNPS and hydroxylamine. The cleaved products were separated by RP-HPLC and larger fragments were subjected to edman degradation to obtain the amino acid sequences (Figure 9). When the sequences were aligned we did not obtain any overlapping sequences however when compared to viperid PLA_2 from the database we could combine to obtained the full sequence. To confirm the complete sequence, we performed peptide mass finger printing using ESI-LC-MS/MS. The protein was digested with trypsin and directly loaded into the ESI-LC-MS/MS. MS sequence of eleven peptides were obtained from the MS/MS

analysis (Table 3). The sequences were identified using the software Proteome Discoverer 3.1 using Sequest program and was found to be homologous to PLA_2 enzyme family.

Table 3: MS/MS sequence of tryptic digested peptides. Ruvitoxin was subjected to tryptic digestion and MS/MS sequences were obtained. The peptide sequence similar to known protein of the database is shown.

MS/MS Sequence	Match to Protein Group Accessions	Charge	MH+ [Da]	Protein family
YMLYPDFLcKGELK	24638087	3	1776.14	
VNGAIVcEK	24638087	1	989.36	
SLLEFGKMILEETGK	24638087	3	1695.85	
GTSCENTRICECDK	24638087	2	1457.00	
QNLNTYSK	24638087	1	967.47	
MILEETGK	24638087	1	920.47	PLA_2
LAIPSYSSYGCYCGWGGK	24638087	3	1911.51	
KYmLYPDFLcK	24638087	2	1494.36	
IcEcDK	298351762;24638087	1	824.27	
AAAIcFR	3914268;298351762;2638087	1	808.40	
IcEcDKAAAIcFRQNLNTYSK	24638087	3	2504.97	

Figure 9: Amino acid sequence of Ruvitoxin. Sequence obtained by N-terminal sequencing, sequencing of peptide obtained by protease cleavage peptides and MS/MS sequencing of tryptic digest fragments. X indicates Cys which were not identified by N-terminal sequencing. The sequences were overlapped to obtain the complete sequence of Ruvitoxin.

On comparison of the peptide sequences with that of the sequence obtained from N-terminal and proteolytically cleaved, we obtained the overlapping sequences (Figure 9). The complete sequence of Ruvitoxin was found to be SLLEFGKMILEETGKLAIPSYSSYGCYCGWGGKGTPKDATDRC

CFVHDCCYGNLPDCNNKSKRYRYKKVNGAIVCEKGTSCENRICECDKAAAICFRQNLNTY SKKYMLYPDFLCKGELVC. The molecular mass of this protein was calculated using online software and found to be 13596.70 Da (from sequence). This mass was found to be in agreement with the observed mass of 13597.62 ± 1.28 Da when analyzed on ESI-MS (Figure 6b).

Figure 10: Sequence alignment of Ruvitoxin with other viperid PLA_2. BLASTp was performed to obtain the homologous sequences. The conserved Cys residues are highlighted in grey and the amino acid substitutions in Ruvitoxin is in red letter. * indicates the active site His residue.

Homology search was done using BLASTp program to find its homology with other snake venom PLA2 enzymes. It was found to be highly similar to viperid PLA_2 enzymes. Ruvitoxin showed maximum similarity of 95.87% to the major Basic phospholipase A_2 VRV-PL-VIIIa (24638087) reported from the venom of *Daboia russellii russellii* (Gowda et al., 1994). There were five amino acid substitutions in this protein which are shown in red letter (Figure 10). The active site His residue was found to be conserved and the 49th amino acid residue was Asp which is involved in catalysis. The conserved Cys residues were also obtained which are involved in forming the disulphide bridges. To understand the phylogenetic relationship, between other PLA_2 enzymes of viperid family, phylogenetic tree was constructed (Figure 11). Ruvitoxin was found to be more closely related to PLA_2 from the venom of *Daboia russellii limitis*. Based on the primary sequence analysis, Ruvitoxin is a novel PLA_2 enzyme isolated from the venom of India *Daboia russelii*. Biochemical and pharmacological characterization was undertaken to understand its role in contributing towards the toxicity of the crude venom.

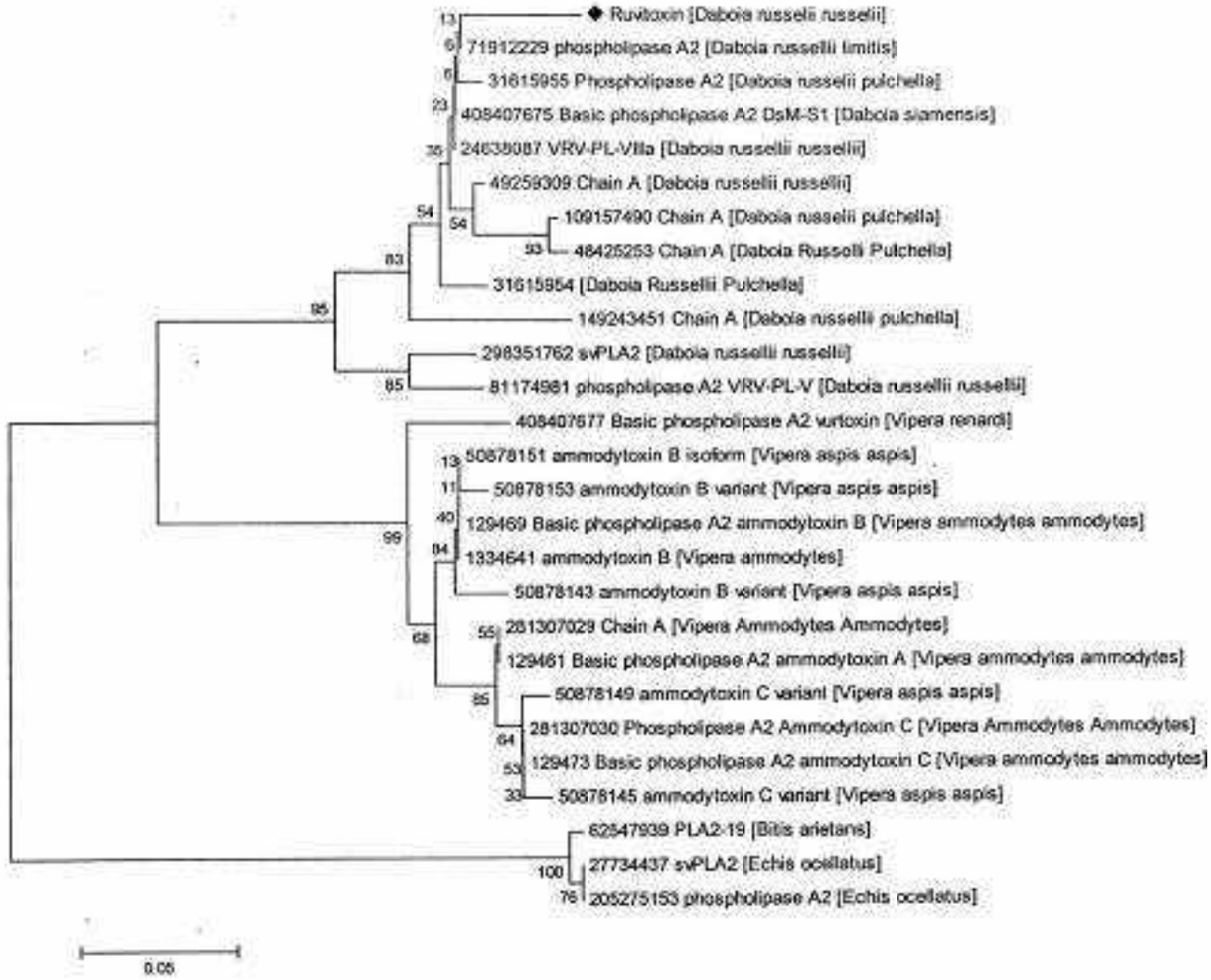

Figure 11: Phyogenetic analysis of Ruvitoxin.

Objective 4: Biochemical and pharmacological characterization of the toxin isolated from the venom of *Daboia russelii*

4.1 Materials

a) Chemicals and reagents:
sPLA2 assay kit was procured from Cayman Chemical Company (MI, USA). Uniplastin for PT was obtained from Tulip Diagnostics (India), AGAPEE kit for CK/LDH analysis was purchased from AGAPPE diagnostics (Switzerland), Polyvalent antivenom manufactured by Bharat Serums and Vaccines Limited (India) was purchased locally. Bovine plasma fibrinogen was obtained from Sigma-Aldrich and all other reagents used were of analytical grade and were either from Merck or Sigma-Aldrich, (USA).

4.2 Methods

a) Determination of protein content: Total protein content of RVV fractions obtained from crude fractionation was determined according to Lowry's method using BSA as standard (LOWRY et al., 1951). Protein estimation of the GFC peak was redone on Nano Drop 2000 spectrophotometer (Thermo Scientific, USA) using BSA as a standard.

b) Phospholipase A_2 (PLA_2) activity: PLA_2 activity was performed using egg yolk phospholipid as substrate according to method of Doley and Mukherjee (Doley and Mukherjee, 2003). 1 to 10µg of GFC Peak II was incubated with 170µl egg yolk substrate for 10mins. Absorbance was checked in a designed programme from 0 min to 10 mins at 740nm in a MultiSkan GO, UV-Vis spectrophotometer (Thermo Scientific, USA). 1 unit of PLA_2 activity is defined as the 0.01decrease in OD at 740nm.

c) Caseinolytic Assay: Caseinolytic assay of RVV GFC fraction II was performed using casein as a substrate (Ouyang and Teng, 1976). 1% (w/v) casein in 20 mM phosphate buffer, containing 150 mM NaCl, pH 8.0 was incubated with 1 to 100µg of GFC peak II protein for 1 h at 37 8C followed by addition of 0.5 ml of 10% TCA. The digested protein in the supernatant was determined using Folin–Ciocalteau's reagent. Amount of tyrosine released was calculated by plotting a standard tyrosine curve.

d) Recalcification time test: Fresh goat blood was collected from local butcher shop with 3.8% tri sodium citrate in 1:9 ratios. The blood was centrifuged at 3000rpm for 20mins at 4°C in a

refrigerated centrifuge (Thermo Scientific, USA). The experiment was done in dose dependant manner. 0.1-50µg of RVV GFC peak II was incubated with 120ul of PPP (platelet poor plasma) and Time of clotting was measured in a coagulation analyzer.

e) Prothrombin time test:

Prothrombin time was measured using Uniplastin (PT reagent) obtained from Tulip Diagnostics (India). Various amount of venom (0.1-10µg) in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 1min and 100µl of PT reagent was added to initiate the clot formation. The clotting time with buffer was considered as normal clotting time. The results are mean ±SD of three experiments.

f) Fibrinogenolytic assay

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng, using bovine fibrinogen (2mg/ ml) dissolved in 50mM Tris HCl buffer, pH 7.5, 0.15M NaCl (Ouyang and Teng, 1976). To 300µl of dissolved fibrinogen, various amount of venom in 150µl of buffer was incubated for different time intervals at 37°C. The incubated mixtures were then run on a 12.5% (w/v) SDS-PAGE according to the method of Laemmeli (Laemmli, 1970). Staining was done with 0.25% (w/v) Coomassie brilliant blue R250 and de-stained till the protein bands were visible.

g) Direct and Indirect hemolytic assay

Preparation of RBC's was done according to the method followed by Das et al., 2013. Various amount of venom were incubated for 60min at 37°C with 150µl of 10% RBC to a final volume of 2ml with 0.9% (v/v) NaCl. However additional 20µl of egg yolk substrate solution was mixed to the reaction mixtures at the time of incubation for indirect hemolytic assay. The tubes were centrifugation at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 10min and the absorbance of the supernatant was measured at 540nm in a MultiSkanGO, UV-Vis spectrophotometer (Thermo Scientific, USA). The hemolysis caused by dH2O was considered as 100%.

h) Bactericidal activity

The bactericidal activity of the crude venom and venom fractions were carried out using both gram positive (*Bacillus sp.*) and gram negative bacteria (*E. coli.*) were spread on fresh LBA (luria bertani

agar) plates and holes of 6 mm (d) were punched on each plate. 50ug/ml of ampicillin was used as the positive control and 20mM TrisCl buffer as the negative control. The venom fractions were put in the respective wells followed by the incubation of the plates at 37^0C for 4 hrs.

i) Animal Studies

Male Swiss albino mice of 40 ± 3 grams used for animal studies were obtained from central animal facility, University of Mysore and housed in well ventilated cages. Animal experiments were performed as per the Ethical Guidelines of Animal Ethical Committee of University of Mysore, Mysore, India.

Edema inducing activity assay: Edema inducing activity of crude *Daboia russelii* venom was determined according to Vishwanath and co-workers (Vishwanath et al., 1987). 15µg of crude venom in 20µl of PBS were injected to right foot pad of male albino mice, control animals received the same amount of PBS. After 1 hour the mice was sacrificed using anesthesia (Barbitone 30mg/kg, i.p.). The legs were removed at ankle joints and weight was taken. Minimum edema dose is defined as the amount of protein required to cause an edema ration of 120%.

Haemorraghic activity: Haemorraghic activity assay was done as described by Kondo and co-workers (KONDO et al., 1960). Three male albino mice were injected (intra dermal) with 15µg crude venom in 30µl of PBS, control animals received same amount of PBS. 3µg of Saw scaled viper venom in 30µl of PBS was used as positive control. The mice were sacrificed after 3 hours post injection using anesthesia (Barbitone 30mg/kg, i.p.). The dorsal surfaces of the skin were removed and inner surface was checked for haemorraghic activity. The minimum hemorrhagic dose (MHD) is defined as the concentration of venom required to induce a hemorrhagic spot of 1 cm diameter from the spot of injection.

4.3 Results and Discussion

There is a tremendous molecular diversity of snake venom PLA_2s, with both active and catalytically inactive forms, that results in a wide spectrum of toxin action, such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, hemorrhagic, and edema-inducing activities (Doley et al., 2009; Kini, 1997). To understand the role of Ruvitoxin in the crude venom,

its biological activities were assayed (Table 4). About 10 µg of Ruvitoxin did not show any direct haemolytic activity on washed human RBC, whereas the same concentration it exhibited indirect haemolysis of 13% indirect haemolytic activity. This protein does not hydrolyse the RBC membrane though it is enzymatically active protein. The indirect hemolysis exhibited by purified PLA_2 in presence of egg-yolk phospholipids was due to formation of phospholipid hydrolysis products like lysophospholipids and free fatty acids which are lytic by themselves (Condrea et al., 1964; Doley et al., 2004). The absence of cytolytic activity is further observed from the bactericidal assay. It did not show bactericidal activity on both gram positive and gram negative bacteria as observed on LB plate by well diffusion and disc diffusion method (Data not shown).

Table 4: Summary of some biochemical activities of Ruvitoxin

Activities	Ruvitoxin (1µg)
PLA_2 activity (µmol/min/ml/mg)	37.53 ± 1.52
Direct haemolysis	Nil
Indirect haemolysis	1.3 %
Proteolytic activity (µmol/min/ml)	Nil

Crude viper venom is pro-coagulant in nature because of large amount of serine proteases present in this venom though PLA_2 is a major family. Ruvitoxin purified from this venom is found to be a strong anticoagulant protein as at 10 ng/ml the plasma did not clot (Figure 12A). PLA_2 are classified into strong, weak or non-anticoagulant enzymes. By systematic and direct comparison of the proteins the region between residues 54 and 77 is responsible for this. It is positively charged in strongly anticoagulant PLA_2 enzymes, but negatively charged in weakly and non-anticoagulant enzymes (Kini and Evans, 1987). Ruvitoxin is found to have positively charged residues in this region which might be responsible for the strong anticoagulant activity. It has been observed that Activated partial thromboplastin time was delayed rather than prothrombin or thrombin time (Figure 12 C and D). Hence the probable target of Ruvitoxin would be the coagulation factors either FVIIa or FX. To dissect out the probable target of Ruvitoxin, inhibition of enzymatic activity of coagulation factors were assayed using chromogenic substrates (Figure 13). It was observed that even at a concentration of 1 micromole of Ruvitoxin, the enzymatic activity of FIXa, FXa, FXIa,

FXIIa and FVIIa was not lost. Hence this protein does not target the active site of these serine proteases. As the protein was inhibiting the intrinsic tenase complex, both extrinsic and intrinsic tenase complex were reconstituted and inhibition was studied. Ruvitoxin at 1 micromole concentration inhibited both the complexes up 90% (Figure 14). As FX is the common factor in these two complexes, Ruvitoxin might be inhibiting the activation of FX to FXa. Phospholipid is one of the important components for the complex formation. PLA_2 enzymatically cleaves phospholipid and the functional complex may not form for activation of FX to FXa. However in absence of phospholipid it has been observed that FX is activated to FXa.

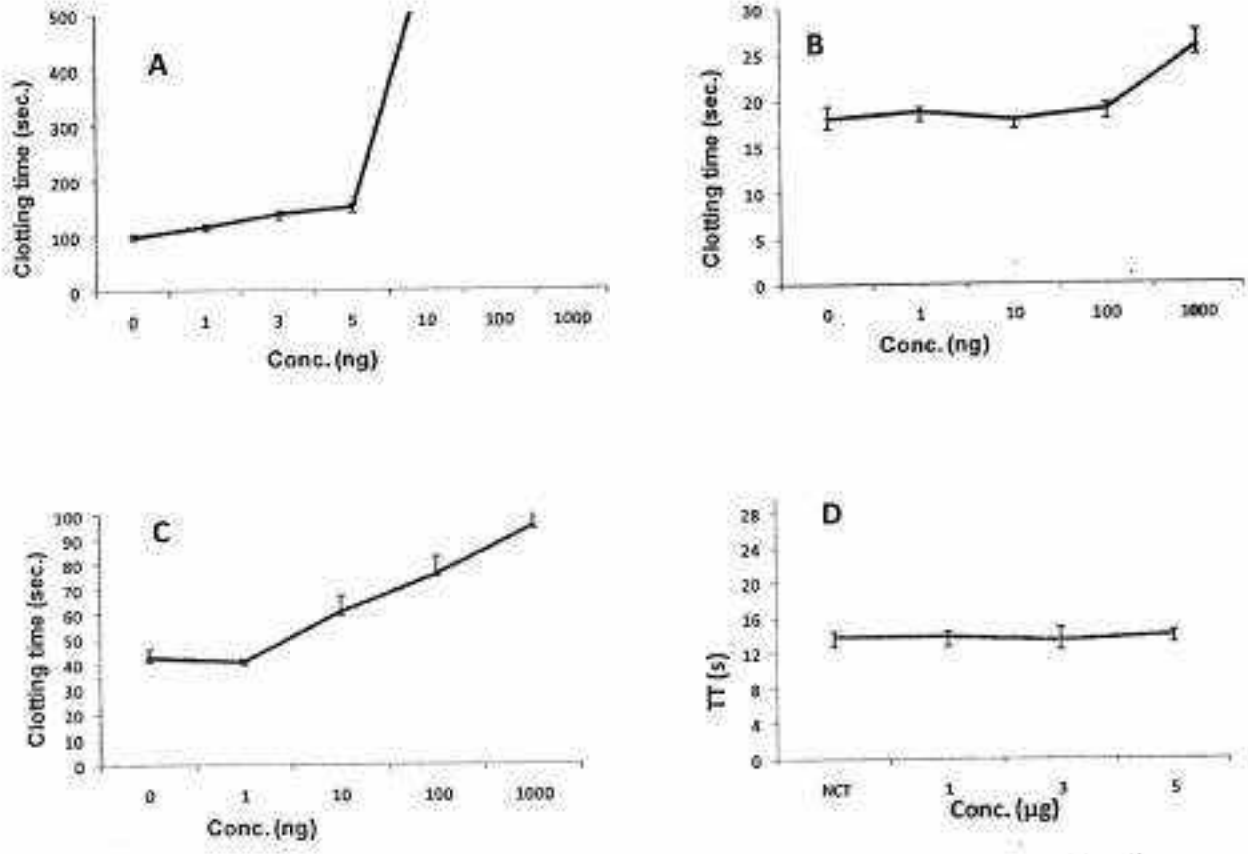

Figure 12: Effect of Ruvitoxin on blood coagulation. (A): Recalcification time; (B): Prothrombin time; (C): Activated partial thromboplastin time; (D): Thrombin time. Goat plasma was used for the assay and the clot formation was monitored using Tulip Coastat-1 coagulo analyser (Tulip, Alto Santa Cruz, Goa, India). Clotting time of the plasma in seconds (sec) in presence of buffer (20 mM, pH 7.4) was considered as normal clotting time (NCT). Results are ±SD of three independent experiments.

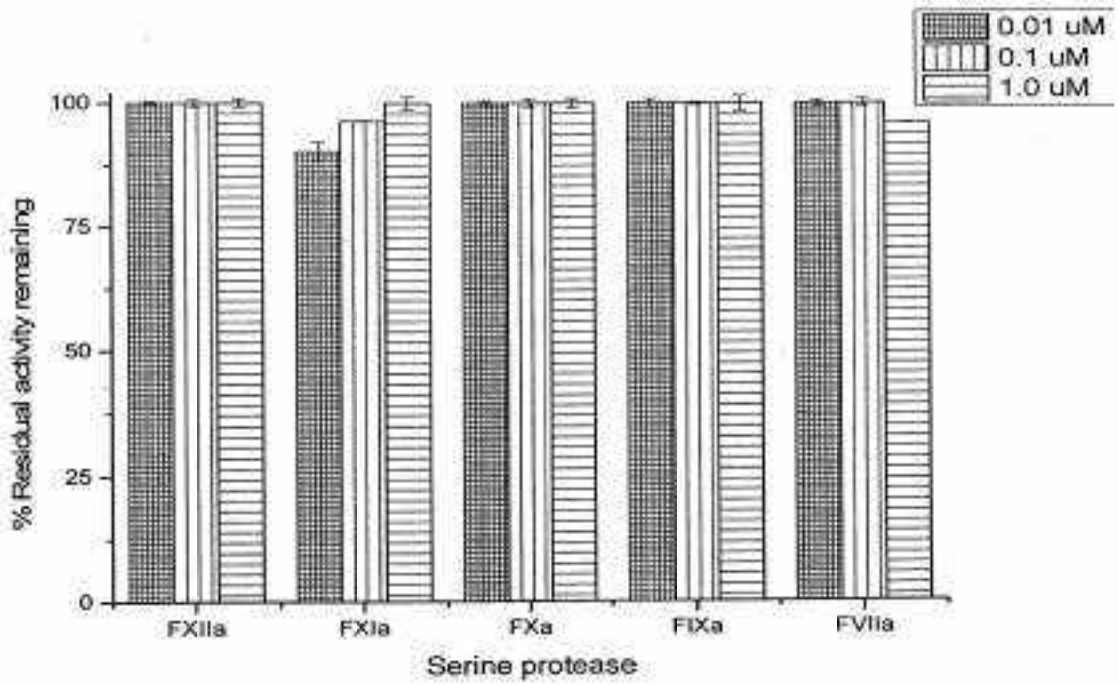

Figure 13: Effect of Ruvitoxin on various coagulation factors. The percent inhibition by Ruvitoxin was assayed using specific chromogenic substrates. Activity of the coagulation factors without Ruvitoxin was considered as 100% activity. Results are ±SD of three independent experiments.

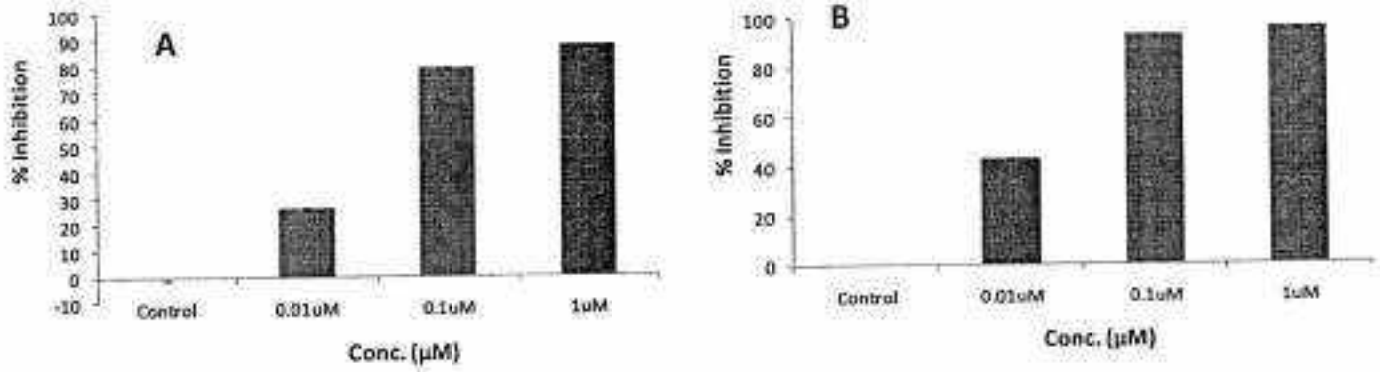

Figure 14: Effect of Ruvitoxin on extrinsic tenase complex (A) and intrinsic tenase complex (B). Percent inhibition by Ruvitoxin was calculated considering 100% activity with only buffer. Results are ±SD of three independent experiments.

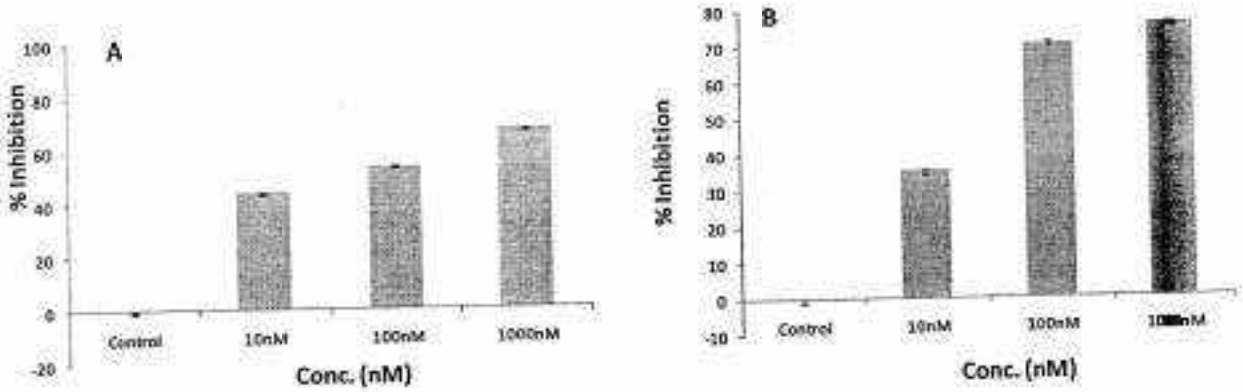

Figure 15: Effect of Ruvitoxin on extrinsic tenase complex (A) and intrinsic tenase complex (B) in absence of phospholipid. Percent inhibition by Ruvitoxin was calculated considering 100% activity with only buffer. Results are ±SD of three independent experiments.

We also tested the role of Ruvitoxin in absence of phospholipid and found that it inhibits the activation of FX to FXa (Figure 15). Hence enzymatic activity of Ruvitoxin does play any role in inhibiting the activation of FX to FXa.

PLA_2 induces pharmacological activities either through enzymatic and non-enzymatic mechanisms. To explain the functional specificity and mechanism of induction of various pharmacological effects, a target model has been proposed which hypothesize the presence of pharmacological sites in this protein independent of the active site (Doley et al., 2009; Kini and Evans, 1989). These pharmacological sites on the PLA_2 molecule recognized the specific 'target sites' and binds with the target molecule forming a non-covalent complex. The affinity between PLA_2 and its target protein is in the low nanomolar range, whereas the binding between PLA_2 and phospholipids is in the high micromolar range (Doley et al., 2009; Kini and Evans, 1989). Hence non-enzymatic mechanism of pharmacological action is highly specific. From the present study we observed that Ruvitoxin exhibits anticoagulant activity by inhibiting the activation of FX to FXa by binding to FX.

Further to confirm the binding, interaction of Ruvitoxin with FX and FXa using Fluorescence emission spectroscopy was studied. On incubation of Ruvitoxin with FX quenching of fluorescence emission was observed (Figure 16A). In presence or absence of Ca^{2+} the quenching was not affected but with increase in incubation time it increased. Interestingly the FXa was also found to be interacting with Ruvitoxin (Figure 16B). It can be concluded that Ruvitoxin binds to FX and blocks

its activation. Moreover it does not bind to the active site of FXa. The probable mechanism of action is shown in figure 16 and figure 17.

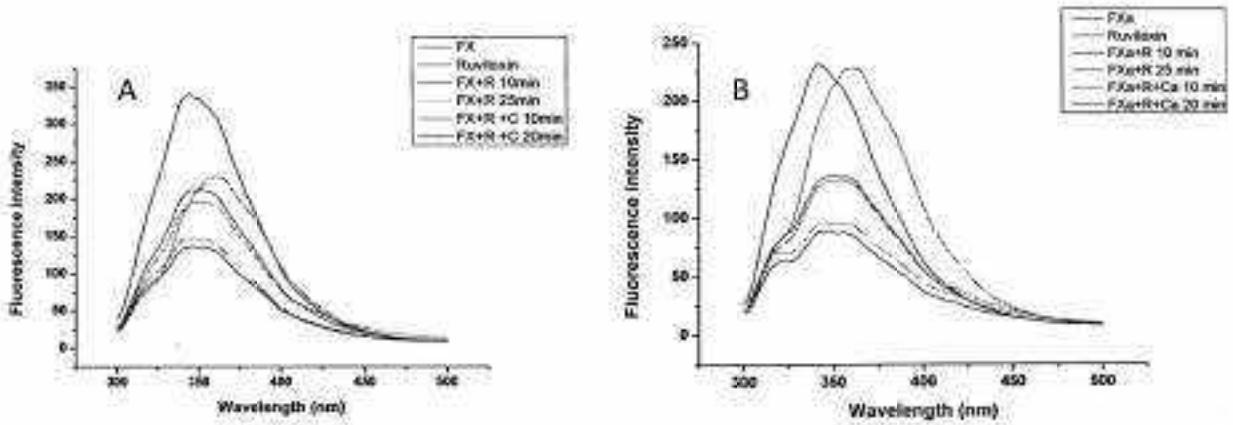

Figure 16: Interaction study of Ruvitoxin with FX and FXa using Fluorescence emission spectroscopy. The interaction was studies at different time interval either in presence or absence of Ca^{2+}.

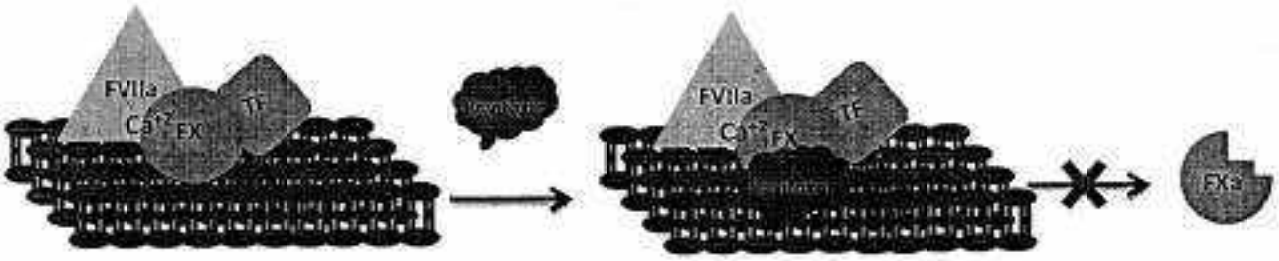

Figure 17: Probable mechanism of inhibition of FX activation by Ruvitoxin during activation of FX to FXa by extrinsic tenase complex.

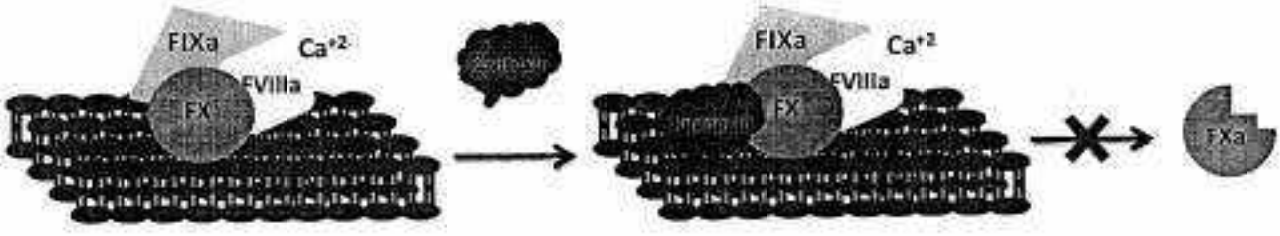

Figure 18: Probable mechanism of inhibition of FX activation by Ruvitoxin during activation of FX to FXa by intrinsic tenase complex.

Major finding of the project

1. This is the first analysis of complete proteome of Indian *Daboia russelii* venom and the protein families present.
2. The absence of daboiatoxin, a major protein of *Daboia russelii* venom has been revealed for the first time.
3. The major protein present in this venom is a novel PLA_2 enzyme.
4. This PLA_2 enzyme is named as Ruvitoxin
5. It is a thermostable protein as evident from the secondary structure analysis using CD
6. It is a strong anticoagulant PLA_2 enzyme
7. It binds to both FX and FXa for its anticoagulant activity
8. Ruvitoxin might be responsible for excess bleeding at the site of bite

Future Prospects

1. Detail study of the molecular interaction of Ruvitoxin with human FX and FXa
2. Design of synthetic peptide based on molecular interaction
3. Toxicity test of Ruvitoxin using experimental animal/cell lines
4. Study the effect of Ruvitoxin on inflammation and myotoxicity using cell culture model
5. Study the effect of crude venom and Ruvitoxin on cancer cell lines

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REVIEW ARTICLE

Geographical variation of Indian Russell's viper venom and neutralization of its coagulopathy by polyvalent antivenom

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Abstract

Indian Russell's viper venoms of four different geographical locations were found to vary in composition, coagulopathy and phospholipase A_2 (PLA_2) activity. Venom from Kerala showed highest procoagulant activity followed by Tamil Nadu, West Bengal and Karnataka whereas PLA_2 activity was highest in venom from West Bengal. The commercial polyvalent antivenom differentially neutralized the aforesaid activities of the crude venoms. Antivenomics study showed the presence of non-immunodepleted and partially immunodepleted proteins in the crude venoms. Thus, Indian Russell's viper venom from different region varies in composition and accentuates the need to design regiospecific antivenoms to confront the problem of envenomation more effectively.

Keywords

Antivenomics, coagulopathy, geographical variation, Indian Russell's viper, *in vitro* neutralization

History

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Introduction

Snake venom is a complex mixture of proteins and polypeptides which varies from species to species and also within the same species. This variation has been mainly attributed to difference in diet, gender, age, season and geographical locations of the snakes (Alape-Giron et al., 2008; Chippaux et al., 1991; Daltry et al., 1996a,b; Jayanthi & Gowda, 1988; Menezes et al., 2006; Minton & Weinstein, 1986; Williams & White, 1992). Clinical symptoms of envenomation like neurotoxicity, myotoxicity, hemotoxicity, anticoagulant, procoagulant, haemorraghic, necrosis, renal damage and muscular paralysis in prey/victims might also vary within the same species due to this variation in venom composition (Hung et al., 2002a; Markland, 1998; White, 2005). The haemostatic system of prey/victim is a common target of all the snakes for capture of prey. The anticoagulant components of venom cause defective coagulation of blood leading to excess blood loss from the bite site and also from gums and internal organs. This in turn causes hypovolemic shock to vital organs like brain, kidney and pituitary glands leading to death (White, 2005). The procoagulant components of venom proteins cause excess clot formation leading to thrombosis in the blood vessels. This is often followed by consumptive coagulopathy which leads to heavy bleeding at later stages and thus compromising the functioning of vital organs like kidney, heart and brain (White, 2005). Some of the venom protein families act as both procoagulant and anticoagulant. For example, snake venom phospholipase A_2 (PLA_2) enzymes hydrolyze the membrane phospholipids of platelets leading to the release of arachidonic acid and platelet aggregation factors which cause platelet aggregation during the process of primary haemostasis (Braud et al., 2000). Some of them act as inhibitors of secondary haemostasis by enzymatically hydrolyzing the membrane phospholipids that are required to form complexes like the prothrombinase, extrinsic tenase and intrinsic tenase (Kini & Evans, 1989). Further, some PLA_2 enzymes interact non-covalently with some clotting factors of these complexes and exhibits anticoagulant activity (Kini, 2006, 2011). Snake venom proteases like metalloproteases and serine protease have been reported to inhibit or activate the components of haemostasis. They cause severe vascular damage by interacting with the extracellular matrix (White, 2005). This in turn interfere the regulation of the coagulation cascade. A schematic representation of the various protein families of snake venom acting as agonist and antagonist to the secondary haemostatic system is shown in Figure 1(a and b).

Daboia russelii, commonly referred as Russell's viper, is one of the medically important snakes of the world (Warrell, 1989). In India, subspecies *Daboia russelii russelii* is found across the country and responsible for majority of the snakebites cases (Warrell, 1989). It is one of the members of the "Big Four" snakes of India (Simpson & Norris, 2007). Russell's viper envenomation mainly causes excess bleeding due to consumptive coagulopathy by haemostatically active

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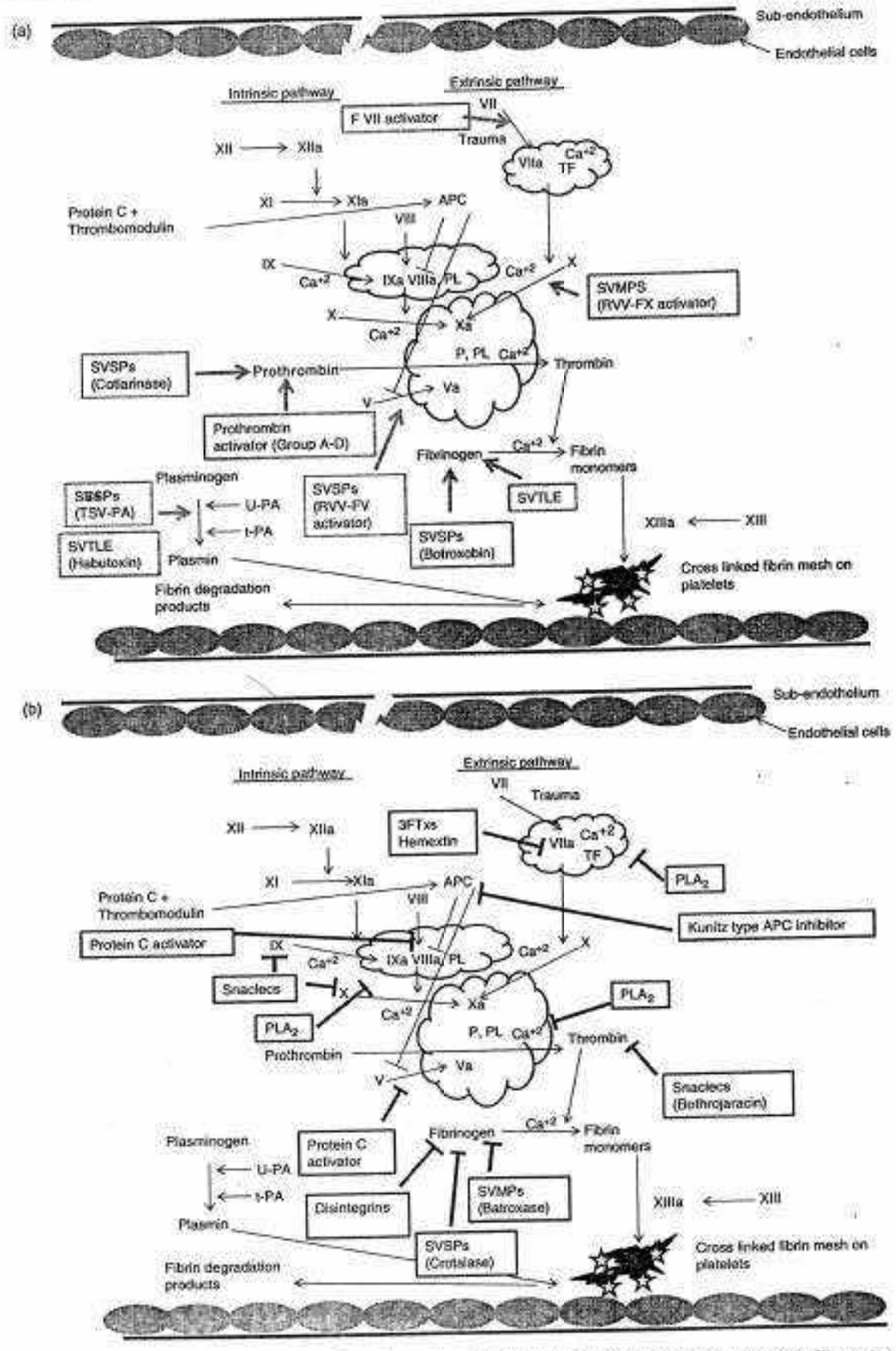

Figure 1. Schematic representation of snake venom proteins acting on haemostatic system. Various snake venom proteins families acting as (a) agonist (green box) and (b) antagonist (red box) of the secondary haemostatic system. Green arrow indicates proteins acting as agonist while proteins acting as antagonist are indicated by "T" shape bars. Examples of proteins belonging to the families are shown in brackets.

proteins which acts immediately (Cheng et al., 2012; Hung et al., 2002b; Phillips et al., 1988; Than et al., 1988). This often leads to hypovolemic shock to the vital organs of the prey/victim leading to severe clinical manifestations. Therefore, immediate medical attention is required in Russell's viper envenomation. In India, Russell's viper victims are treated with polyvalent antivenom and in many instances, adverse effect of antivenom therapy is being reported. This might be due to the variation in the venom composition between the venom used for raising the antibodies and venom present in the victim's body.

In India, Gowda and others studied the Russell's viper venom variation and toxicity from different regions, namely: western, northern, southern and eastern region (Jayanthi & Gowda, 1988; Prasad et al., 1996; Shashidharamurthy et al., 2002). Although variation in venom composition has been carried out but variation in venom composition with respect to coagulopathy has not been assessed. Hence, we reinvestigated the venom variation from four different geographical locations of India using proteomic approaches. Neutralization of coagulopathy of the crude venoms by polyvalent antivenom was also investigated using *in vitro* inhibition studies and antivenomics approach. In the present study we have selected geographically close regions as well as a far-off region to understand the variation in Indian Russell's viper venom composition.

Compositional analysis of Indian Russell's viper venom

Snake venom collection

Crude venom was obtained from four different states of India with typical climatic and geographical variations. The three geographically close states, namely Tamil Nadu, a land of coastal area; Karnataka, a land of hills and plateaus; Kerala, with typical humid equatorial climate surrounded by hills and coastal areas; and West Bengal, with completely distinct conditions of mixed climatic conditions comprising of hills, plateaus and delta.

The crude venom obtained from Tamil Nadu is designated as RvTN (Russell's viper Tamil Nadu), this venom was procured from Irula Snake catchers Society, Tamil Nadu; venom from West Bengal is designated as RvWB (Russell's viper West Bengal), which was obtained from Kolkata Snake Park; venom from Kerala is designated as RvKE (Russell's viper Kerala), which was milked from the snake kept in Agadantantra snake park, Ayurveda Medical college, Thiruvananthapuram, and venom from Karnataka is designated as RvKA (Russell's viper Karnataka), it was collected from Hassan, Western Ghats (Milked Personally).

SDS–PAGE analysis of crude venom

Crude venoms (15 μg) after reduction with β-mercaptoethanol were loaded on 12.5% SDS–PAGE along with standard protein markers (Thermoscientific; Lafayette, CO) and performed according to the method of Laemmli (1970). The gels were stained with 0.25% (w/v) coomassie brilliant blue and destained till the bands appeared. All the four crude venoms have shown protein bands in the molecular mass range of 170–10 kDa with varying intensity of protein bands (Figure 2). The SDS–PAGE profile of RvTN has shown the presence of 9 protein bands with two very high-intensity protein bands at 70 and 15 kDa. RvKA has shown the presence of 13 protein bands with major bands at 15 and 10 kDa, while no prominent band was observed at 70 kDa. On the other hand, RvKE has revealed the presence of 11 protein bands with very intense bands at 100, 25 and 15 kDa. The RvWB has shown the presence of 12 protein bands with major bands at 70, 35, 25 and 15 kDa. The analysis has revealed that all the four crude venoms consist of a major protein band at 15 kDa, but with different intensities. Thus, SDS–PAGE analysis of the four venoms revealed that they differ in composition and expression level of venom proteins in the glands.

Gel filtration chromatography analysis

Crude venoms were subjected to gel filtration chromatography on a Shodex column pre-equilibrated with 20 mM Tris–Cl pH 7.4. Elution was carried out with the same buffer at a flow rate of 0.5 ml min^{-1} using a Waters HPLC system (Milford, MA) and monitored at 215 and 280 nm. All the four geographically isolated crude venoms had different elution profiles. The crude venom of RvWB has 10 peaks of which the 1st and the 5th peaks were found to be prominent. The crude venom of RvKE has only 6 protein peaks with the 1st and the 6th peak as the major peaks. RvKA has 13 peaks of which the 1st, 4th, 7th, 9th and the 10th peaks were found to be prominent. RvTN has 10 peaks of which peak 1st, 3rd and 5th are the prominent peaks. The gel filtration profile documents the variation in venom composition with respect to protein families as well as its expression level (Figure 2).

Reverse phase high-performance liquid chromatography analysis

To further understand the variation in the venom composition, 200 μg of each crude venom was loaded on a Jupiter C_{18} columns pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The proteins were eluted by a linear gradient of 80% (v/v) acetonitrile (AcCN) containing 0.1% (v/v) TFA at a flow rate of 0.8 ml min^{-1} over 180 min. The elution was monitored at 280 and 215 nm. The Rp-HPLC profiles of the four venoms were different (Figure 3).

PLA_2 activity

Phospholipase A_2s are hydrolytic enzymes ubiquitously present in all snake venoms that induce multiple pharmacological effects (Harris, 1985; Kini, 1997). This group of enzymes exhibit anticoagulant activity either through enzymatic or non-enzymatic mechanisms (Kini, 2006). PLA_2 activity of the four crude venoms of Russell's viper was measured using egg yolk as substrate by the method of Joubert & Taljaard (1980). The analysis revealed that 1 μg of RvWB has PLA_2 activity of ~58.18 ± 0.02 U while the same amount of RvKA, RvKE and RvTN, has only 18.7 ± 0.5, 7.12 ± 0.011 and 5.0 ± 0.18 U, respectively. Hence, venom from the Eastern region has the most hydrolytically active PLA_2 enzymes followed by venom from Karnataka, Kerala and Tamil Nadu.

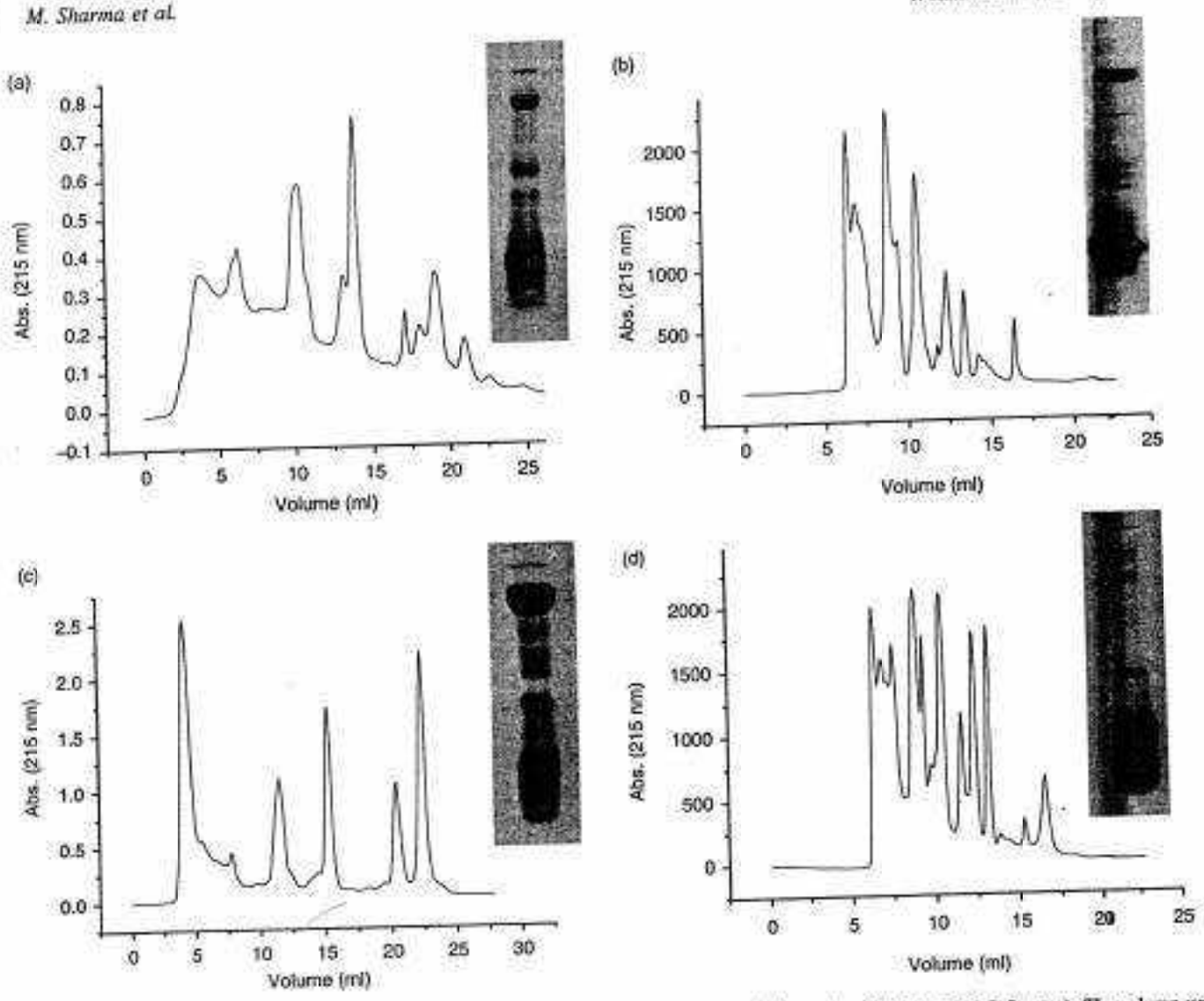

Figure 2. Gel filtration chromatography profile of crude venoms of Russell's viper on a shodex column (Waters HPLC System). The column was pre-equilibrated with 20 mM Tris–Cl pH 7.4 and 50 µg of sample was loaded. Elution was carried out at flow rate of 0.5 ml min^{-1} and monitored at 215 nm. (a) Elution profile of RvWB, inset 12.5% SDS–PAGE. (b) RvTN, (c) RvKE, (d) RvKA.

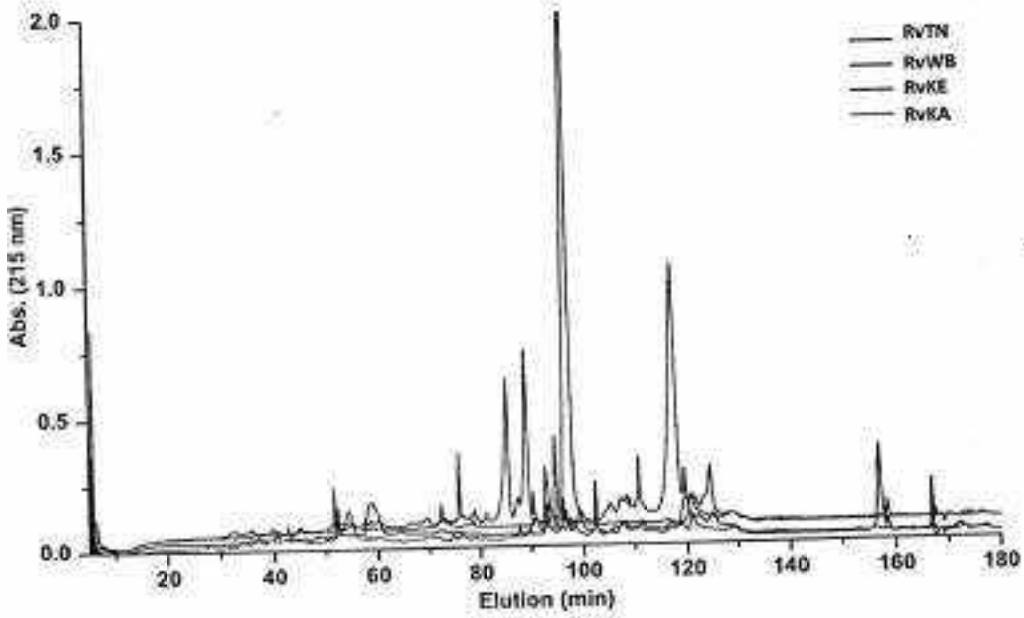

Figure 3. Comparative analysis of RvWB, RvTN, RvKE and RvKA on Rp-HPLC. Rp-HPLC was performed on a Jupiter C_{18} column (Phenomenex) pre-equilibrated with 0.1% (v/v) TFA. About 200 µg of crude venom was loaded on the column and eluted with a linear gradient of 80% (v/v) AcCN containing 0.1% (v/v) TFA over 180 min at a flow rate of 0.8 ml min^{-1}. The elution was monitored at 215 nm.

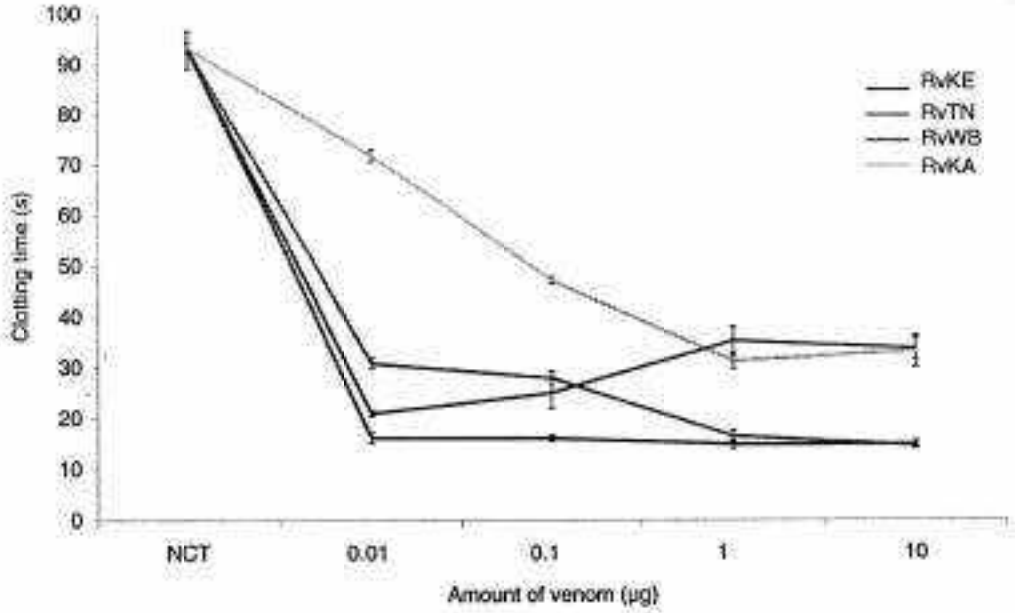

Figure 4. Comparative analysis of the recalcification time of RvTN, RvWB, RvKE and RvKA. About 150 µl of goat plasma was pre-incubated with various amount of crude venom in 50 µl of 20 mM Tris–Cl pH 7.4 at 37 °C. About 100 µl of 50 mM of $CaCl_2$ was added to initiate the clot formation and the time taken to form the clot was recorded on a Coastat-1 coagulation analyser (Tulip Diagnostics; Goa, India). About 20 mM Tris–Cl pH 7.4 instead of crude venom was used to determine the control clotting time. Experiments were repeated thrice and the means values were used to plot the graph.

Recalcification time test

The effect of crude Russell's viper venoms on recalcification time was analyzed using goat plasma in a Coagstat-1 (Tulip Diagnostics) coagulometer. All the crude venoms exhibited procoagulant activity compared to the control clotting time of 93 s. The venom from Tamil Nadu and Kerala reduced the clotting time to 21 and 16.23 s at 0.01 µg ml^{-1}. With increase in concentration there was no change in the clotting time of Kerala venom however, there was a slight increase in clotting time of Tamil Nadu venom when tested up to 10 µg. The venom from Karnataka and West Bengal showed procoagulant activity in a dose dependent manner and at 10 µg the clotting time was 33.5 and 14.7 s, respectively. The increase in clotting time of Tamil Nadu venom might be due to the presence of higher concentrations of anticoagulant components in its venom (Figure 4).

Neutralization by polyvalent antivenom

Indian polyvalent antivenom (Bharat serums and vaccines limited, B. No. A5310028) raised against viz: *Daboia russelli* (Russell's viper), *Naja naja* (Spectacled cobra), *Bungarus caeruleus* (Common krait), *Echis carinatus* (Saw-scaled viper) commonly referred to as "Big four" was used for the neutralization study.

Various concentrations of polyvalent antivenom were pre-incubated with venom at 37 °C for 1 h. The percentage neutralization of activity was calculated considering the activity of the crude venom in the absence of polyvalent antivenom as 100%. About 100 µg of the polyvalent antivenom could neutralize the PLA_2 activity of RvWB upto 94%, RvKE upto 67%, RvTN upto 55% and RvKA upto 24% (Figure 5). For complete neutralization of the PLA_2 activity of 1 µg of RvWB 250 µg of polyvalent antivenom is required whereas for RvTN and RvKE, 500 µg is required and for RvKA, 1000 µg of polyvalent antivenom is required. Similarly, to neutralize the procoagulant effect of the crude Russell's viper venoms higher amounts of polyvalent antivenom is required. About 100 µg of polyvalent antivenom could neutralize the procoagulant effect on goat plasma by ~76% (RvTN) and ~66% (RvKA), respectively. However, at the same concentration the antivenom could not neutralize the procoagulant effect of RvWB and RvKE (Figure 6). This shows the absence of specific antibodies for the epitopes of the RvWB and RvKE venom procoagulant proteins in the polyvalent antivenom. This in turn reflects the inefficacy of the available antivenom to combat the envenomation effects of Russell's viper in West Bengal or Kerala due to difference in geographical variation.

Immunodepletion of venom proteins

Antivenomics is a new generation proteomics approach to analyze the immunoreactivity of antivenoms with venom proteins both qualitatively and quantitatively (Calvete et al., 2009; Pla et al., 2012). To check the immunoreactivity of commercially available polyvalent antivenom, it was immobilized on CNBr-activated support (Sigma). The resin was washed several times to remove any unbound antibodies. Crude venom was passed through the column, the flow-through (non-retained fraction) was reloaded onto the column several times before loading on to a Jupiter C_{18} Rp-HPLC column pre-equilibrated with 0.1% (v/v) TFA. The fractions were eluted by a linear gradient of 80% (v/v) AcCN and 0.1% (v/v) TFA at a flow rate of 0.8 ml min^{-1}. The separation was monitored at 215 and 280 nm (Figure 7). The elution profile of the flow-through fraction was compared with the crude venom profile to identify the depleted, partially depleted and non-depleted venom proteins. The flow-through fraction of RvTN showed the presence of five peaks while one partially

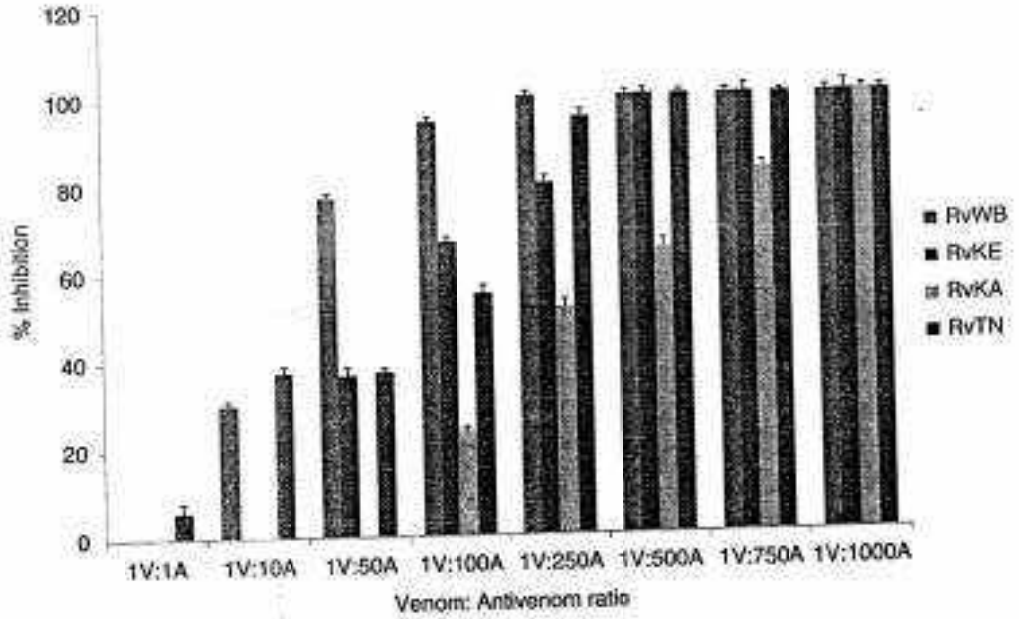

Figure 5. Neutralization of phospholipase A_2 activity of crude venoms of RvTN, RvWB, RvKE and RvKA by commercially available polyvalent antivenom. With different amount of antivenom, 1 µg of crude venoms of Russell's viper were incubated at 37 °C for 1 h. To this, 200 µl of egg yolk adjusted to 1 O.D. were added. The absorbance of the reaction was monitored at 740 nm for 10 min in a Thermo Scientific spectrophotometer. One unit of PLA_2 activity is defined as the amount of protein which produces a decrease in 0.01 absorbance units in 10 min at 740 nm. Experiments were repeated thrice and the means values were used to plot the graph.

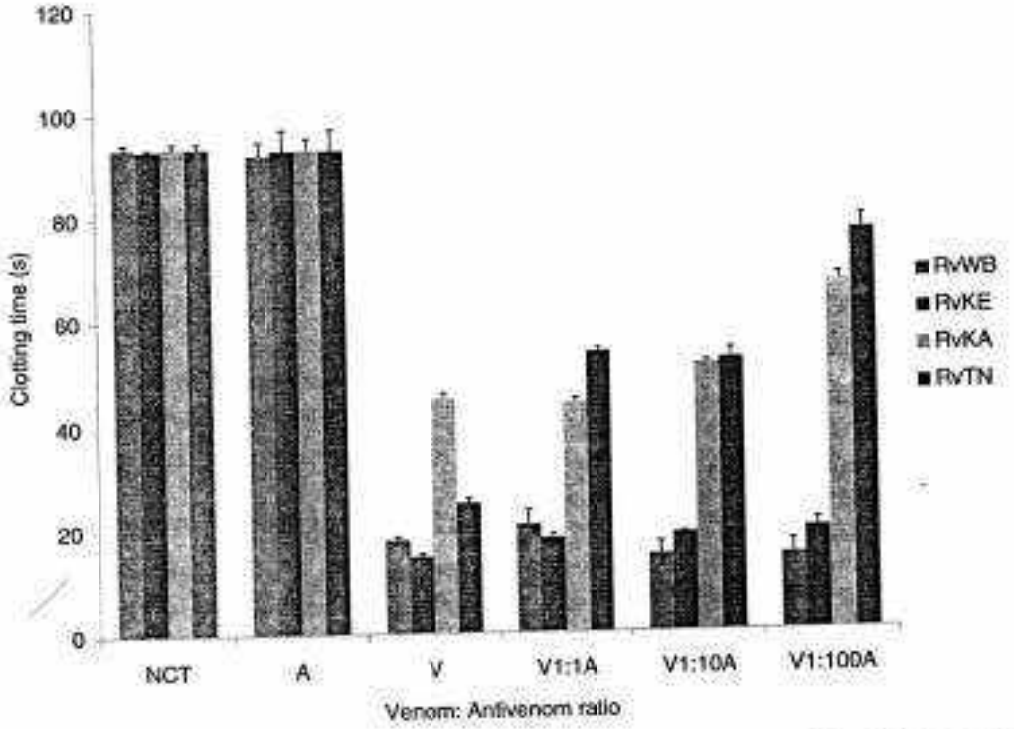

Figure 6. Neutralization of recalcification time of RvTN, RvWB, RvKE and RvKA by commercially available polyvalent antivenom. Various amount of polyvalent antivenom were incubated with 1 µg of crude venoms at 37 °C for 1 h. The pre-incubated mixture was added to 150 µl of goat plasma and incubated at 37 °C for 2 min. The clot formation was initiated by adding 100 µl of 50 mM of $CaCl_2$ and the time taken to form the clot was recorded on a Coastat-1 coagulation analyser. About 20 mM of Tris-Cl pH 7.4 without crude venom and antivenom was used to determine the control clotting time. Experiments were repeated thrice and the means values were used to plot the graph.

depleted peak was observed at 97.5 min. This protein exhibited anticoagulant activity and required higher amount of polyvalent antivenom to completely neutralize its activity when studied under *in vitro* conditions (data not shown). On the other hand, the elution profile of flow-through fraction of RvKA, RvWB and RvKE contained four different non-depleted protein peaks with no partially depleted proteins.

Discussion

Variation in the snake venom composition based on geographical location is well documented in the literature.

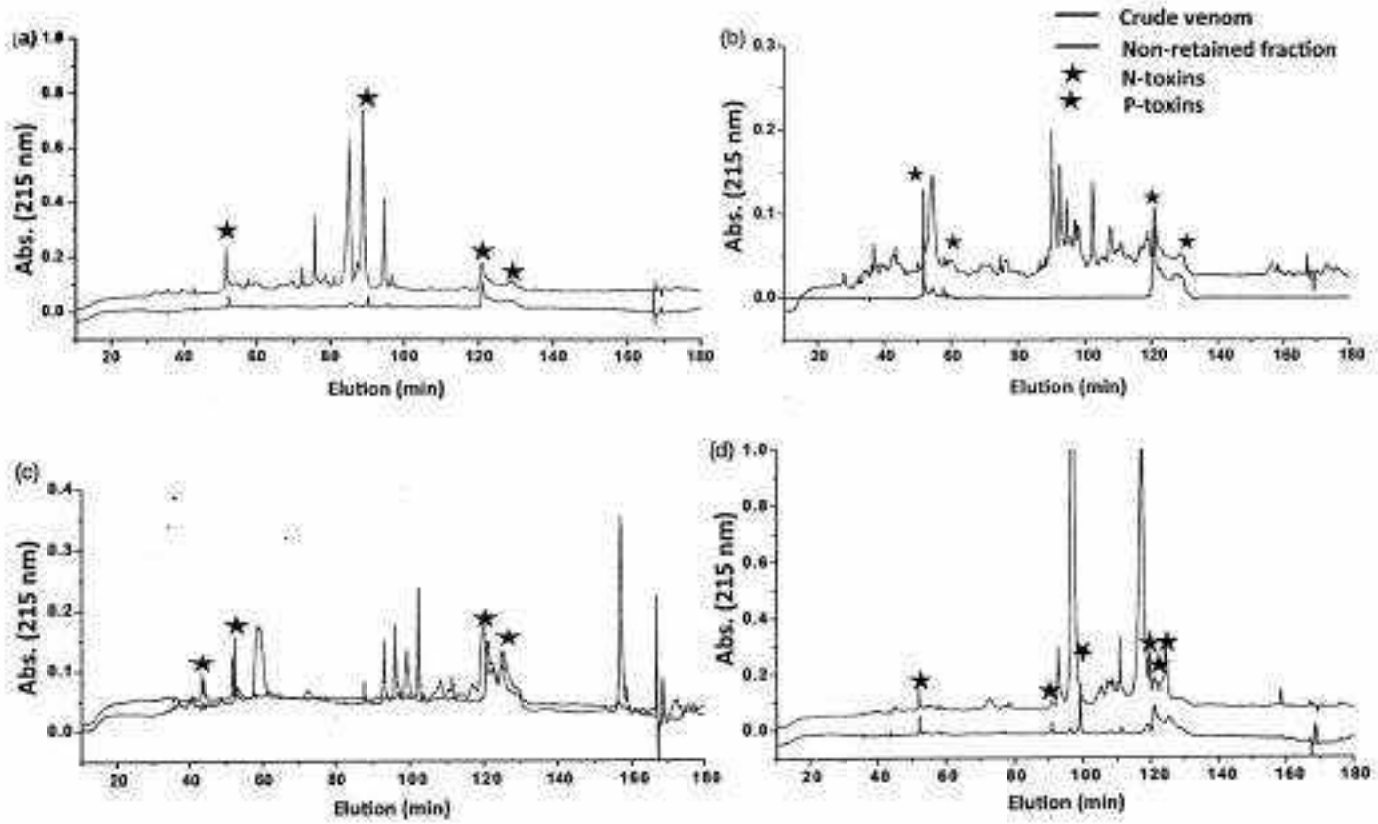

Figure 7. Rp-HPLC profile of non-retained fraction of crude Russell's viper venoms. (a) RvKA, (b) RvWB, (c) RvKE and (d) RvTN. Rp-HPLC was performed on a Jupiter C_{18} column (Phenomenex, Torrance, CA) pre-equilibrated with 0.1% (v/v) TFA. The non-retained fractions of crude venom was loaded on the column and eluted with a linear gradient of 80% (v/v) AcCN containing 0.1% (v/v) TFA over 180 min at a flow rate of 0.8 ml min^{-1}. The elution was monitored at 215 nm.

Indian Russell's viper venom from northern, southern, western and eastern region has been found to differ significantly (Prasad et al., 1999). Basic PLA_2 activity was found to be more prominent in venom of northern and southern regions while acidic proteins are abundant in eastern and western origin venoms. Proteolytic activity and trypsin inhibitory activity were more pronounced in the venom of western and northern regions compared to other region venoms. Moreover, eastern Russell's viper was the most lethal compared to the venoms of the other three regions (Jayanthi & Gowda, 1988; Prasad et al., 1999). Comparison of Russell's viper venom from Indian and Myanmar origin showed variation in the composition (Tsai et al., 2007). PLA_2 enzymes (two acidic and two basic) isolated from *Daboia siamensis* and *Daboia russelii* differs in toxicity and lethality on model animals (Tsai et al., 2007). This suggests variation in the biological activities of the similar proteins in venom of same species but geographically different origin. Moreover, envenomation by *Daboia russelii siamensis* from Taiwan exhibits both neurotoxic and haemotoxic effects on victims leading to systemic thrombosis and neuromuscular blocking (Hung et al., 2002a) whereas *Daboia russelii* envenomation in India shows symptoms of bleeding, renal failure, nausea, convulsions, hypotension, myonecrosis and edema with no neurotoxic effects (Mukherjee et al., 2000).

The SDS–PAGE, gel filtration and Rp-HPLC profile of the crude venoms from four different geographical locations of India showed significant variation in the venom composition. The differences in intensity of bands and peaks reveal that expression of venom proteins is different in all the venoms. Venoms from Kerala and Karnataka showed the presence of more low molecular mass proteins compared to Tamil Nadu and West Bengal venoms. Gel filtration elution profile of the venoms from Tamil Nadu, Kerala and Karnataka were similar whereas the venom from West Bengal was different. The former origins are geographically close and the snakes are likely to have similar diet which might be responsible for the similarity in venom protein composition to some extent. However, the Rp-HPLC elution patterns of the venoms showed different retention time of venom proteins. Two distinct proteins peaks were observed in venom of Tamil Nadu, which is absent in all other venoms demonstrating the variation in venom composition though they belong to the same species. This difference in the venom composition would contribute to differences in clinical symptoms during envenomation which would require different strategies to neutralize. PLA_2 activity and recalcification time of the crude venoms further demonstrates that these geographically close snakes of the same species are different in venom composition and expression level. The recalcification time of all the venom analyzed in the present study showed procoagulant activity

however they differ in their mode of action. The venoms of Tamil Nadu and Kerala showed the presence of fast acting procoagulant proteins whereas the other two venoms were slow to exhibit their activity.

Antivenom therapy is the only available therapy for the treatment of snakebite patients. However on many occasions, it has been observed that polyvalent antivenom failed to neutralize the toxic effect of venom (Isbister et al., 2008; Phillips et al., 1988). During antivenom therapy, about 10–20 vials of polyvalent antivenom is administered (Saravu et al., 2012) which might lead to anaphylactic reactions in patients (Caron et al., 2009; Stone et al., 2013). This is due to the unwanted antibodies present in the commercially available Indian polyvalent antivenom as it contains only ~25% of the antibodies to a particular snake (Simpson & Norris, 2007). It has been observed that polyclonal antibodies raised against southern Russell's viper showed significant difference in diffusion and cross-reactivity pattern with other geographical regions. Moreover, these antibodies were incapable in providing any sort of protection against the lethality by venoms of other regions (Prasad et al., 1999). Further, it has been observed that antibodies raised against the same species of *Naja naja* were also ineffective in neutralizing the toxic effect of venom from other geographical locations (Shashidharamurthy & Kemparaju, 2007). This failure is most likely associated with venom variation due to the geographic location and also due to the presence of unique toxins. Hence, regiospecific antivenom needs to be raised for treatment of snakebite patients from a particular geographical location. In the present study the comparative analysis of Rp-HPLC profile of crude venom and non-retained fraction after immunodepletion demonstrated that the polyvalent antivenom is ineffective in complete depletion of the venom components. This is due to the variation in venom composition, as venom from a particular geographical location is used for the production of antivenom. Expression of venom proteins differs greatly among the species as well as within the same species, which might also be responsible for non-immunodepletion (Lomonte & Carmona, 1992; Rodrigues et al., 1998; Saad et al., 2012). Apart from this the non-immunogenicity might be also responsible for the presence of these proteins in the flow-through fraction. Interestingly, highly hydrophobic proteins in all the venoms were not depleted by the polyvalent antivenom. Thus, the variation and non-immunogenecity of venom proteins need to be considered while producing antivenoms.

Conclusion and future prospective

The availability of food differs with different geographical locations for a snake. In order to adapt itself for survival, it has to induce various patho-physiological symptoms in prey for immediate immobilization and subjugation. Evolutionarily venom protein families have undergone accelerated evolution followed by point mutation to incorporate wide array of toxins with diverse biological functions to target their prey (Chijiwa et al., 2000; Nakashima et al., 1995; Ogawa et al., 1996; Zupunski et al., 2003). This evolutionary mechanism has resulted in variation in the venom composition among the snake species and also within the same species. The effective neutralization of these pharmacological effects in victims would be successful only when the detailed composition of venom proteins is understood and specific antivenoms are raised. The present study documents the variation in venom composition within the same species of Russell's viper which are from different geographically locations.

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Declaration of interest

The authors declare no conflicts of interest

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RESEARCH ARTICLE

Biochemical and Biological Characterization of *Naja kaouthia* venom from North-East India and its Neutralization by Polyvalent antivenom

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ABSTRACT

This study describes biochemical and biological properties of *Naja kaouthia* (Indian monocled cobra) venom of North-East India. The LD_{50} of the crude venom was found to be 0.148mg/kg and neurotoxicitic symptoms like paralysis of lower limbs and heavy difficulty in breathing at sub-lethal dose in mice was observed. The venom exhibited PLA_2, indirect hemolytic and myotoxic activities but showed weak proteolytic and low direct hemolytic activities. It did not exhibit any hemorrhage when injected intradermally to mice. Anticoagulant activity was prominent when recalcification, prothrombin and activated partial thrombinplastin time were tested on platelet poor plasma. Rotem analysis of whole citrated blood in presence of venom showed delay in coagulation time and clot formation time. Fibrinogen of whole citrated blood was depleted by venom when analyzed in Sonoclot. Crude venom at 10µg and after 16hr of incubation was found to degrade α chain of fibrinogen. Neutralization study showed that Indian polyvalent antivenom could neutralize some of the biochemical and biological activities as well as its fibrinogenolytic activity.

KEYWORDS: *Naja kaouthia*, haemostasis, thromboelastometry, myotoxicity, polyvalent antivenom

INTRODUCTION

Snakebite envenoming is a neglected tropical disease (WHO), which requires immediate attention. It is estimated that globally 2.5 million people are bitten by snakes each year with ~85,000 deaths (Gutierrez et al, 2010); in India, approximately 35,000 to 40,000 people die of snakebites annually (Chippaux, 1998; Kasturiratne et al, 2008). According to recent National Mortality Survey data, the incidence of snakebite cases is likely to be more than 50,000 per year in India (Mohapatra et al, 2011). However, these data may be far from the truth as most of the incidences happen in rural areas and these deaths mostly remain unreported. In India the "Big Four", *Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus* are considered to be medically important snakes and are responsible for most of the deaths. Recently, it has been reported that hump-nosed pit viper (*Hypnale hypnale*) from Kerala, is capable of causing lethal envenomation (Joseph et al, 2007). Hence, in addition to the "Big Four", there might be other medically important snakes in specific geographical locations, which need attention. This is important for clinical diagnosis for treatment and for production of effective antivenoms. In India, polyvalent antivenom is raised against the "Big Four" venoms but these snakes may not be present throughout the country. Moreover, administration of this polyvalent antivenom has well documented limitations (Offerman et al, 2001; Lalloo and Theakston, 2003; Williams et al, 2007).

Naja kaouthia is recognized phenotypically with the presence of O-shaped or monocellate hood pattern. They are widely distributed in Nepal, North East India, Bangladesh, Myanmar, Thailand and Peninsular Malaysia (Whitaker, 1978; Viravan et al, 1992; Mukherjee and Maity, 2002).

According to WHO, it belongs to Category 1 of venomous snakes. The symptoms of cobra bite are general neurotoxicity leading to flaccid paralysis and death by respiratory failure, and also severe hypertension (Agarwal et al, 2006; Halesha et al, 2013). Symptoms of coagulopathy have also been reported in victims of *Naja kaouthia* of Asian origin (Khandelwal et al, 2007). The *Naja kaouthia* venom of North-East India origin has not been explored though venom of West Bengal (India) origin have been studied extensively (Mukherjee and Maity, 2002; Lalloo and Theakston, 2003; Mukherjee, 2007; Debnath et al, 2010; Sekhar and Chakrabarty, 2011). Hence, some work on biochemical and biological characterization of the *Naja kaouthia* venom and its *in vitro* neutralization by Indian polyvalent antivenom has been undertaken previously.

MATERIALS AND METHODS

Reagents and kits

$sPLA_2$ assay kit was procured from Cayman Chemical Company (MI, USA). NEOPLASTINE® CL PLUS and APTT reagent were obtained from STAGO (France). AGAPEE kit for CK/LDH analysis was purchased from AGAPPE diagnostics (Switzerland), Glass beads gbACT+ kit was obtained from Sienco, Inc. (USA). Polyvalent antivenom manufactured by Bharat Serums and Vaccines Limited (India) was purchased locally. Bovine plasma fibrinogen was obtained from Sigma-Aldrich and all other reagents used were of analytical grade and were either from Merck or Sigma-Aldrich, (USA).

Animals

Male Swiss albino mice of 40±3gm were obtained from central animal facility, University of Mysore. All animal were housed in well ventilated cages and experiments were carried out according to the Animal Ethical Committee Protocol (University of Mysore, Mysore, India, Proposal no. UOM/IAEC/25/2011).

Collection of snake venom, preparation and storage

Adult *Naja kaouthias* were captured from Jamugurihat, district Sonitpur, Assam, North-East India in the, month of May from its natural habitat and venom was extracted by allowing the snake to bite into a sterile beaker covered with para-film. The crude venom was immediately desiccated using dehydrated silica gel and stored in -20°C until further use. The permission for milking of snakes was obtained from Principal Chief Conservator of Forest (Wild Life) and Chief Wild Life Warden of Assam, India (WL/FG.27/tissue Collection/09 dated 07/10/2011).

Determination of protein content

Total protein content of *Naja kaouthia* venom was determined according to Lowry's method using BSA as standard (Lowry et al, 1951).

Phospholipase A_2 (PLA_2) activity

PLA_2 activity was assayed using $sPLA_2$ assay kit according to the manufacturer's protocol (Cayman Chemical Company, MI, USA). Briefly, in a 96-well microtitre plate, 10µl of venom (0.1mg/ml), 10µl DTNB (5, 50-dithio-bis-(2-nitrobenzoic acid)) and 5µl assay buffer were added. The reaction was initiated by adding 200µl of substrate solution (diheptanoyl Thio-PC). After gentle shaking, the optical density was measured every minute at 405nm using MultiSkan GO multi plate reader (Thermo Scientific, USA) for 10min. Assay buffer was used as blank and bee venom PLA_2 (0.01mg/ml) was used as a positive control. Tests were carried out in triplicate and mean values were taken. The activity was expressed as micromoles of diheptanoyl Thiol-PC hydrolyzed per min per mg of enzyme.

Caseinolytic assay

Digestion of casein was evaluated according to the method of Ouyang and Teng (Ouyang and Teng, 1976). Briefly, 1% (w/v) casein in 20mM Tris-Cl, pH 7.4, was incubated with various amounts of venom protein (1, 5, 10, 50 and 100µg) for 1hr at 37°C. Reaction was stopped by addition of ice cold 10% (v/v) TCA and centrifuged for 10min at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R). The digested protein in the supernatant was determined according to Lowry's method (Lowry et al, 1951). Tyrosine curve was used to determine the protease activity and one unit of protease activity is defined as *n* mole equivalent of tyrosine formed per min per ml.

LD_{50} determination

Toxicity of the venom was analyzed according to the method of Meier and Theakston (Meier and Theakston, 1986). Briefly, various amount of freshly dissolved venom (0.05 to 1mg/kg) in saline was injected intraperitoneally to eight male Swiss albino mice in a final volume of 150µl and the controls were injected with saline alone. The animals were carefully monitored for 24hr and their survival time was recorded and LD_{50} was determined.

Edema inducing activity

The procedure of Yamakawa et al, (Yamakawa et al, 1976) as modified by Vishwanath et al, (Vishwanath et al, 1988) was followed. Mice weighing 20–30gm were injected with varying amount of venom (2–15µg) in a total volume of 20µl saline into intra plantar surface of right hind foot pad. Respective left foot pad received 20µl of saline and served as vehicle. Control mice were injected with 20µl saline into intra plantar surface of both hind foot pads. After 45min the mice were anesthetized (barbitone, 30mg/kg, i.p.) before sacrifice and hind limbs were removed at the ankle joint and weighed individually. The increase in weight due to edema is expressed as the ratio of the weight of edematous limb to the weight of vehicle (saline injected) limb x100. The amount of venom required to cause an edema ratio of 120% (20% above the basal level) is defined as minimum edema dose (MED).

Hemorrhagic activity

Hemorrhagic activity was assayed as described by Kondo et al, (Kondo et al, 1960). Various amount of venom (2–15µg) in 30µl saline were injected intradermally into mice and control mice received saline instead of venom sample. After 3hr, mice were sacrificed using anesthesia (barbitone, 30mg/kg, i.p.). The dorsal surface of the skin was removed and the inner surface was observed for hemorrhagic lesions. *E. carinatus* venom was used as positive control. The minimum hemorrhagic dose (MHD) is defined

as the concentration of venom that induce a hemorrhagic spot of 1cm diameter from the spot of injection.

***In-vivo* myotoxicity**

For myotoxicity, release of serum creatine kinase (CK) and lactate dehydrogenase (LDH) in the blood were determined using AGAPPE kit (AGAPPE diagnostics, Switzerland). Group of six male albino mice were injected (i.m) with 15µg crude venom (40µl) and control received 40µl of saline. After 3hr, mice were anesthetized and 0.5ml of blood samples was drawn using cardiac puncture. The serum obtained by centrifugation was diluted with saline at 1:20 ratio. The CK and LDH activity were measured in 10µl of plasma according to the manufacturer's protocol and were expressed in Units/liter (U/l). The results are mean ±SD of three experiments.

Collection of Blood and Platelet Poor Plasma (PPP) preparation

Fresh goat blood was collected in citrated tube (0.11M tri sodium citrate) at 1:9 ratios (citrate: blood) from local butcher's shop. Human blood was collected from healthy donors (27Yr) who had not taken any medication for last 48hr. 9ml of blood was drawn with 20 gauge 3/4" needle and immediately transferred to a plastic tube containing 1ml of 0.11M tri sodium citrate (Suntravat et al, 2010). The tubes were centrifuged at 3000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 15mins to separate the red blood cells (RBC) and platelet poor plasma (PPP) and used within 4hr of collection.

Direct and indirect hemolytic activity

The RBC pellet obtained from the blood (as described above) was washed 4–5 times and re-suspended in 0.9% (w/v) saline to a final concentration of 10% (v/v). Various amount of venom were incubated for 60min at 37°C with 150µl of 10% RBC to a final volume of 2ml with 0.9% (v/v) NaCl. The tubes were centrifugation at 5000rpm (Thermo Scientific, USA, Heraeus Multifuge X1R) for 10min and the absorbance of the supernatant was measured at 540nm in a MultiSkanGO, UV-Vis spectrophotometer (Thermo Scientific, USA). The hemolysis caused by dH_2O was considered as 100%. For Indirect hemolytic, 20µl of egg yolk substrate solution was added to the reaction mixtures at the time of incubation and hemolysis was measured as described for direct hemolytic activity. The results are mean ±SD of three experiments.

Fibrinogenolytic activity

Fibrinogenolytic activity was assayed according to the method of Ouyang and Teng, using bovine fibrinogen (2mg/ml) dissolved in 50mM Tris HCl buffer, pH 7.5, 0.15M NaCl (Ouyang and Teng, 1976). To 300µl of dissolved fibrinogen, various amount of venom in 150µl of buffer was incubated for different time intervals at 37°C. The incubated mixtures were then run on a 12.5% (w/v) SDS-PAGE according to the method of Laemmeli (Laemmli, 1970). Staining was done with 0.25% (w/v) Coomassie brilliant blue R250 and destained till the protein bands were visible.

***In-vitro* coagulant assays**

Recalcification time

Recalcification time of human PPP was measured using coagulation analyzer (STAGO, France). Various amount of venom in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 3min and 50µl of 25mM $CaCl_2$ was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are as mean ±SD of three experiments.

Prothrombin time (PT) test

Prothrombin time was measured using PT reagent (NEOPLASTINE® CL PLUS) obtained from STAGO (France) according to the manufacturer's protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50µl of PBS was pre-incubated with 50µl of human PPP at 37°C for 1min and 100µl of PT reagent was added to initiate the clot formation. The clotting time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

Activated partial thrombin time (APTT) test

Activated partial thrombin time was determined using APTT reagent obtained from STAGO (France) according to the manufacturer's protocol on a coagulation analyzer (STAGO, France). Various amount of venom in 50µl PBS was incubated with 50µl of human PPP and 50µl of APTT reagent for 3min at 37°C. The clot formation was initiated by adding 50µl of 25mM $CaCl_2$. The clot formation time with PBS was considered as normal clotting time. The results are mean ±SD of three experiments.

Whole citrated blood analysis

Thromboelastometry analysis

To quantify the CT (clotting time, in seconds), CFT (clot formation time, in seconds) and MCF (maximum clot firmness, in mm) of the whole citrated blood, Rotem® Analyzer (ROTEM®Pentapharm GmbH Diagnostic Division; Munich, Germany) was used. For the analysis, blood samples from healthy volunteers were collected in 0.11M tri sodium citrate at 9:1 (blood: citrate) ratio. Various amount of venom in 20µl of PBS was mixed with 20µl of 200mM $CaCl_2$, to this reaction mixture, 320µl of whole citrated blood was added and clot formation was observed over 30min. Clot formation function with only PBS was considered as control. The results are mean ±SD of three experiments.

Sonoclot analysis

A glass bead activated test tube (gbACT+ Kit obtained from Sienco, Inc, USA) was used to monitor clot detection, clot rate and platelet function (clot retraction) in a Sonoclot Coagulation and Platelet Function Analyzer (Sienco, Inc, USA). Various amount of venom in 20µl of PBS was added to 320µl of citrated human blood followed by 20µl 200mM $CaCl_2$. The head assembly of the analyzer was closed 10s after the start button was pressed. Data were acquired and analyzed with Signature Viewer software (Sienco, Inc.). The results are mean ±SD of three experiments.

Neutralization studies

For neutralization studies, various amount of polyvalent antivenom was pre-incubated with 1µg of *Naja kaouthia* venom in a final volume of 20µl for 1hr at 37°C and assays were performed as described above. The percentage inhibition was calculated by considering the activity in absence of polyvalent antivenom as 100%. The results are mean ±SD of three experiments.

RESULTS

Biological characterization

The biochemical and biological activities of the crude venom are listed in Table 1. The median lethal dose (LD_{50}) was found to be 0.148mg/kg when injected intraperitoneally to experimental mice. When sub-lethal dose of venom was injected to mice, neurotoxic symptoms like difficulty in movement; breathing and frequent drinking of water were observed followed by death after 40min. The amount of CK and LDH released after injection of 15µg of venom was found to be 6.605U/l and 26.38U/l respectively in the plasma. The CK was 10 times more than observed for the control mice (0.63U/l), however, the LDH was found to be only 3U more. The minimum edema dose (MED) of the venom was found to be 11.25µg. No direct hemolytic activity was observed up to 10µg of venom but when the amount was increased up to 100µg, it exhibited 1.4% RBC hemolysis. For indirect hemolytic activity, 23% hemolysis was observed for 1µg of venom. The venom showed weak proteolytic activity when tested on casein. The amount of tyrosine liberated was 0.14±0.02 moles by 100µg of venom in 1min. PLA_2 activity of the venom was 7.584µmol/min/mg when assayed using $sPLA_2$ assay kit. However no haemorraghic spot was observed when 3µg of venom was injected intradermally (Figure 1).

In-vitro coagulation activities

The venom showed anticoagulant activity in dose dependent manner. When recalcification time of human plasma was tested with 1µg venom, the plasma did not form clot up to 500s whereas the normal clotting time was 126.5sec (Figure 2). Prothrombin time increased dose dependently and at 0.1µg, clot formation was not observed up to 500sec. The APTT test on plasma did not increase significantly up to 0.1µg but when the amount was increased to 1µg venom the plasma did not form clot (Figure 2).

In Rotem® Analyzer, coagulation time (CT) for 0.1µg venom was 634±15sec and for the control it was observed to be 503±10sec. When the amount of the venom was increased to 1 and 10µg, clot formation was not observed which is depicted by a straight line (Data not shown). The clot formation time (CFT) in presence of 0.1µg of venom was recorded to be 266±10sec, whereas the CFT for control plasma was only 87±3s (Table 2). Maximum clot firmness (MCF) value at 0.1µg of venom was 61±1.3mm, whereas for the control the value was 65±2mm. However, at higher concentration of venom the blood clot did not form. Hence, the values were not measurable in the Rotem® analyzer (Table 2).

The activated clotting time (ACT) increases dose dependently and at 10µg of venom it was recorded to be 591±1.3sec in Sonoclot Coagulation and Platelet Function Analyzer. At 0.1µg venom the clot rate was similar to normal clot rate (normal range 9–35sec) but with increase in concentration, the clot rate decreased which might be due to depletion of fibrinogen. However, up to 1.0µg the platelet function was found to be normal but at 10µg the platelet function was not observed (Table 2). Lower amount of venom did not show any digestion of fibrinogen (data not shown). However, when the amount of venom was increased to 10µg, clear digestion of α chain of fibrinogen was observed after 16hr of incubation (Figure 3A).

Neutralization studies

Effect of polyvalent antivenom on some of the biochemical and biological properties of *Naja kaouthia* venom are shown in Table 3. At 1:1 ratio, the polyvalent antivenom could

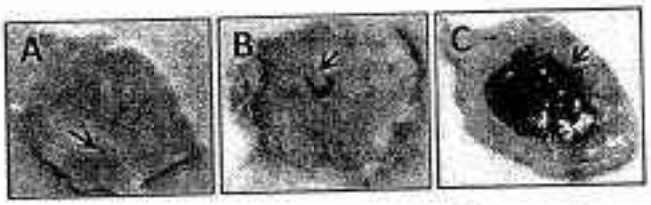

Figure 1. Haemorraghic activity of *Naja kaouthia* venom. **A.** Control (30µl of saline), **B.** *Naja kaouthia* venom (15µg), **C.** Saw scaled viper venom (3µg) (Positive control), the arrow indicates site of injection.

Table 1. Some biochemical and biological activities of *Naja kaouthia* venom

Parameters	Activity
LD_{50}	0.148 mg/kg
PLA_2 activity assay	7.9±0.24Ψ
Direct hemolytic assay (100µg venom)	1.4±0.51%
Indirect hemolytic assay (1µg venom)	23.0 ±3%
Caseinolytic activity (100µg venom)	0.14±0.02*
Creatine kinase (CK) (15µg i.m. injection)	6.6±0.2 U/l
Lactate dehydrogenase (LDH) (15µg i.m. injection)	26.3±2.3U/l
Minimum edema dose (MED)	11.2±0.18 µg
Haemorraghic activity (up to 15µg)	NA

*Normal CK and LDH values are 0.63 U/l and 23.39 U/l respectively; Ψµmol of diheptanoyl Thiol-PC hydrolyzed/min/mg; *n moles of tyrosine formed/min; NA= No Activity. Results are mean ±SD (n=3)

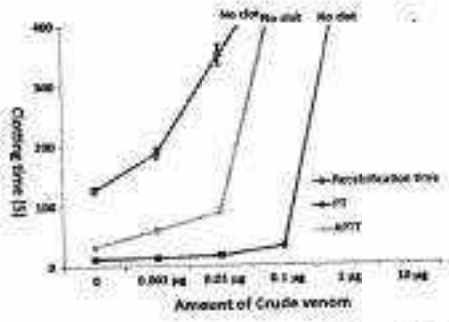

Figure 2. Dose dependent anticoagulant activity of *Naja kaouthia* venom on human plasma. Effect of crude venom on Recalcification time, Prothrombin Time test (PT) and Activated Partial Thrombin Time test (APTT). The results are mean ±SD of three experiments.

Table 2. Anticoagulant activity of *Naja kaouthia* venom on whole citrated blood. Results are expressed as mean ±SD of three experiments.

Parameters	PBS	Crude venom (µg/ml)		
		0.1	1.0	10
Thromboelastometry analysis				
Coagulation time (CT) (s)	503±10	634±15	>1200	>1200
Clot formation time (CFT) (s)	87±3	266±10	NCF	NCF
Maximum clot firmness (MCF) (mm)	65±2	61±1.3	NCF	NCF
Sonoclot analysis				
Activated clotting time (ACT) (s) (range: 128–213)	176±5.2	215±7.4	243±6.3	591±10
Clot rate (CR)(range: 9.0–35)	23±0.5	23±0.32	16±0.21	1.2±0.2
Platelet function (range: 3–5)	2.8±0.01	3.8±0.02	3.3±0.01	0

*NCF: No clot formation, the results are expressed as mean ± SD

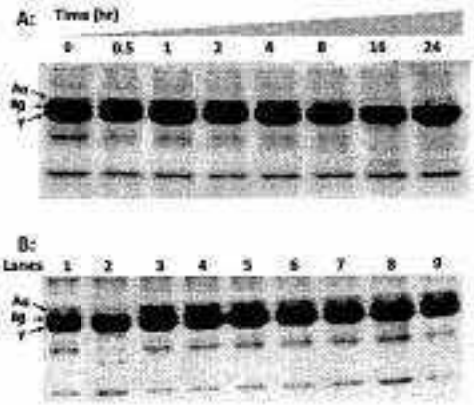

Figure 3. A. Fibrinogenolytic activity of *Naja kaouthia* venom. SDS-PAGE of bovine fibrinogen (reduced) after incubation with 10µg crude *Naja kaouthia* venom at various time intervals. **B.** Inhibition of fibrinogenolytic activity of *Naja kaouthia* by polyvalent antivenom. The venom:polyvalent antivenom (1:1, w/w) mixture was pre-incubated for 1hr at 37°C. This mixture was incubated with 300µl of fibrinogen (2mg/ml) for 24hr and aliquots were withdrawn at different time interval and fractionated in 12.5% (w/v) SDS-PAGE. **Lane 1.** Undigested fibrinogen (control). **Lane 2.** Fibrinogen incubated with only venom; **Lane 3.** After 0.5hr; **Lane 4.** 1hr; **Lane 5.** After 2hr; **Lane 6.** After 4hr; **Lane 7.** After 8hr; **Lane 8.** After 16hr; and **Lane 9.** After 24hr.

not neutralize the PLA_2 activity of the venom but at 1:100 ratios, 97.38 ± 4.8% inhibition was observed. Inhibition of the indirect hemolytic activity of venom was also observed similar to the PLA_2 activity. When the concentration of the polyvalent antivenom was increased by 100 times, indirect hemolytic activity was completely neutralized. Recalcification time of the venom was neutralized up to 49.34% at 1:1 ratio and with 10 times increase in polyvalent antivenom, 92.03% neutralization was observed. Similarly, the APTT and PT was also brought to the normal clotting time when the polyvalent antivenom was 10 times excess of the venom concentration. Moreover, degradation of α chain of fibrinogen by venom was inhibited by polyvalent antivenom at 1:1 ratio (Figure 3B).

DISCUSSION

The patho-physiological effect post-snakebite envenomation varies greatly among the various species and even within species due to variation in the venom proteins and biological activities (Glenn et al, 1983; Minton and Weinstein, 1986; Daltry et al, 1996; Saravia et al, 2002; Menezes et al, 2006). These variations affect the clinical manifestation of envenomation and require specific consideration for treatment. Hence understanding the biochemical and biological properties of snake venom from a particular geographic location is important.

The LD_{50} of the *Naja kaouthia* venom was found to be 0.148mg/kg, whereas those for cobra venoms of Thailand and Kolkata origin were reported to be 0.23mg/kg and 0.7mg/kg, respectively (Mukherjee and Maity, 2002; Leong et al, 2012). Though the route of injection was different (Kolkata origin venom given via tail vein injection) in these experiments, the lethal dose of North East origin venom was less than that of the other geographical locations suggesting it might be more lethal. However, the comparative study with indistinguishable experimental conditions would be necessary to differentiate these venoms. In mice the venom did not induce haemorraghic activity and venom of Kolkata origin is reported to be devoid of such activities. The haemorraghic is mainly caused by metalloproteases, which are abundantly found in viper venom (Kamiguti et al, 1996; Chakrabarty et al, 2000; Mukherjee, 2008). Moreover, the edema inducing activity was not found to be significant. Hence this venom might not induce inflammation and tissue damage at the site of bite. Interestingly, the venom at 100µg showed only 1.4% hemolysis of RBC, whereas at the same amount Kolkata venom activity is reported to be 39.0% (Mukherjee and Maity, 2002). The membrane damaging activity is mainly contributed by the low molecular weight proteins which might be absent in this venom. The indirect hemolytic activity of the venom in presence of the egg yolk is due to PLA_2 enzymes. The lysophospholipids and free fatty acids formed during the catalysis of phospholipids by PLA_2 enzyme exhibits this activity as they are lytic in nature (Condrea et al, 1964). The presence of various PLA_2 isoenzymes and neurotoxins in *Naja kaouthia* venom have been

Table 3. *In vitro* neutralization of whole venom activity by polyvalent antivenom

Activity	% inhibition by polyvalent antivenom		
	1:1	1:10	1:100
PLA_2 activity	0	40.0±5.0	97.38 ± 4.8
Indirect hemolytic	11.96±2.12	68.15±0.15	100
Recalcification time	49.34±5.01	92.03±3.0	96.52±2.81
PT	36.44±5.8	78.19±3.86	99±1.76
APTT	32.33±6.44	92.1±5.83	100
Fibrinogenolytic	α chain present	α chain present	α chain present

*The results are expressed as mean ± SD (n=3)
Values indicate % inhibition at each venom:antivenom (µg:µg) ratio

reported by various workers (Joubert and Taljaard, 1980; Meng et al, 2002; Qiumin et al, 2002; Doley et al, 2004). When the crude venom was analyzed for the PLA_2 activity using diheptanoyl Thiol-PC as substrate, the amount of substrate hydrolyzed product was 7.9±0.24µmol/min/mg suggesting the presence of enzymatically active PLA_2 in the venom. PLA_2 is one of the major constituent in the elapid venom, which confers multiple toxicity to the prey or victim such as membrane damaging, neurotoxicity, edema and prolongation of coagulation time (Kini and Evans, 1989; Doley et al, 2004). Hence the myotoxicity, neurotoxicity and edema induced by this venom are due to the presence of large amount of PLA_2 enzyme in the venom. The observed differences in the biochemical and biological activities in the venoms of Indian origin might be due to variation in the venom composition and content due to difference in geographical locations. Both venoms were collected during summers; however, in the present study, the ages of the snakes were unknown as they were captured from the wild. Detailed analysis of *Naja kaouthia* venoms from different locations of India need to be carried out to decipher the differences in the venom composition as well as the presence of unique toxins.

Snake venom proteins affect the haemostasis process of victim/prey either by prolonging or shortening the clotting time. Elapid venoms are anticoagulant in nature due to the presence of large amount of strong and weak anticoagulant PLA_2 enzymes. Moreover, non-enzymatic protein from elapid venom like Cardiotoxins from *Naja nigricollis crawshawii* and Hemextin A and hemextin AB complex from *Hemachatus haemachatus* venom are also reported to be anticoagulant in nature (Kini et al, 1988; Banerjee et al, 2005). The venom significantly delayed the recalcificaion time, PT and APTT of plasma under *in vitro* condition, which is due to strong anticoagulant proteins present in the venom. The plasma did not form clot at 0.01, 0.1 and 1µg concentration of venom when tested for recalcificaion time, PT and APTT, respectively. This suggests that the anticoagulant activity of the venom is most likely to affect all the pathways. Venom PLA_2 enzymes inhibit activation of FX to FXa which leads to disruption in the formation of prothrombinase complex, which is required for blood coagulation (Stefansson et al, 1990; Kerns et al, 1999; Kini, 2005). The higher amount of venom required in case of PT and APTT for non-coagulation of blood might be due to the addition of extra phospholipids during these tests; however, this needs to be verified. The venom proteins, especially the PLA_2 enzymes, hydrolyze the phospholipids which are required for the prothrombinase complex formation. The Sonoclot and Rotem analysis also demonstrated that the *Naja kaouthia* venom is anticoagulant in nature. The whole citrated blood analysis by sonoclot clearly indicated the depletion of fibrinogen in the reaction when pre-incubated with venom. The lower value of MCF by Rotem analysis indicates decreased platelet number or function, decreased fibrinogen level or fibrin polymerization disorders, or low activity of factor XIII. Recently, Nk a metalloprotease, which cleaves the α- chain, as well as a low molecular protein with fibrin(ogen)olytic activity have been reported (Wijeyewickrema et al, 2007; Debnath et al, 2010). The weak proteolytic activity towards casein and higher amount of venom and time required for complete degradation of α chain of bovine serum fibrinogen might be due to presence of these proteins in lower amount. Hence anticoagulant activity of *Naja kaouthia* might not be only due to degradation of phospholipids or α chain of fibrinogen but action of different venom proteins which might be acting enzymatically or non-enzymatically on coagulation factors and complexes.

Polyvalent antivenom is currently used by the medical practitioners for the treatment of snakebite patients in India. The Indian polyvalent antivenom is prepared using the venoms of four major poisonous snake species viz: *Naja naja, Daboia russelii, Echis carinatus* and *Bungarus caeruleus*. In most of the cases, it has been observed that the efficacy is highly reduced when antivenoms raised against venom from a particular geographic region is used to treat victims from another region (Shashidharamurthy et al, 2002; Shashidharamurthy and Kemparaju, 2007). The polyvalent antivenom could neutralize some of the biochemical and biological activity partially at 1:10 ratio (venom: polyvalent antivenom) and complete neutralization was observed when the dose of the polyvalent antivenom was increased to 10 fold. The partial inhibition might be due to the antibodies of *Naja naja* proteins present in the polyvalent antivenom, which recognizes the *Naja kaouthia* venom proteins. Present study documents that the polyvalent antivenom can neutralize some of tested biochemical and biological activities of *Naja kaouthia* venom under *in vitro* condition.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

CFT; clot formation time
CT; Coagulation time
MCF; Maximum clot firmness
ACT; Activated clotting time

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Unveiling the complexities of *Daboia russelii* venom, a medically important snake of India, by tandem mass spectrometry

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ABSTRACT

Composition of Indian Russell's viper (*Daboia russelii russelii*) venom, a medically important snake and member of 'Big' four snakes of India was done by gel filtration chromatography followed by tandem mass spectrometry. The MS/MS analyses of tryptic digested gel filtration peaks divulged the presence of 63 different proteins belonging to 12 families. Phospholipase A_2 (PLA_2), serine proteases, metalloproteases, cysteine-rich secretory proteins, L-amino acid oxidase, C-type lectin-like proteins, kunitz-type serine protease inhibitor, disintegrin, nucleotidase, phosphodiesterase, vascular endothelial growth factor and vascular nerve growth factor families were identified. PLA_2 enzymes with isoforms of N-, S- and H-type based on their first N-terminal amino acid residue were observed. The venom is also found to be rich in RVV-X, RVV-V and thrombin-like enzymes. Homologues of disintegrins with RGD and RTS motifs were also observed. The high percentage of PLA_2 and proteases in the venom proteome could be responsible for the observed coagulopathy, haemorrhage and edema which can be correlated with the clinical manifestations of Russell's viper envenomation. This is the first proteomic analysis of Indian *D. russelii* venom which might assist in understanding the pathophysiological effects of viper envenomation. Such study will also be important for developing more effective antivenom for viper bite management.

1. Introduction

The incidences of snake envenomation in tropical countries, such as India, are among the most neglected health issues leading to thousands of mortality and morbidity cases every year. The management of the snakebites and awareness programs initiated by the government and the public health sector in India and elsewhere are insufficient and ineffective (Gutiérrez et al., 2010; Warrell, 2011; Warrell et al., 2013; Bawaskar, 2014). It has been estimated that every year around 35,000 to 50,000 or more people die in India due to snakebites, majority of which are inflicted by cobras, kraits, saw scaled viper and Russell's viper (Warrell, 1999).

Russell's viper (*Daboia russelii russelii*) is widespread in Southeast Asia including India, Pakistan, Bangladesh, Sri Lanka, Myanmar, Thailand, Taiwan and Indonesia (Warrell, 1989). It is one of the most important venomous snakes of India which causes significant number of mortality and morbidity (Warrell, 1989). Pathophysiological manifestations of Russell's viper envenomation include coagulopathy, pain, swelling, myonecrosis, renal failure and neurotoxicity (Simpson and Norris, 2007). In India, polyvalent antivenom is raised against the "Big four" snake venoms (namely *D. russelii*, *Naja naja*, *Echis carinatus* and *Bungarus caeruleus*) which is the only available treatment for viper envenomation. However, at times, administration of antivenom is accompanied by some anaphylactic reactions like nausea, vomiting, hypotension, respiratory discomfort and low body temperature (Singh et al., 2001; Deshpande et al., 2013). This could be due to the presence of large repertoire of non-specific antibodies (small portion of polyvalent antibodies are against specific snake venom). Alternatively, due to non-immunogenicity of venom toxins, some toxins might not be neutralized by antivenom (Pla et al., 2012; Gutiérrez et al., 2013; Calvete et al., 2014). This is further complicated by intraspecies variations in the venom composition that are mostly attributed to differences in age, gender, seasonal changes and

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geographical location (mostly due to prey availability) (Minton and Weinstein, 1986; Williams et al., 1988; Jayanthi and Gowda, 1988; Chippaux et al., 1991; Daltry et al., 1996a, 1996b; Menezes et al., 2006; Alape-Girón et al., 2008; Sharma et al., 2014). Such variations attribute to inefficacy of antivenom produced from a particular geographical region against the venom toxins of other locations. Our recent antivenomics studies have shown that Indian Russell's viper venom from different geographical locations consists of distinct non-immunodepleted and partially immunodepleted proteins (Sharma et al., 2014). These factors together make the post-administration task tedious for the clinicians (Theakston et al., 1990; Singh et al., 2001; Deshpande et al., 2013). Hence, comprehensive understanding of the variations in venom composition and identification of the non/partially immunodepeleted proteins present in the venom becomes important. Although variations in the venom composition of Russell's viper from different geographical regions of India has been reported (Jayanthi and Gowda, 1988; Prasad et al., 1999; Sharma et al., 2014), detailed proteomics or transcriptomics analyses have not been carried out. So far, the research is mainly focused on isolation and characterization of individual toxins, such as phospholipase A_2 (PLA_2) (Bhat et al., 1991; Gowda et al., 1994; Prasad et al., 1996; Chandra et al., 2000; Chakraborty et al., 2002; Saikia et al., 2013), L-amino acid oxidase (LAAO) (Mandal and Bhattacharyya, 2008; Chen et al., 2012), snake venom metalloproteases (SVMP) (Kole et al., 2000; Chakraborty et al., 2002; Mukherjee, 2008; Chen et al., 2008), snake venom serine protease (SVSP) (Bhattacharjee and Bhattacharyya, 2013), and phosphodiesterase (PDE) (Mitra and Bhattacharyya, 2014). As a result data regarding venom composition and details of toxin profile are still missing.

Tandem mass spectrometry is one of the most powerful techniques of the present era, widely used for analysing proteomes. Venom composition revealed by such techniques unveils the complexities of the venom proteome and assist in understanding the symptoms/signs of envenomation triggered by these toxins (Risch et al., 2009; Bernardes et al., 2013; Malih et al., 2014). Here we report the proteome profile of *D. r. russelii* venom from South India using biochemical and proteomic techniques. This study provides an overview of various toxins present in this crude venom.

2. Materials

2.1. Venom procurement and chemicals

The lyophilized crude venom of *D. r. russelii* was procured from Irula Snake Catchers Society, Tamil Nadu, India. Although the exact geographical location of individual snakes is not available, the snakes were from within a small region of Tamil Nadu State and venoms were pooled from a few individuals. Dithiothreitol (DTT) was obtained from Gold biotechnology (Olivette, MO, USA). Protease Max from Promega (Madison, WI, USA), trypsin, iodoacetamide (IAA) and ammonium bicarbonate from Sigma (St. Louis, MO, USA), acetic acid from VWR (Radnor, PA, USA), PageRuler™ prestained protein marker from Thermo Scientific (Waltham, MA, USA). All other chemicals used were of analytical grade and obtained from either Merck Millipore (Billerica, MA, USA) or Sigma (St. Louis, MO, USA).

2.2. Animals

Swiss albino mice of both sexes were obtained from central animal facility, University of Mysore. The animals were housed at 25 ± 3 °C on a 12 h light/dark cycle with access to food and water and were used for in-vivo studies. Experiments were performed as per the approved guidelines from Animal Ethical Committee Protocol (University of Mysore, Mysore, India, Proposal no.UOM/IAEC/25/2011).

3. Methods

3.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). 20 μg of Crude venom of *D. r. russelii* was treated with β-mercaptoethanol and loaded into the gel. The electrophoretic run was set at 15 mA until sample enters the stacking gel and 25 mA for the resolving gel. Gel was stained with 0.25% of Coomassie Brilliant Blue R-250 in methanol:water:acetic acid (40:50:10) for 3 h followed by destaining for 4 h using methanol:water:acetic acid (40:50:10).

3.2. Gel filtration chromatography of crude D. russelii venom

The crude venom of *Daboia r. russelii* (100 mg) was dissolved in 2.5 ml of 50 mM Tris–HCl (pH 7.4) and filtered through a 0.2 μm nylon syringe filter. The clear venom filtrate (500 μl) was loaded onto a Hiload 16/600 Superdex 75 pg gel filtration column pre-equilibrated with 50 mM Tris–HCl (pH 7.4). Proteins were eluted at a flow rate of 1 ml/min under isocratic condition with the same buffer using Äkta Purifier HPLC system (GE Healthcare, Uppsala, Sweden). Elution was monitored at 215 nm and 280 nm and 0.8 ml fractions were collected.

3.3. ESI-LC-MS/MS of gel filtration peaks

For ESI-LC-MS/MS analysis of gel filtration peaks, proteolysis of the fractions were carried out using trypsin with Protease Max surfactant (Promega) according to the manufacturer's instructions. Each gel filtration fraction (~50 μg) was dissolved in 50 μl of MilliQ water. To this 41.5 μl of 50 mM of ammonium bicarbonate, 2 μl of 1% Protease Max and 1 μl of 0.5 M DTT were added. The reaction mixtures were incubated at 56 °C for 20 min. Then 2.7 μl of 0.55 M IAA was added and incubated in dark for 15 min. Finally 1 μl of 1% Protease Max and 1.8 μl of Trypsin (1 μg/μl in 50 mM acetic acid) were added and the reaction mixtures were incubated at 37 °C for 3 h. To stop the reaction 0.5 μl of 100% trifluoroacetic acid (TFA) was added and incubated at room temperature for 5 min. The reaction mixtures were centrifuged at 12,000 rpm for 10 min.

The tryptic digests of individual samples were loaded onto Accela LCQ Fleet Ion Trap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) for MS/MS analysis. Each sample (~80 μl) was injected into a Hypersil Gold C_{18} column (50 × 2.1 mm, 1.9 μm, Thermo Scientific, Waltham, MA, USA) pre-equilibrated with 0.1% formic acid. Elution was carried out at a flow rate of 200 μl/min with a linear gradient of 100% acetonitrile (AcCN) in 0.1% formic acid. The gradient starts from 0% to 40% (in 38 min) and then from 40% to 80% (in 18 min) of AcCN. The eluent from liquid chromatography (LC) column was directly fed to the mass spectrometer. Ion polarity of the system was set to positive ionization mode. Spectra were obtained in MS/MS mode and MS/MS scan range was set from 500 to 2000 *m/z*. Oxidation of methionine residues and S-carbamidomethylation of cysteine residues were set as modification. The MS/MS spectra was analysed by the software Proteome Discoverer 3.1 using Sequest program. Based on sequence similarity, the peptide fragments were assigned to the proteins in the NCBI database. Analysis and identification of the proteins and peptides were validated by the parameters like Sequest protein score (−2–105), coverage (~8–86%) (calculated by the Sequest program) and the presence of at least one unique peptides. Peptides with lower score and coverage were manually validated by NCBI BLASTp search.

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3.4. Phospholipase A_2 (PLA_2) activity

PLA_2 activity of the crude venom was assayed using the $sPLA_2$ assay kit (Cayman, MI, USA) according to the manufacturer's protocol. Thio-ester bond at *Sn*-2 position of the substrate, diheptanoyl thio-phosphatidylcholine is cleaved by PLA_2 enzymes releasing free thiols that are measured by 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB). Different concentrations of crude venom ranging from 0.01 to 1.0 μg/ml were added to assay buffer containing DTNB. The reaction was initiated by adding 200 μl of 1.66 mM substrate solution. The optical density was measured after every minute at 414 nm for 10 min at room temperature (~25 °C) using a UV–Vis MultiSkan GO multi plate reader (Thermo Scientific, Waltham, MA, USA). The specific activity of the venom was expressed in micromoles of phosphatidylcholine hydrolysed per min per mg of enzyme. Bee venom PLA_2 enzyme was taken as the positive control for the assay.

3.5. Proteolytic activity

The proteolytic activity of crude venom was determined according to the method of Ouyang and Teng (1976) using casein as substrate. In brief, different concentrations of crude venom ranging from 0.01 to 10 μg/ml were incubated with 1% casein (substrate) for 90 min at 37 °C in a reaction volume of 1.02 ml. The reaction was stopped by the addition of 500 μl of 10% ice cold trichloroacetic acid (TCA). The mixture was centrifuged at 5000 rpm for 10 min to remove the undigested protein. The amount of digested proteins in the supernatant was quantified by Lowry's method.

3.6. Fibrinogenolytic activity

The fibrinogenolytic activity of the crude venom was assessed using bovine plasma fibrinogen (2 mg/ml) dissolved in 50 mM Tris-Cl, pH 7.5, 0.15 M NaCl (Ouyang and Teng, 1976). 300 μl of 2 mg/ml fibrinogen (Sigma, USA) and 1 μl of 2.2 ng/μl of crude venom were incubated for 24 h at 37 °C and analysed on SDS-PAGE. Thrombin (3 μl of 10 units/ml) was used as the positive control.

3.7. Preparation of platelet poor plasma

Fresh goat blood was collected in a sterilized tube containing 3.2% of tri-sodium citrate at a ratio of 1:9 (citrate:blood). The platelet poor plasma (PPP) was obtained after centrifugation of the whole blood at 3000 rpm for 20 min (Condrea et al., 1981). PPP was used for the following (described below) clotting assays. The clot formation was monitored using Tulip Coastat-1 coagulo analyser (Tulip, Alto Santa Cruz, Goa, India). Clotting time of plasma with Tris buffer (20 mM, pH 7.4) was considered as the control clotting time.

3.7.1. Recalcification time

Recalcification time of crude venom on PPP was measured as follows. Briefly, various concentrations of crude venom (0.01–66.67 μg/ml) were pre-incubated with 50 μl of PPP at 37 °C for 2 min. The clotting was initiated by the addition of 50 μl of 50 mM $CaCl_2$.

3.7.2. Prothrombin time (PT)

Prothrombin time of crude venom was evaluated using Uniplastin (PT reagent) (Tulip Diagnostics Pvt. Ltd., Alto Santa Cruz, Goa, India) according to the manufacturer's specifications. Briefly, various concentrations of crude venom (0.01–6.67 μg/ml) were pre-incubated with 50 μl of PPP at 37 °C for 2 min and 50 μl of PT reagent was added to initiate the clot formation.

3.7.3. Activated partial thromboplastin time (APTT)

APTT of crude venom was determined using Liquecelin (APTT reagent) (Tulip Diagnostics, India) according to the manufacturer's instructions. In brief, various concentrations of crude venom (0.01–6.67 μg/ml) were incubated with 50 μl of PPP and 50 μl of APTT reagent for 3 min at 37 °C. The clot formation was initiated by adding 50 μl of 25 mM $CaCl_2$.

3.8. Edema inducing activity

The edema inducing activity of crude venom was measured according to the protocol developed by Vishwanath et al. (1988). Briefly, 20 μl of 0.75 μg/μl of crude venom dissolved in phosphate buffer saline (PBS) was injected into the right foot pad of male albino mice weighing 20–30 g. Control leg of the mice received equal volume of PBS. After 1 h of injection, the mice were euthanized by injecting barbitone (30 mg/kg, i.p.). The feet of the sacrificed mice were removed from the ankle joints and weighed.

3.9. Haemorraghic activity

The haemorraghic activity of the crude venom was determined according to the method of Kondo and co-workers (KONDO et al. 1960). Briefly, three male albino mice were injected intradermally in the back with 30 μl of 0.5 μg/μl of crude venom dissolved in PBS. Control mice were injected with equal volume of PBS. As a positive control 30 μl of 0.1 μg/μl of saw scaled viper venom in PBS was injected into the mice. After 3 h mice were euthanized by injecting barbitone (30 mg/kg, i.p). The dorsal skin was removed and the inner surface was analysed for haemorrhagic damage.

4. Results and discussion

The cocktail of proteins and polypeptides in snake venom is responsible for the pathophysiological manifestation in prey/victims. Hence, elucidation of venom composition helps in deciphering its pharmacological profile. Such an understanding clarifies the complexity of the venom proteome and helps in the identification of major proteins as well as the trace protein components present in the venom. This in turn may provide strategies to design specific and effective antivenoms to combat clinical challenges of snake envenomation. Further, comparative analysis of the proteome profiles along with the experimental evaluation will help in correlating the probable mechanism of action of the venom proteins on various physiological systems of the prey or the victim. Such comprehensive information about the complete venom composition will supplement the existing knowledge of characterization of some of the toxins isolated from *D. russelii* venom of Indian origin.

In the present study, the complexity of *D. russelii* venom of Indian origin was analysed using biochemical and proteomic techniques. The abundance of high and low molecular weight proteins in the crude venom was confirmed by SDS-PAGE (Fig. 1). The prominent protein bands at 95, 55 and 14 kDa indicate the presence of LAAO, SVMP and PLA_2 enzymes in the venom. To evaluate the venom complexity, the crude venom was subjected to gel filtration chromatography which resolved it into 8 distinct protein peaks (Fig. 2). Individual peaks were subjected to tryptic digestion followed by ESI-LC-MS/MS. A thorough analysis of the peptide fragments provided a comprehensive overview of different proteins present in the crude venom (Fig. 2, Table 1). Based on the sequence homology search, 63 different proteins were identified which belong to 12 distinct snake venom protein families. The relative distribution of isoforms in each protein family, which describes the relative diversity of protein toxins, was determined considering the

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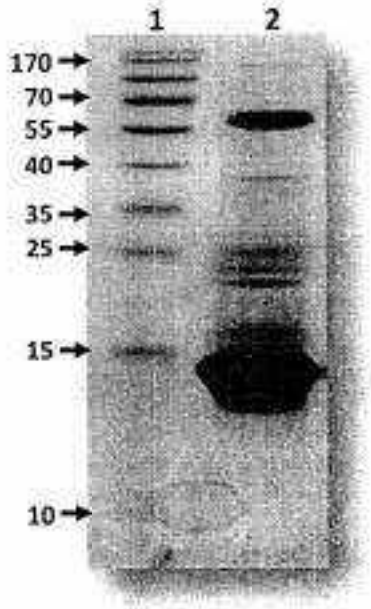

Fig. 1. Gel electrophoresis of crude *Daboia russelii* venom. Crude *Daboia russelii* venom (20 μg) reduced with β-mercaptoethanol and SDS-PAGE was run on a 12.5% gel. **Lane 1:** PageRuler™ prestained protein marker. MW of standard proteins is marked in kDa. **Lane 2:** Crude venom.

total number of proteins identified in the proteome by MS/MS as 100% (Fig. 3).

PLA_2 enzymes were found to be one of the major enzymatic families of this venom (Fig. 3). The members of this protein family are reported to induce wide range of pharmacological effects including myotoxicity, neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathic, haemorrhage, edema, tissue damage and convulsion (Kini, 2003). In viperid venom, PLA_2 enzymes have been reported to exist either as monomers or as complexes, such as vipoxin and viperotoxin F (for a review, see (Doley and Kini, 2009)). Vipoxin from the venom of *Vipera ammodytes meridionalis* is a heterodimeric complex with a PLA_2-like natural inhibitor (Chain A) and a highly toxic basic enzymatically-active PLA_2 (Chain B) (Mancheva et al., 1987). In this proteome, five peptide fragments including three unique peptides similar to chain A of vipoxin were observed (Accession 1408314, Table 1). One of the peptide fragments homologous to this acidic subunit with the substitution of His 48 to Gln 48 at the active site was also found. However, no peptides homologous to toxic basic PLA_2 (Chain B) was observed in the proteome. Similarly, RV-4 (enzymatically active) and RV-7 (non-toxic acidic subunit) are the two subunits of viperotoxin F, the major toxic component of *Vipera russelii formosensis* (Wang et al., 1992). Six peptide sequences similar to RV-7 subunit (Accession. No. 400714) from *D. russelii siamensis* were found in the venom, while the active form, RV-4 was absent. The absence of Chain B of vipoxin and RV-4 subunit of viperotoxin F indicates the absence of functional vipoxin and vipertoxin F in *Daboia russelii* venom from South India. However, both the subunits of these toxin complexes are reported in the venom of *D. russelii siamensis* from Myanmar (Risch et al., 2009). Similarly, daboiatoxin, a major PLA_2 toxin in *D. russelii siamensis* from Myanmar which shows myotoxic, neurotoxic and cytotoxic activity (Maung et al., 1995), was not identified in our venom sample. These differences might be due to the difference in the geographical origin of *D. russelii* and validate their classification as distinct subspecies. It would be interesting to analyse and validate whether the inactive forms of PLA_2 might interact with some other PLA_2 components of the venom from South India and impose distinct synergistic functional

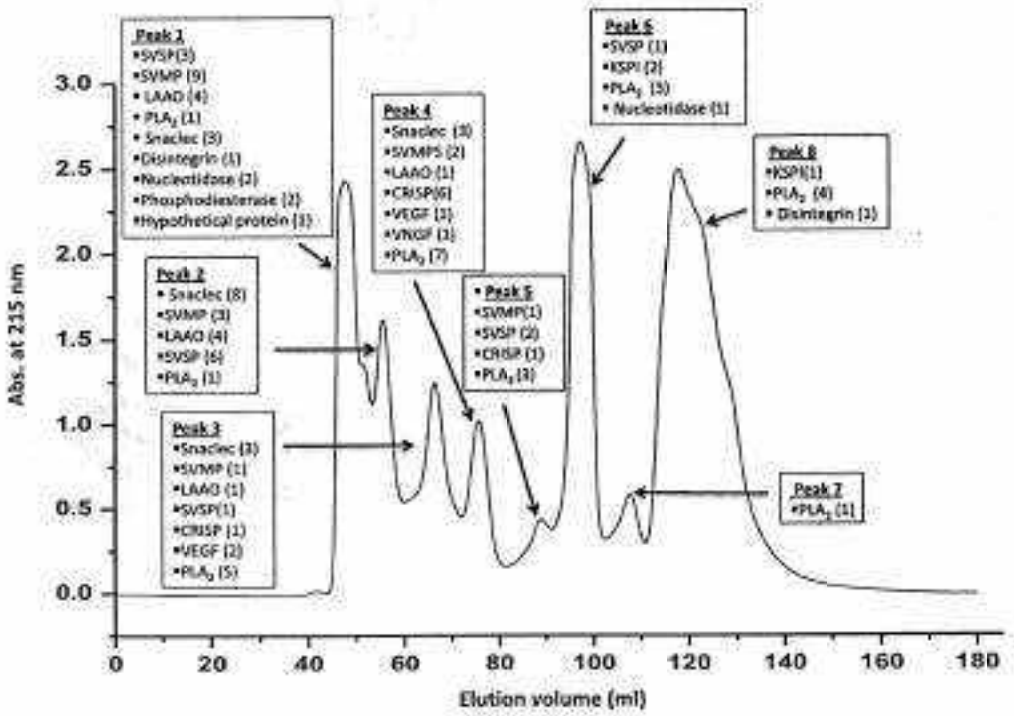

Fig. 2. Gel filtration profile of crude *Daboia russelii* venom on a Superdex 75 column. Crude venom (20 mg in 0.5 ml) was loaded into the Superdex 75 column pre-equilibrated with 50 mM Tris–HCl (pH 7.4). Proteins were eluted with the same buffer at a flow rate of 1 ml/min and monitored at 215 nm. Protein peaks were pooled and named as Peak1 to Peak10. **Insets:** Snake venom protein families identified by tandem mass spectrometry in the gel filtration peaks are shown.

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Table 1
List of various snake venom protein families as identified by ESI-LC MS/MS in the gel filtration fractions of crude *Daboia russelii* venom. The number of similar peptides and the unique peptides identified from different snake venom families are described in the Supplementary Fig. 1.

Sl. No.	MS/MS derived sequence	No. of peptides	Peptide ion m/z	MH+ [Da]	z	Coverage (%) & sequest score	Accession no.	Protein	Homology with protein from	Protein family
Gel filtration Peak 1										
1.	ARNEcDVPEHcTGQSAEcPR	2	792.19	2373.57	3	37.00 & 103.27	300079900	Factor X activator heavy chain	*Daboia russelii russelii*	SVMPs
	ASDLMTRKSHDNALLFTDMR	1	774.82	2321.47	3					
	cILYPPLR	1	1032.53	1031.53	1					
	KSHDNALLFTDMR	1	517.07	1548.22	3					
	LKPGAEcGNGLecYQcK	1	1008.63	2015.26	2					
	NEcDVPEHcTGQSAEcPR	1	1074.22	2146.45	2					
	NPCNmHYScmDQHKGMVDPGTKcEDGK	1	1057.98	3170.93	3					
	SVGIVQVQGNR	1	579.67	1157.35	2					
2.	ARDEcDVPEHcTGQSAEcPR	1	792.19	2373.57	3	49.41 & 97.67	162329887	Chain A, Crystal Structure of Russell's Viper Venom Metalloproteinase	*Daboia siamensis*	SVMPs
	DEcDVPEHcTGQSAEcPR	1	1074.58	2147.16	2					
	GYcYNGDcPIMR	1	1506.45	1505.45	1					
	KSHDNALLFTDMR	1	517.07	1548.22	3					
	LKPGAEcGNGLecYQcK	1	1008.63	2015.26	2					
	LVSTSAQFNK	1	548.58	1095.16	2					
	NPCNmHYScmDQHKGMVDPGTKcEDGK	1	1057.98	3170.93	3					
3.	KGSHLVSLHSR	1	611.46	1220.91	2	59.35 & 22.04	251205	Coagulation factor X activating enzyme light chain, RVV-X-light chain	*Daboia russelii*	SVMPs
	SMTcNFIAPVVcK	1	764.63	1527.27	2					
	VLDcPSGWLSYEQHcYK	1	1073.16	2142.32	2					
4.	DcQNPCCDAATCKLTPGAEcGNGLCCEKCK	2	1064.96	3191.89	3	25.98 & 15.15	73621852	Coagulation factor X-activating enzyme heavy chain	*Macrovipera lebetina*	SVMPs
	ENGRKIPcAPQDIK	1	813.92	1625.85	2					
	GSYYGYcR	1	513.99	1025.98	2					
	KNcIcNDSScIMSAVLSSQPSK	1	1244.35	2486.70	2					
	LFSNcSNHDYRR	1	1570.43	1569.43	1					
	QcISLFGSRATVAEDSCPQENQK	1	874.01	2619.04	3					
	TAVIMAHELGHNLGMYHDR	1	722.97	2165.90	3					
5.	EcMEHPNNGHLVSVESmEEAEPVAK	1	969.87	2906.62	3	33.54 & 10.99	300490458	Factor X activator light chain 2	*Daboia siamensis*	SVMPs
	TWEAAER	1	863.39	862.39	1					
	FcmEHPNNGHLVSVESmEEAEPVAK	1	975.75	2924.27	3					
	MWFNR	1	754.41	753.41	1					
	YLPVcKVPPEc	1	706.68	1411.37	2					
6.	IKDKEQIcSSEWSDGSSVSYDNLGK	1	950.67	2849.01	3	41.77 & 9.12	300079896	Factor X activator light chain 2	*Daboia russelii russelii*	SVMPs
	cEEPYPPVcK	1	665.26	1328.53	2					
	ESGYRMWFNHK	1	728.11	1454.22	2					
	mWFNHKCEEPYPPVcKVPPEC	1	886.62	2656.86	3					
7.	KcFGLEK	1	882.36	881.36	1	24.05 & 5.64	73621141	Coagulation factor X-activating enzyme light chain 2	*Macrovipera lebetina*	SVMPs
	GTGYRSWFNLNCEEPYPPVCKVPPNC	1	1007.77	3020.32	3					
	YFcYR	1	809.34	808.34	1					
8.	DEcDVTEHcTGQSAEcPR	1	1076.02	2150.04	2	22.83 & 9.68	83523646	Group III snake venom metalloproteinase	*Echis ocellatus*	SVMPs
	IPcAPQDVK	1	1028.36	1027.36	1					
	NQcISLPGSR	1	1182.43	1181.43	1					
	VFSSCSYDDYRMYLAK	2	975.07	1948.14	2					
9.	DLQTFcYPSIIQK	1	807.62	1613.24	2	54.56 & 98.63	395406796	L-amino-acid oxidase	*Daboia russelii russelii*	LAAO
	EGWYANLGPMR	1	648.09	1294.18	2					
	HIVIVGAGMSGLSAAYVLAGAGHK	1	1140.60	2279.20	2					
	IFFAGEYTANAHGWIDSTIK	1	1121.94	2241.89	2					
	KDLQTFcYPSIIQK	2	871.42	1740.85	2					
	LNEPVQETENGWYFIK	1	1009.67	2017.33	2					
	SAGQLYQESLGK	1	1281.45	1280.45	1					
	VTVTYQTTQK	1	585.54	1169.08	2					
10.	KFWEDDGIQGGK	1	691.10	1380.19	2	32.74 & 43.72	347602327	L-amino-acid oxidase	*Echis ocellatus*	LAAO
	RFDEIVGGMDQLPTSMYR	1	1056.70	2115.41	2					
	VYFAGEYTANAHGWIDSTIK	1	748.54	2242.63	3					

(continued on next page)

Table 1 (continued)

Sl. No.	MS/MS derived sequence	No. of peptides	Peptide ion m/z	MH+ [Da]	z	Coverage (%) & sequest score	Accession no.	Protein	Homology with protein from	Protein family
11.	EYLIK	1	666.37	665.37	1	29.31 & 28.94	538250837	L-amino acid oxidase	*Protobothrops flavoviridis*	LAAO
	HDDIFAYEK	1	1138.40	1137.40	1					
	SAGQLYEESLR	1	627.03	1252.06	2					
12.	HDDIFAYEK	1	1138.40	1137.40	1	22.49 & 27.10	10120762	Chain H, of L-amino acid oxidase	*Calloselasma rhodostoma*	LAAO
	LNEPSQENDNAWYFIK	1	673.78	2018.35	3					
	SAGQLYEESLGK	1	641.86	1281.72	2					
	RFDEVDGmDK	1	671.09	1340.19	2					
13.	cTERQAcCQDYEDTcVLPTQSWScSK	1	1072.02	3213.07	3	38.66 & 66.52	586829527	Phosphodiesterase	*Macrovipera lebetina*	Phosphodiesterase
	DPYTFDSEGIVR	1	725.31	1448.62	2					
	FGPVSGEIIMALQMADR	1	918.72	1835.44	2					
	GKNEVTSFENIEVYNLMcDLLK	1	873.30	2616.90	3					
	NPFYNPSPAK	1	568.78	1135.55	2					
14.	NLHNcVNLILLADHGMEEIScDR	2	908.92	2723.77	3	33.73 & 35.20	538250853	Phosphodiesterase	*Protobothrops flavoviridis*	Phosphodiesterase
	TFLPIPVNPVN	1	1261.53	1260.53	1					
	VNLMVDQQWMAVR	1	795.90	1589.80	2					
	VRDVELLTGLNFYSGLK	1	642.64	1924.92	3					
15.	STHIAPLSLPSSPPSVGSVcR	1	1126.31	2250.62	2	18.22 & 22.01	380875417	Serine protease VLSP-3	*Macrovipera lebetina*	SVSPs
	WDKDIMLIK	1	1162.40	1161.40	1					
	DIMLIK	1	733.41	732.41	1					
16.	FFcLSNK	1	916.36	915.36	1	20.70 & 26.99	311223824	Serine beta-fibrinogenase-like protein precursor	*Daboia siamensis*	SVSPs
	TSTYIAPLSLPSSPPR	1	844.95	1687.90	2					
	WDKDIMLIK	1	1162.40	1161.40	1					
17.	TLcAGILQGGIDTcK	1	1608.43	1607.43	1	10.12 & 22.05	380875421	Beta-fibrinogenase	*Macrovipera lebetina*	SVSPs
	DIMLIK	1	733.41	732.41	1					
18.	AWNEGTNcFVIK	1	737.54	1473.09	2	57.43 & 22.60	73620113	Snaclec 5	*Daboia siamensis*	Snaclec
	GSHLLSLHNIAEADFVLK	1	655.99	1964.99	3					
	NHWSHMDcSSTHNFVcK	1	716.78	2147.35	3					
	QDcLSDWSFYEGYcYK	1	1061.43	2120.85	2					
19.	TWFNLScGDDYPFVcK	1	670.70	2009.11	3	26.28 & 12.33	218526485	Snaclec A14	*Macrovipera lebetina*	Snaclec
	NcPGLEK	1	868.35	867.35	1					
	AFDEPKR	1	863.49	862.49	1					
	NVIER	1	631.40	630.40	1					
	SGDAEK	1	607.20	606.20	1					
20.	WSDGVNLDYK	1	599.444	1196.89	2	20.67 & 11.74	300490464	Dabocetin beta subunit	*Daboia russelli russelli*	Snaclec
	QDcLSDWSFYEGYcYK	1	1061.43	2120.85	2					
	VFNEK	1	637.35	636.35	1					
21.	LRQGAQCAEGLCCDQcRFmK	1	778.84	2333.52	3	50.56 & 4.81	50365991	Adinbitor	*Glaydius brevicaudus*	Disintegrin
	MHMEAGEEcDCGSPGNPCCDAATCKLR	1	962.66	2884.98	3					
	MHMEAGEECDCGSPGNPcCDAATCK	1	872.90	2615.71	3					
22.	cTGQDcYGGVAR	1	673.05	1344.11	2	7.41 & 6.54	338855300	Ecto-5' nucleotidase	*Crotalus adamanteus*	Nucleotidase
	VLGVLNVIFDDK	1	730.89	1459.77	2					
	AEVNK	1	561.21	560.21	1					
	EVVKFMNSLR	1	612.72	1223.43	2					
23.	FHEcNLGNLIcDAVIYNNLR	1	812.79	2435.38	3	8.33 & 3.31	586829529	5'-nucleotidase, partial	*Macrovipera lebetina*	Nucleotidase
	DIPEDQVVK	1	522.43	1042.86	2					
	AQVNK	1	561.29	560.29	1					
24.	AcSGENGEQATSQNNSGDNER	1	742.74	2225.21	3	9.62 & 2.35	387016758	Hypothetical protein LOC100554767	*Crotalus adamanteus*	Hypothetical like protein
	QLPCmHEFHPHCIDRWLSENSTcPICR	1	1126.14	3375.43	3					
	RHPSITLDLQVRR	1	796.08	1590.18	2					
25.		1	1007.56	2013.12	2	71.01 & 2.13	50874332	Ammodytin1(C) variant	*Vipera berus berus*	PLA_2
	YmLYSLLDcGEESEQc	2	674.08	2019.23	3					
	CCFVHDCCYGRVNGCDPK	1	1005.95	3014.87	3					
	VAAIcFGENmNTYDK	1	583.98	1748.94	3					
	KTGIPGImSYTYYGcYCGWGGK	1	847.50	2539.50	3					
Gel filtration Peak 2										
26.	DLQTFcYPSIQK	1	807.58	1613.17	2	56.94 & 78.87	395406796	L-amino-acid oxidase		LAAO

	FDEIVGGMDQLPTSMYR	1	980.70	1959.39	2				*Daboia russelli russelli*	
	HIVIVGAGMSGLSAAYVLAGAGHK	1	760.87	2279.62	3					
	IPFAGEYTANAHGWIDSTIK	1	748.27	2241.82	3					
	KPWEDDGIQQGK	1	691.16	1380.31	2					
	LNEPVQETENGWYFIK	1	1009.81	2017.61	2					
	NPLEEcFREDQYEEFLEIAK	1	849.86	2546.59	3					
	SAGQLYQESLGK	1	641.69	1281.38	2					
	VTVLEASERPGCR	1	686.47	1370.94	2					
	VTVTYQTTQK	1	585.06	1168.12	2					
	YPVKPSEAGK	1	539.15	1076.29	2					
27.	FDEIVGGMDKLPTSMYR	2	654.31	1959.93	3	20.24 & 41.31	75570145	L-amino-acid oxidase	*Gloydius blomhoffii*	LAAO
	HDDIFAYEK	1	570.14	1138.27	2					
	SAGQLYEESLGK	1	642.22	1282.44	2					
28.	EGWYANLGPMR	1	647.94	1293.87	2	19.21 & 34.56	347602330	L-amino-acid oxidase	*Vipera ammodytes ammodytes*	LAAO
	FDEIVGGMDQLPTSMYR	2	980.70	1959.39	2					
	VTVLEASER	1	503.10	1004.20	2					
29.	EGWYANLGPMR	1	647.94	1293.87	2	21.39 & 30.57	538259837	L-amino acid oxidase	*Protobothrops flavoviridis*	LAAO
	HDDIFAYEK	1	570.14	1138.27	2					
	SAGQLYEESLR	1	627.73	1253.47	2					
30.	FDFPWIGLR	1	601.58	1201.16	2	54.00 & 49.10	300490464	Dabocetin beta subunit	*Daboia russelli russelli*	Snaclec
	QDcLSDWSPYEGYcYK	1	1061.73	2121.47	2					
	SSEEMDFVIR	1	607.18	1212.37	2					
	TTDNQWLR	1	518.03	1034.07	2					
	TWEDAEK	1	880.36	879.36	1					
	WSDGVNLDYK	1	599.40	1196.81	2					
31.	GFDcFPGWSSYEGYcYK	1	708.79	2123.38	3	59.20 & 2.48	3023231	Alboaggregin-A subunit beta	*Trimeresurus albolabris*	Snaclec
	AWSGKSYcLVSK	1	693.46	1384.91	2					
	mNWEDAESFcR	1	731.62	1461.24	2					
	TTNNEWLSMDCSR	1	779.25	1556.50	2					
32.	NPFIcK	1	779.33	778.33	1	27.92 & 24.74	123899657	Dabocetin subunit alpha	*Daboia siamensis*	Snaclec
	TWEDAEK	1	880.36	879.36	1					
	YHAWIGLR	1	508.94	1015.88	2					
33.	FGSVWIGLNDPWHNcNWEWSDNAR	1	987.90	2960.70	3	48.00 & 19.28	300490484	P31 beta subunit	*Daboia siamensis*	Snaclec
	GSHLASIHSSEEEAPVSK	1	958.72	1915.44	2					
	IPWFNRGcEKPVSFVcK	1	742.46	2224.39	3					
	RPYcTVMMVLKPDR	1	817.95	1633.91	2					
34.	AWNEGTNcPVFK	1	737.53	1473.07	2	56.08 & 16.42	73620113	Snaclec 5	*Daboia siamensis*	Snaclec
	GSHLLSLHNIAEADFVLK	1	655.93	1964.79	3					
	QDcLSDWSPYEGYcYK	1	1061.73	2121.47	2					
	TWEDAEK	1	880.36	879.36	1					
35.	DGIYVWIGLR	1	596.97	1191.94	2	34.18 & 15.14	300490478	P31 alpha subunit	*Daboia russelli limitis*	Snaclec
	FcTEQANSGHLVSIK	1	846.62	1691.25	2					
	WDYVNcAEHYK	1	757.51	1513.03	2					
36.	TWFNLScGDDYPFVcK	1	1005.84	2009.68	2	17.09 & 8.76	300490470	P68 alpha subunit	*Daboia siamensis*	Snaclec
	AFDEPK	1	707.36	706.36	1					
	TWFNLScGDDYPFVcKFPPRc	1	890.02	2667.05	3					
	FPPR	1	516.45	515.45	1					
	FIKNCFGLEKESDYR	2	889.45	2665.34	3					
37.	KGSHLVSLHSR	1	611.72	1221.45	2	24.66 & 8.59	73620112	Snaclec 4	*Daboia siamensis*	Snaclec
	VFTEEmNWADAEKFCTEQKK	1	817.79	2450.36	3					
	MEWSDR	1	824.36	823.36	1					
38.	DIMLIK	1	733.42	732.42	1	32.03	311223824	Serine beta-fibrinogenase-like protein precursor	*Daboia siamensis*	SVSPs
	FFcLSNK	1	916.41	915.41	1					
	IMGWGAITSPNETFPGVTHcANINILPYSVcR	1	1193.23	3576.69	3					
	TSTYIAPLSLPSSPPR	1	563.60	1687.80	3	15.47				
39.	DIMLIR	1	761.49	760.49	1	23.61 & 11.51	90116798	Thrombin-like enzyme elegaxobin-1	*Protobothrops elegans*	SVSPs
	NMEIYLGVHSK	1	646.35	1290.71	2					
	NMEIYLGVHSKK	1	710.12	1418.24	2					
40.	TSTHIAPLSLPSSPPSVGSVcR	1	751.25	2250.75	3	17.44 & 8.67	380875417	Serine protease VLSP-3	*Macrovipera lebetina*	SVSPs

(continued on next page)

Table 1 (continued)

Sl. No.	MS/MS derived sequence	No. of peptides	Peptide ion m/z	MH+ [Da]	z	Coverage (%) & sequest score	Accession no.	Protein	Homology with protein from	Protein family
	DIMLIK	1	733.42	732.42	1					
	SSELVIGGDECNINEHR	1	937.24	1872.48	2					
41.	SIIAGNTAATcPP	1	637.65	1273.29	2	17.44 & 7.51	13959617	Snake venom serine protease 1	*Trimeresurus gramineus*	SVSPs
	FGAHSQKVLNEDEQIRNPK	1	737.10	2210.10	3					
	NNEVLDKDImLIK	1	521.01	1560.04	3					
42.	FHcSGTLLNEEWVLTAAHCDmENmQIYLGVHDK	1	1298.61	3892.83	3	32.31 & 7.35	381141431	Serine protease VLSP-1	*Macrovipera lebetina*	SVSPs
	DIMLIR	1	761.49	760.49	1					
	KAYGGLPEK	1	963.46	962.46	1					
	TLCAGVLQGGIDTCLADSGGPLIcNGQFQGIVAWGR	1	1216.93	3647.79	3					
43.	KDDENDKDIMLIR	1	536.08	1605.23	3	11.63 & 4.69	406609998	Hypothetical like protein	*Gloydius blomhoffii*	SVSPs
	SSELVIGGDECNINEHR	1	937.24	1872.48	2					
44.	VLDcPSGWLSYEQHcYK	1	1072.35	2142.70	2	27.64 & 9.44	251205	RVV-X-light chain	*Daboia russelii*	SVMPs
	KGSHLVSLHSR	1	611.72	1221.45	2					
	MEWSDR	1	824.36	823.36	1					
45.	DQLQQNGQPcQNNR	1	851.18	1700.36	2	24.39 & 45.24	300079900	Factor X activator heavy chain	*Daboia russellii russellii*	SVMPs
	DScFQENLK	1	571.74	1141.47	2					
	NEcDVPEHcTGQSAEcPR	1	1074.16	2146.32	2					
	NQcISLPGSR	1	592.07	1182.14	2					
46.	cEEPYPPVcK	1	665.33	1328.65	2	32.28 & 13.27	300079896	Factor X activator light chain 2	*Daboia russellii russellii*	SVMPs
	EEFRKcPVLQK	1	742.59	1483.18	2					
	FcMEHPNNGHLVSIESMEEAEFVAK	1	969.75	2906.26	3					
	VPPEc	1	602.28	601.28	1					
47.	EAVHSYAIYGcYcGWGGQGR	1	764.95	2291.86	3	43.44 & 7.36	1408314	Phospholipase A_2 acidic subunit	*Vipera aspis*	PLA_2
	AVCECDRAAAICLGENVNTYDK	1	787.11	2358.34	3					
	CCFAQDCcYGR	1	664.10	1326.20	2					
Gel filtration Peak 3										
48.	AAAIcLGENVNTYDK	1	820.68	1639.36	2	80.33 & 31.58	1408314	Phospholipase A_2 acidic subunit	*Vipera aspis*	PLA_2
	CcFAQDCcYGR	2	691.917	1381.83	2					
	EAVHSYAIYGcYcGWGGQGR	1	764.781	2291.34	3					
	NLFQFGDmILQK	1	735.999	1469.10	2					
	NYEYYSISHcTEESEQc	1	1100.68	2199.37	2					
49.	AAAIcLGQNVNTYDK	1	547.12	1638.36	3	69.57 & 22.98	400714	Acidic phospholipase A_2RV-7	*Daboia siamensis*	PLA_2
	EVVHSYAIYGcYcGWGGQGR	1	774.15	2319.46	3					
	NYEYYSISHcTEESEQc	1	1100.68	2199.37	2					
50.	ccFVHDccYGKLWSCSPK	1	770.266	2307.80	3	33.33 & 11.15	123907686	Basic phospholipase A_2 Tpo-G6D49	*Trimeresurus puniceus*	PLA_2
	GTWCEKQIcEcDKAAAIcFR	1	816.331	2445.10	3					
	NGAIVcARGTWCEKQIcECDK	2	1221.11	2440.23	2					
51.	ccFVHDCCYGNLPDcNPK	1	734.72	2201.15	3	68.61 & 5.97	408407675	Basic phospholipase A_2DsM-S1	*Daboia siamensis*	PLA_2
	LAIPSYSSYGCYcGWGGKGTPK	1	784.90	2351.71	3					
	VNGAIVcEKGTScENR	1	898.40	1794.81	2					
	YmLYPDFLCKGELRC	1	934.10	1866.20	2					
52.	CCLVHDCcYTRVGDCSPK	1	687.16	2058.47	3	46.67 & 4.99	40889259	Chain A, Acidic Phospholipase A_2	*Echis carinatus*	PLA_2
	EAAICLGENVNTYDK	1	547.70	1640.11	3					
	VGDCSPKmTLYSYRFENGDICDNK	1	962.35	2884.05	3					
53.	FDFFWIGLR	1	601.52	1201.04	2	51.33 & 26.92	300490464	Dabocetin beta subunit	*Daboia russellii russellii*	Snaclec
	QDcLSDWSFYEGYcYK	1	1061.63	2121.26	2					
	SSEEMDFVIR	1	607.56	1213.12	2					
	TTDNQWLR	1	517.85	1033.70	2					
	WSDGVNLDYK	1	599.75	1197.50	2					
54.	NPFIcK	1	779.36	778.36	1	38.31 & 18.80	123899657	Dabocetin subunit alpha	*Daboia siamensis*	Snaclec
	YHAWIGLR	1	508.96	1015.91	2					
	YHEWITLPcGDKNPFIcK	1	760.33	2277.10	3					
55.	FDYK	1	573.402	572.40247	1	**37.33 & 15.35**	**300490484**	**P31 beta subunit**	***Daboia siamensis***	**Snaclec**
	FGSVWIGLNDPWHNcNWEWSDNAR	1	987.923	2960.76999	3					
	GSHLASIHSSEEEAFVSK	1	958.684	1915.36894	2					
	IFWFNRGcEK	1	679.656	1357.31120	2					

56.	EDDYEEFLEIAKNGLK	1	638.52	1912.56	3	26.19	395406796	L-amino-acid oxidase	*Daboia russelii russelii*	LAAO
	BGWYANLGPmRVPEK	1	588.35	1762.05	3					
	LNEFVQETENGWVYFK	1	1009.83	2017.67	2					
	SAGQLYQESLGK	1	641.43	1280.87	2	24.15				
57.	DEcDVPEHcTGQSAEcPR	2	716.74	2147.22	3	20.84 & 8.97	162329887	Chain A, Russell's Viper Venom Metalloproteinase	*Daboia siamensis*	SVMPs
	DQLQQNGKPeQNNR	1	850.92	1699.84	2					
	NPcNMHYScMDQHK	1	608.54	1822.63	3					
	YKPKCIFNPPLR	1	738.67	1475.35	2					
58.	TLcAGILEGGKDSCR	1	791.37	1580.73	2	6.44 & 4.06	82117246	Full = Beta-fibrinogenase brevinase	*Gloydius blomhoffii*	SVSPs
	TLCAGILEGGK	1	531.40	1060.80	2					
59.	SLLQQDSCQDAGmQSKcSASCFCQNK	1	961.837	2882.51	3	41.82 & 5.05	190195337	Cysteine-rich secretory protein Da-CRPa	*Deinagkistrodon acutus*	CRISP
	SVDFDSESPR	1	570.742	1139.48	2					
	VLEGIKcGENIYMSPNPMK	1	1090.99	2179.97	2					
	YTNcKSLLQQDSCQDAGMQSKcSASCFCQNK	1	1178.63	3532.88	3					
60.	cSGccTDESMK	1	668.472	1334.94	2	56.94 & 32.54	327478537	Snake venom vascular endothelial growth factor toxin	*Daboia russelii russelii*	VEGF
	FMEHTAcEcRPR	2	798.092	1594.18	2					
	HTADIQIMR	1	543.665	1085.33	2					
	MEVMKFmEHTACEcRPR	1	724.447	2170.34	3					
	WKQGEPEGPK	1	579.19	1156.38	2					
61.	QQGEVISFLTVYER	1	835.685	1669.37	2	19.27 & 6.28	327488518	Vascular endothelial growth factor A	*Vipera ammodytes ammodytes*	VEGF
	QENHcEPCSERR	1	773.384	1544.77	2					
	CSCKFTDSRcK	1	1335.28	1334.28	1					
Gel filtration Peak 4										
62.	cGENIYMSPYPMK	2	796.09	1590.18	2	53.56 & 69.14	190195321	Cysteine-rich secretory protein Dr-CRPK	*Daboia russelii*	CRISP
	clLNHSPYNSR	1	1361.59	1360.59	1					
	RPEIQNEIVDLHNSLR	1	967.78	1933.56	2					
	RSVTPTASNMLK	1	653.69	1305.38	2					
	WTAIIHEWHK	1	661.46	1320.92	2					
63.	KPEIQNEIVDLHNSLR	1	953.80	1905.59	2	61.09 & 28.64	190195307	Cysteine-rich secretory protein Ch-CRPKa	*Crotalus horridus*	CRISP
	SLVQQAGcEDKQQSDcSAICFCQNKII	1	1062.84	3185.53	3					
	SVDFDSESPRKPEIQNEIVDLHNSLR	1	1009.40	3025.20	3					
64.	KPEIQNKIVDLHNFLR	1	655.69	1964.08	3	54.30 & 24.21	190195319	Cysteine-rich secretory protein Cv-CRP	*Crotalus viridis*	CRISP
	mEWYPEAAANAER	2	777.78	1553.55	2					
	SGPPCGDCPSACDNGLCTNPCTK	1	747.93	2240.80	3					
65.	DPKYGVGAVPSNAATGHYTQIVWYK	1	925.08	2772.24	3	28.96 & 12.38	1778013	Prepro-cysteine-rich venom protein	*Protobothrops mucrosquamatus*	CRISP
	mEWYPEAAANAER	2	777.78	1553.55	2					
	SYRGGcAAAYCPSSK	1	790.05	1578.10	2					
	GGcAAAYcPSSK	1	615.52	1229.03	2					
66.	clLDHSPYNSR	1	1363.45	1362.45	1	46.15 & 21.81	190195323	Cysteine-rich secretory protein Dr-CRPB	*Daboia russelii*	CRISP
	mEWYPEAAANAER	2	777.78	1553.55	2					
	SVDFDSESPR	1	570.71	1139.43	2					
67.	IVDLHNSLR	1	534.61	1067.23	2	22.08 & 11.43	190195329	Cysteine-rich secretory protein Pg-CRP	*Cerrophidion godmani*	CRISP
	mEWYPEAAANAER	2	777.78	1553.55	2					
	YTNcNSLVQKSGCQDTWmQSNcPAICPCQNK	1	1216.06	3645.19	3					
68.	AAAIcLGENVNTYDK	1	820.60	1639.21	2	86.07 & 23.82	1408314	Phospholipase A_2 acidic subunit	*Vipera aspis*	PLA_2
	EAVHSYAIYGcYcGWGGQGR	1	764.78	2291.33	3					
	NLFQFGDmILQK	1	735.66	1469.31	2					
	NYEYYSISHcTEESEQc	1	1100.56	2199.13	2					
69.	AAAIcLGQNVNTYDK	1	820.14	1638.29	2	69.67 & 18.33	37927199	Chain G, of Rv4RV7 complex	*Daboia siamensis*	PLA_2
	NLFQFGEMILQK	1	735.35	1468.71	2					
	NYEYYSISHcTEESEQc	1	1100.56	2199.13	2					
70.	VNGAIVcEQGTScENR	1	898.08	1794.16	2	58.68 & 14.85	298351762	Basic phospholipase A_2	*Daboia russelii russelii*	PLA_2
	ccPVHDCCYGNLPDcNPK	1	734.95	2201.86	3					
	LAVPPYSSYGcYcGWGGK	1	691.81	2072.43	3					
	RVNGAIVcEQGTScENR	1	651.18	1950.54	3					
71.	NLFQFAEMIVK	1	670.94	1339.88	2	27.27 & 6.27	3914259	Basic phospholipase A_2 RVV-VD	*Daboia russelii russelii*	PLA_2
	GKPQDATDRccPVHDccYEKVK	1	925.11	2772.33	3					
72.	SPIFSYGDYGcYcGWGGK	1	692.49	3074.47	3	37.50 & 2.39	129506	Acidic phospholipase A_2	*Cerastes cerastes*	PLA_2
	VAAIcFGENVNTYDKK	1	915.12	1828.24	2					

(continued on next page)

Table 1 (continued)

Sl. No.	MS/MS derived sequence	No. of pepcides	Peptide Ion m/z	MH+ [Da]	z	Coverage (%) & sequest score	Accession no.	Protein	Homology with protein from	Protein family
	CcFVHDcCYGR	1	710.63	1419.27	2					
73.	ccPVHDccYGKATGCDPKK	1	769.94	2306.81	3	13.67 & 2.39	403399517	Acidic phospholipase A_2 Tgc-B5	*Trimeresurus gracilis*	PLA_2
74.	ccPVHDccYGKLAK	1	910.15	1818.30	2	57.38 & 2.01	223635543	Basic phospholipase A_2	*Crotalus durissus ruruima*	PLA_2
	DATDRCcFVHDCCYGK	1	631.73	1892.18	3					
	GTWCEEQICPcDRVAAECLR	1	791.42	2371.26	3					
75.	YHEWITLPcGDSNPFICK	1	760.66	2278.98	3	37.01 & 17.16	123809657	Dabocetin subunit alpha	*Daboia siamensis*	Snaclec
	cFGLNK	1	739.34	738.34	1					
	TWEDAEK	1	879.34	878.34	1					
76.	TWEDAEWFCTK	1	708.56	1415.13	2	10.13 & 6.14	82129809	Crotocetin-1	*Crotalus durissus terrificus*	Snaclec
	cFGLK	1	625.37	624.37	1				*Daboia russellii russellii*	Snaclec
77.	TTDNQWLR	1	517.88	1033.76	2	48.00 & 11.53	300490464	Dabocetin beta subunit		
	KTWEDAEK	1	504.39	1006.79	2					
	VFNEK	1	637.31	636.31	1					
78.		1	997.28	1992.56	2	41.98 & 10.75	335892642	Venom nerve growth factor 2	*Daboia russelii*	VNGF
	GNVVTVMVDVNLNNNVYK	1	647.37	1292.74	2					
	INTAcVcVISR	1	597.15	1788.45	3					
	HWNSYcTTTDTFVR	1	800.46	2398.37	3					
	THEALKTSRNTDQHYPAPNK	1	770.36	2308.09	3					
79.	SAGQLVQESLGK	1	641.50	1280.10	2	23.41 & 9.39	395406795	L-amino-acid oxidase	*Daboia russelii russellii*	LAAO
	LNEPVQETENGWYFIK	1	1009.73	2017.47	2					
	HNIVGAGMSGLSAAYVLAGAGHK	1	760.66	2278.98	3					
	EGWYANLGPMR	1	648.10	1294.21	2					
	VTVLEASERPGGR	1	686.64	1371.29	2					
80.	cSGccTDESMK	2	668.50	1335.00	2	43.75 & 8.07	327478537	Snake venom vascular endothelial growth factor toxin	*Daboia russelii russellii*	VEGF
	cTPVGKHTADIQIMRMNPR	2	1113.64	2225.29	2					
	HTADIQIMR	1	543.66	1085.32	2					
	QGEPEGPKEPR	1	613.25	1224.51	2					
81.	ATVAEDAcFQFNRLGSDYGYcRK	2	910.39	2728.17	3	36.27 & 7.34	297593790	Metalloproteinase	*Echis carinatus sochureki*	SVMPs
	DECDmADLcNGQSDEcPK	2	682.86	2045.58	3					
	DENKGmVEPGTKcENGK	1	955.70	1909.40	2					
	NGHPcQNNNGYcYNGK	1	949.62	1897.25	2					
82.	ARNECDVPEHCTGQSAECPR	1	735.19	2202.56	3	22.94 & 5.53	300079900	Factor X activator heavy chain	*Daboia russelii russellii*	SVMPs
	cEDGKVcNNK	1	612.52	1223.05	2					
	DQLQQNGQPcQNNR	1	851.12	1700.23	2					
	GMVDFGTK	2	805.37	804.37	1					
	LPSNcSIHDYQRYLTR	1	691.81	2072.43	3					
Gel filtration Peak 5										
83.	LAIPSYSSYGcYcGWGGK	1	676.48	2026.43	3	76.86 & 45.27	24638087	Basic phospholipase A_2VRV-PL-VIIIa	*Daboia russelii russelii*	PLA_2
	MILEETGK	1	921.38	920.38	1					
	VNGAIVcEKGTScENR	1	898.045	1794.09	2					
	YMLYPDFLcK	2	675.90	1349.80	2					
84.	LAVPIYSSYGcYcGWGGK	1	1037.08	2072.16	2	66.94 & 36.61	298351762	Basic phospholipase $A_2$3	*Daboia russelii russelii*	PLA_2
	RVNGAIVcEQGTScENR	1	976.28	1950.57	2					
	SLLEPGMMILEETGK	1	850.14	1698.29	2					
85.		1	820.14	1638.27	2	67.39 & 5.24	400714	Acidic phospholipase A_2 RV-7 (Viperotoxin non-toxic acidic component)	*Daboia siamensis*	PLA_2
	AAAIcLGQNVNTYDK	1	1336.56	2671.12	2					
	TATYSYSFENGDIVcGDNDLcLR	1	1076.51	3226.53	3					
	EVVHSYAIYGcYcGWGGQGRAQDATDR	1	1026.42	3076.25	3					
86.	IPcAPEDVK	1	514.81	1027.63	2	16.29 & 10.04	123896981	Zinc metalloproteinase-disintegrin-like Eoc1	*Echis ocellatus*	SVMP
	IQNDADSSASISACNGLKGHFK	1	755.25	2262.76	3					
	LYcFDNLPEHK	1	718.95	1435.91	2					
	MPQcILIKPSR	1	672.77	1343.55	2					
87.	EKPFcLSSK	1	1146.44	1145.44	1	21.15 & 7.21	13959855	Venom serine proteinase-like protein 2	*Macrovipera lebetina*	SVSP
	FFcLSSK	1	889.316	888.31	1					
	FYcAGTLINQEWVLTAAR	1	1057.87	2113.75	2					
	NVPNEDQQIRVPK	1	769.85	1537.70	2					

	TLCAGILQGGIDSCK	1	739.94	1477.87	2					
88.	WDKDIMLIK	1	582.21	1162.43	2	16.88 & 6.22	297593764	Serine protease	*Echis carinatus sochureki*	SVSP
	TLcAGVLEGGIDScK	1	790.62	1579.24	2					
	VPAERVFCVSSKTYTR	1	606.29	1815.87	3					
89.	RPEIQNEIVDLHNSLR	1	967.74	1933.48	2	11.72 & 4.86	190195321	Cysteine-rich secretory protein Dr-CRPK	*Daboia russelii*	CRISP
	cPAScFcHNEII	2	754.89	1507.77	2					
Gel filtration peak 6										
90.	KYmLYPDFLcK	3	748.16	1494.32	2	82.64 & 72.67	24638087	Basic phospholipase A_2 VRV-PL-VIIIa	*Daboia russelii russelii*	PLA_2
	LAIPSYSSYGcYcGWGGK	3	676.18	2025.53	3					
	QNLNTYSK	1	968.43	967.43	1					
	VNGAIVcEK	1	991.39	990.39	1					
	SLLEFGKMILEETGK	1	848.59	1695.18	2					
	VNGAIVcEKGTScENR	2	898.15	1794.30	2					
91.	IcECDKAAAICFRR	1	829.30	1656.61	2	81.82 & 45.98	298351762	Basic phospholipase A_2 3	*Daboia russelii russelii*	PLA_2
	IYmLYPDFLcK	3	740.44	1478.89	2					
	LAVPFYSSYGcYcGWGGK	1	1037.08	2072.16	2					
	SLLEFGmMILEETGK	1	858.14	1714.28	2					
	VNGAIVcEQGTScENR	2	898.15	1794.30	2					
92.	CcPVHDccYGKVNGCNPK	1	721.11	2160.33	3	32.79 & 6.21	3914268	Acidic phospholipase A_2	*Trimeresurus gramineus*	PLA_2
	EVCECDKAAAIcFRDNK	1	987.01	1972.02	2					
	NVAGR	1	516.37	515.37	1					
93.	FFNGLDKDIMLIR	1	767.17	1532.34	2	47.03 & 26.89	134129	Factor V activator RVV-V alpha	*Daboia siamensis*	SVSP
	RPVTYSTHIAPVSLPSR	1	941.69	1881.39	2					
	WcEPLYPWVPADSR	1	889.10	1776.19	2					
94.	cKEFIYGGcHGNANKFPSR	1	748.64	2242.91	3	65.56 & 15.16	159883540	Trypsin inhibitor-5 precursor	*Daboia siamensis*	KSPI
	cRQTcGASAKGRPT	1	775.39	1548.79	2					
	EFIYGGcHGNANK	1	734.58	1467.16	2					
	FcYLPADPGEcLAFIMR	1	646.89	1937.69	3					
95.	FcHLPVDSGIcR	1	731.39	1460.78	2	26.19 & 2.25	123913154	Kunitz protease inhibitor 4	*Daboia russelii russelii*	KSPI
	DQcRHTcGGK	1	609.77	1217.54	2					
96.	HANFPILSANIRPK	1	527.25	1578.80	3	9.86 & 8.65	538250847	5′ nucleotidase, partial	*Protobothrops flavoviridis*	Nucleocidase
	ASGNPILNK	1	514.50	1026.99	2					
	ETPVLSNPGPYLEFRDEVEELQNHANK	1	1042.91	3125.74	3					
Gel filtration Peak 7										
97.	IYmLYPDFLcK	2	740.38	1478.76	2	76.86 & 50.08	298351762	Basic phospholipase A_2	*Daboia russelii russelii*	PLA_2
	LAVPFYSSYGcYcGWGGK	1	1037.13	2072.26	2					
	RVNGAIVcEQGTScENR	2	651.08	1950.26	3					
	SLLEFGmMILEETGK	2	857.89	1713.78	2					
Gel filtration Peak 8										
98.	SLLEFGmmILEETGK	3	866.11	1730.22	2	86.78 & 104.89	298351762	Basic phospholipase A_2	*Daboia russelii russelii*	PLA_2
	LAVPFYSSYGcYcGWGGK	2	1037.36	2072.72	2					
	IYmLYPDFLcK	2	740.28	1478.56	2					
	RVNGAIVcEQGTScENR	2	976.225	1950.45	2					
99.	VNGAIVcEKGTScENR	2	1795.25	1794.25	1	82.64 & 59.37	24638087	Basic phospholipase A_2 VRV-PL-VIIIa	*Daboia russelii russelii*	PLA_2
	LAIPSYSSYGcYcGWGGK	1	1014.34	2026.68	2					
	KYMLYPDFLcK	3	740.28	1478.56	2					
100.	FAIIAYSNYGcYcGWGGK	1	696.8767	2087.63	3	21.01 & 3.70	13936543	Phospholipase A_2	*Echis coloratus*	PLA_2
	ccPVHDccYGR	1	767.965	1533.93	2					
101.	SPIPSYGDYGcYcGWGGK	2	692.2833	2073.85	3	30.83 & 2.33	129506	Acidic phospholipase A_2	*Cerastes cerastes*	PLA_2
	ccPVHDccYGR	1	767.965	1533.93	2					
	mIFKMTGK	1	973.32	972.32	1					
102.	TSVSSHYcTGR	1	628.6	1255.20	2	74.42 & 8.67	123916448	Disintegrin CV short precursor	*Cerastes vipera*	Disintegrin
	CTTGPCCRQcK	1	629.085	1256.17	2					
	LKPAGTTcWR	1	596.145	1190.29	2					
103.	FcYLPADPGEcMAYIR	1	982.81	1963.62	2	37.78 & 4.57	159883524	Trypsin inhibitor-4 precursor	*Daboia siamensis*	KSPI
	SPYYDSESK	1	563.925	1125.85	2					
	cRQTcRAPR	1	603.185	1204.37	2					

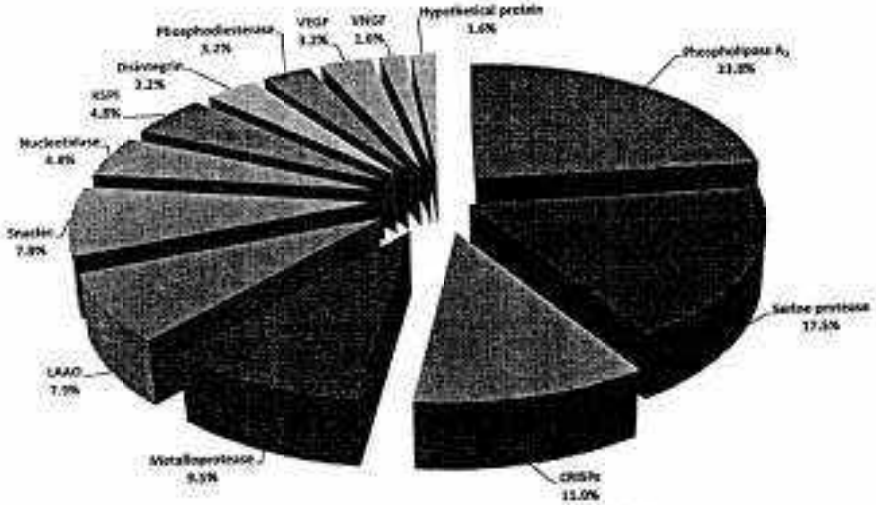

Fig. 3. Relative distribution of isoforms of toxins in various protein families in *Daboia russelii* venom. Peptide sequences were obtained by ESI-LC MS/MS analysis. Protein families were assigned based on the sequence homology identified by the Proteome Discoverer with the Sequest program.

characteristics. VRV-PL-VIIIa, a basic PLA_2 enzyme reported as the major toxin in *D. russelii* venom was found in this venom (Gowda et al., 1994). VRV-PL-VIIIa exhibits diverse pharmacological effects like neurotoxicity, myonecrosis, *in-vivo* organ damaging activity and anticoagulant effect (Kasturi and Gowda, 1989). In the present analysis we have observed six peptide fragments of this toxin in gel filtration peak 5, 6 and 8. Peptide fragments homologous to ammodytin I1 (C) (Accession No. 50874332, Table 1) a presynaptic neurotoxic PLA_2 from the venom of *Vipera berus berus* was observed (Jan et al., 2007).

PLA_2 enzymes of *D. russelii* were classified into N-type and S-type based on the N-terminal residues asparagine or serine (Tsai et al., 1996). The N-type PLA_2 is reported to be present in *D. r. siamensis*, *D. r. russelii* and *D. r. formosensis* venoms while the S-type is found in *D. r. pulchella* venom (Tsai et al., 1996). However, in the South Indian *D. russelii* venom, both the S-type and N-type PLA_2 enzymes were observed along with peptide fragments homologous to PLA_2 with (13936543, 50874332 and 223635543) histidine (H) at the N-terminus (Supplementary Fig. 1). This suggests the lack of correlation between different types of PLA_2 enzymes (classified based on N-terminal amino acid residue) and subspecies of Russell's viper. The observed anticoagulant, PLA_2 activity (48.89 ± 3.85 μmol/min/ml/mg) and edema in experimental animals (15 μg of crude venom induced 208.3% edema in the injected leg of experimental mice) (data not shown) could be attributed to the presence of large number of PLA_2 isoenzymes.

SVSPs are the second most abundant proteins in this venom proteome. The abundance of these enzymes correlates with the proteolytic and fibrinogenolytic activities of the crude venom (data not shown). Homologue of elegaxobin (Accession No. 90116798) from *Protobothrops elegans* (Oyama and Takahashi, 2000), RVV-Vα from *Daboia siamensis* were observed. RVV-V converts coagulation factor V to Va (co-factor in the prothrombinase complex) by cleaving at the Arg^{1545}-Ser^{1546} bond (Nakayama et al., 2011). Apart from these, three isoforms of β-fibrinogenase similar to proteins reported from *D. siamensis*, *Macrovipera lebetina* and *Gloydius blomhoffii* were observed in the present study (Table 1).

SVMPs are classified into three broad classes. These are, namely PI, PII (a, b, c, d) and PIII (a, b, c and d) based on the difference in the domain structure (Fox and Serrano, 2005; Fox and Serrano, 2008; Fox and Serrano, 2009). During envenomation SVMPs cause both local and systemic injuries which include pathological effects like myonecrosis, haemorrhage, edema formation and blistering (Fox and Serrano, 2010). Viperidae venoms are rich source of these proteins; which induce the most profound damaging effects to the basement membrane of the blood vessel capillaries leading to extravasation of endothelial cells including excess bleeding (Gutierrez and Ownby, 2003; Fox and Serrano, 2010). Hence, insufficient blood supply and loss of basement membrane integrity might hamper skeletal muscle regeneration (Grant et al., 1988; Gutierrez and Rucavado, 2000). The formation of the haemorrhagic spot of 1.5 cm in the dorsal skin of the experimental animals suggests the effect of these SVMPs at the site of injection (data not shown). Six isoforms of heavy chain and three light chain (both α and β chain) of RVV-X (coagulation factor X activating enzyme) were observed (Table 1) suggesting that the presence of this protein complex in the crude venom. RVV-X belongs to the PIIId family of metalloprotease with a heavy chain and two light chains (Takeya et al., 1992). The two light chains are connected to each other via an inter-chain disulphide bond between Cys79 of chain A and Cys77 of chain B. While the light chain is connected to the heavy chain via another disulphide bond between Cys133 of chain A and Cys339 of heavy chain (Takeda et al., 2007). It converts coagulation factor X to Xa by cleaving at the Arg^{51}-Ile^{52} of FX leading to severe coagulopathy in victim/prey (Fujikawa et al., 1972).

Presence of large number of these SVMPs and SVSPs in the venom could be responsible for the observed procoagulant and proteolytic activities. The crude venom has exhibited procoagulant effect on goat plasma in a dose dependent manner (Fig. 4) and its proteolytic activity was observed to be 1.37×10^{-4} μg/min/ml/mg when casein was used as the substrate. These enzymes together may be responsible for consumptive coagulopathy in the victim. However, the thrombus formed by SV-TLE is readily dissolved by the plasmin due to absence of proper cross-linked fibrins leading to excess blood loss at the site of injury (Phillips et al., 2010).

LAAO (57–68 kDa, monomeric form) are homodimeric high molecular weight proteins (Tan and Fung, 2010). These enzymes cause oxidation of hydrophobic L-amino acids releasing α–keto acid, ammonia and H_2O_2 (Du and Clemetson, 2002; Chen et al., 2012). The liberated H_2O_2 causes various pathologies, such as edema formation, ADP or collagen induced platelet aggregation inhibition or activation, apoptosis, antibacterial effect, antiparasitic, anticoagulant, haemolytic and haemorrhagic effects (Suhr and Kim, 1996; Du and Clemetson, 2002; Chen et al., 2012). LAAO from *A. h. blomhoffii* delays the activated partial thromboplastin time (intrinsic pathway of blood coagulation) especially by targeting

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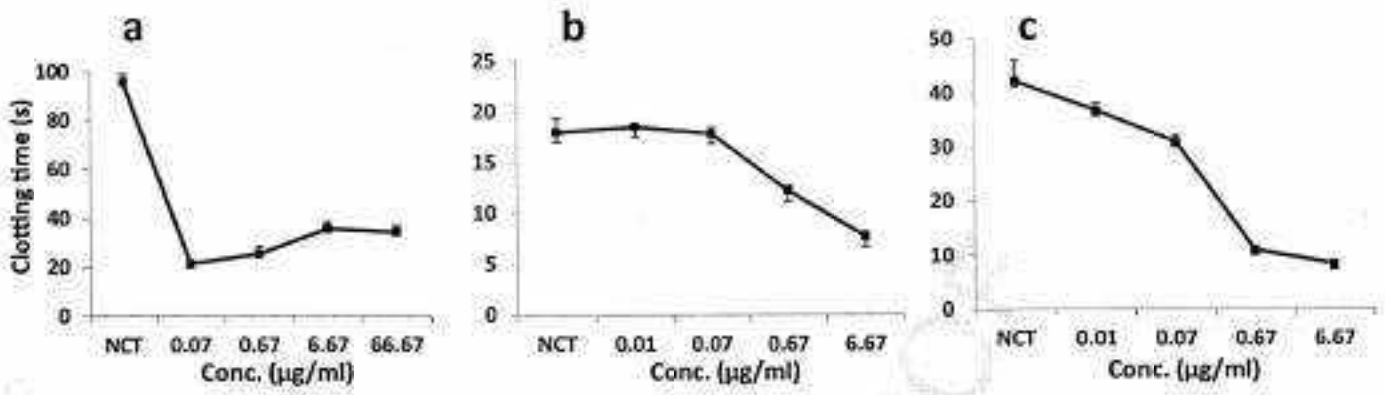

Fig. 4. Effect of crude *Daboia russelii* venom on blood coagulation. a: Recalcification time; **b:** Prothrombin time; **c:** Activated partial thromboplastin time. The clot formation was monitored using Tulip Coastar-1 coagulo analyser. Clotting time of the plasma in the presence of Tris buffer (20 mM, pH 7.4) was considered as normal clotting time (NCT). Each point represents the average ± SD of three independent experiments.

factor IX (Sakurai et al., 2003). Five isoforms of LAAO identified in the proteome were found to be similar with protein sequences previously reported (Table 1). Most of these proteins are reported to inhibit ADP and collagen induced platelet aggregation (Samel et al., 2006; Chen et al., 2012). Thus, LAAO together with SVSPs, SVMPs and PLA_2 might act synergistically on the haemostatic system of the prey or victim.

PDEs are high molecular weight (>90 kDa) (Dhananjaya et al., 2010) proteins reported to act as endonucleases on both double and single stranded RNA and DNA releasing 5'-mononucleotides (Dhananjaya et al., 2010). In this proteome, analogous sequence of PDEs reported previously from *M. lebetina* (Accession No. 586829527) and *Protobothrops flavoviridis* (Accession No. 538259853) (Aird et al., 2013) were identified (Table 1). Pharmacologically, PDEs inhibit platelet aggregation, decrease the mean arterial pressure and hinder locomotion (Russell et al., 1963).

Nucleotidases belong to the metallophosphatase superfamily of proteins (Aird et al., 2013). Presence of homologous peptides suggest their presence in the venom and they might be involved in the release of adenosine (purines) which cause hypotension and paralysis leading to prey immobilization and digestion (Aird, 2002, 2005).

CRISP are the largest group of non-enzymatic proteins (11%) identified in *D. russelii* venom. Several peptides similar to Da-CRPa Dr-CRPK, Ch-CRPKa, Cv-CRP, Dr-CRPB, Pg-CRP and a prepro CRISP from crotalinae and viperinae subfamily were identified (Table 1). They are reported to inhibit cyclic nucleotide gated ion channels in photoreceptor and olfactory cells (Brown et al., 1999), potassium activated smooth muscle contraction (Osipov et al., 2005) and vascular smooth muscle contraction (Ito et al., 2007).

Snaclecs are the non-enzymatic proteins abundantly found in viperid venom. Peptide sequence homologous to α and β subunits of dabocetin documented earlier from subspecies of *D. russelii* were identified (Table 1). α dabocetin is reported to bind to glycoprotein Ib of platelet to induce inhibitory effect on ristocetin-induced platelet aggregation (Zhong et al., 2006). Similarly peptide sequences analogous to α and β subunits of P31 (Accession No. 300490484) and α subunit of P68 (Accession No. 300490470), crotocetin-1 (Accession No. 82129809) and snaclec A14 (Accession No. 218526485) were also observed. Moreover, β subunit of alboaggregin-A (Accession No. 3023231) reported earlier from *Trimeresurus albolabris* is a strong activator of platelet, binding via GPIb α and GP VI of platelets was also identified in this proteome (Andrews et al., 1996; Asazuma et al., 2001; Dormann et al., 2001). The abundance of snaclecs in this proteome might be the cause of enhanced coagulopathic disorders along with SVMPS, SVSPs and PLA_2 enzymes in the envenomed victims.

KSPI are the low molecular weight proteins having 50–60 amino acid residues with a conserved Kunitz motif typical to bovine pancreatic trypsin inhibitor (BPTI) (Mourao and Schwartz, 2013; Morjen et al., 2014). Upon envenomation, KSPI may inhibit trypsin and chymotrypsin (Guo et al., 2013), potassium and calcium ion channel (Schweitz et al., 1994; Stotz et al., 2000) as well as exhibit diverse pharmacological effects on the victim like fibrinolysis (Qiu et al., 2013) and anticoagulation (Earl et al., 2012). We have identified three isoforms of KSPI having sequence similarity with trypsin inhibitor precursor-4 and -5 reported earlier from *D. siamensis* (Guo et al., 2013) and KSPI-4 from *D. r. russelii* (Table 1).

Disintegrin are the polypeptides rich in cysteine residues liberated by the proteolytic cleavage of multidomains of metalloproteases in the venom (Calvete et al., 2005). They are found in the viperidae family and are known to cause inhibition of integrin receptors (Calvete, 2005). Two peptide fragments homologous to adinbitor (Accession No. 50365991), a disintegrin with RGD motif from *Gloydius brevicaudus* were observed in the venom. Disintegrin with RGD motif are reported to inhibit angiogenesis and platelet aggregation. Sequences similar to disintegrin CV with RTS integrin inhibitory motif studied previously from *Cerastes vipera* were also observed in the crude venom (Sanz et al., 2006). These observed peptides are found to have sequence similarity with Russellistatin reported from *D. russelii* (Sanz-Soler et al., 2012). Disintegrin CV is reported to inhibit cell adhesion and migration via collagen I and II, anti-angiogenic and interact with α1 and β1 of integrin (Sanz et al., 2006). The integrin receptor inhibitory loop with RGD motif is antagonist to β1and β3 ($\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_{IIb}\beta_3$) integrins while the inhibitory loop with RTS is specific to α1β1 (Calvete, 2005; Calvete et al., 2010).

Peptide sequence similar to VNGF-2 reported earlier from *D. russelii* of Taiwan was observed. VNGFs are reported to cause apoptosis, vascular permeability and wound healing (Li et al., 1980; Otten et al., 1984). A few peptide sequences identified in the proteome were similar to Vegf toxin and Vegf-A described earlier from snakes of viperinae family (Yamazaki et al., 2003, 2009). These proteins are reported to cause nitric oxide induced hypotension, angiogenesis, capillary permeability, cell proliferation, migration and anti-apoptosis (Yamazaki et al., 2009).

Apart from these well-known protein families, peptide sequences similar to a hypothetical protein reported in the transcriptome of *Crotalus adamanteus* (Accession No. 387016758) was also observed in the venom proteome (Rokyta et al., 2012). It will be interesting to understand the role of this protein in the whole venom during envenomation.

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5. Conclusion

Analysis of the proteome of Indian *D. russelii* by using tandem mass spectrometry has enabled an in-depth understanding of the potential toxic components in this venom. This correlates with the clinical manifestations of Russell's viper envenomation. The protein profile shows clear distinction with *D. r. siamensis* from Myanmar although they belong to the same species. The absence of daboiatoxin (the major lethal component of Myanmar viper) in *D. r. russelii* and the absence of CRISPs, VNGF, KSPI, nucleotidase and phosphodiesterase in *D. r. siamensis* clearly indicate the variation of venom composition in these subspecies. This study reports the presence of nucleotidase, CRISPs, disintegrins, VNGF and VEGF in Indian *D. r. russelii* venom for the first time. Such proteomics studies on the venoms of Indian *D. r. russelii* from other regions need to be carried out for better understanding of the venom variation. This will help in designing better quality, regio-specific antivenoms which might reduce the occurrence of anaphylactic reactions in the victim.

Conflict of interest

The authors declare no conflict of interest.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2015.06.027.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.06.027.

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PLOS ONE

RESEARCH ARTICLE

Daboxin P, a Major Phospholipase A2 Enzyme from the Indian *Daboia russelii russelii* Venom Targets Factor X and Factor Xa for Its Anticoagulant Activity

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Abstract

In the present study a major protein has been purified from the venom of Indian *Daboia russelii russelii* using gel filtration, ion exchange and Rp-HPLC techniques. The purified protein, named daboxin P accounts for ~24% of the total protein of the crude venom and has a molecular mass of 13.597 kDa. It exhibits strong anticoagulant and phospholipase A_2 activity but is devoid of any cytotoxic effect on the tested normal or cancerous cell lines. Its primary structure was deduced by N-terminal sequencing and chemical cleavage using Edman degradation and tandem mass spectrometry. It is composed of 121 amino acids with 14 cysteine residues and catalytically active His48 -Asp49 pair. The secondary structure of daboxin P constitutes 42.73% of α-helix and 12.36% of β-sheet. It is found to be stable at acidic (pH 3.0) and neutral pH (pH 7.0) and has a Tm value of 71.59 ± 0.46°C. Daboxin P exhibits anticoagulant effect under *in-vitro* and *in-vivo* conditions. It does not inhibit the catalytic activity of the serine proteases but inhibits the activation of factor X to factor Xa by the tenase complexes both in the presence and absence of phospholipids. It also inhibits the tenase complexes when active site residue (His48) was alkylated suggesting its non-enzymatic mode of anticoagulant activity. Moreover, it also inhibits prothrombinase complex when pre-incubated with factor Xa prior to factor Va addition. Fluorescence emission spectroscopy and affinity chromatography suggest the probable interaction of daboxin P with factor X and factor Xa. Molecular docking analysis reveals the interaction of the Ca^{+2} binding loop; helix C; anticoagulant region and C-terminal region of daboxin P with the heavy chain of factor Xa. This is the first report of a phospholipase A_2 enzyme from Indian viper venom which targets both factor X and factor Xa for its anticoagulant activity.

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Introduction

Haemostasis, one of the most important physiological processes of vertebrates, involves four crucial steps for sustaining equilibrium, namely, (i) vasoconstriction, to reduce blood flow from the site of injury (ii) platelet activation, aggregation and adherence leading to the platelet plug formation at the injured site (iii) initiation of coagulation cascade involving the extrinsic, intrinsic and the common pathway, forming fibrin mesh on the platelet plug and (iv) fibrinolysis leading to the dissolution of the clot formed, in order to restore normal blood flow [1]. Any malfunction in this vital process leads to two major pathophysiological conditions, haemorrhage or thrombosis, at large. Interestingly, the components of the haemostatic system of the prey/victim are one of the most vulnerable targets in snake envenomation. Owing to this, there has been a quest amongst the venom researchers to unfold the underlying mechanism and explore the therapeutic potentiality of these venom proteins since the last few decades. Recent trend of research reveals a pursuit for direct inhibitors of factor Xa (FXa) and thrombin from the venom of snakes and saliva of hematophagous animals as one of the most thrived components for anticoagulant and antithrombotic drug discovery [2–5].

Daboia russelii russelii (Daboia r. russelii), a venomous snake of the Indian subcontinent, contains an intricate blend of various biologically active protein families. Consumptive coagulopathy is one of the most manifested systemic pathological condition observed in viper envenomated victims [6]. The procoagulant venom proteins like RVV-X and RVV-V activators accelerate the blood-coagulation cascade and the simultaneous down regulation of activated protein C causes severe depletion of the coagulation factors thereby depriving the victim of the serine proteases shortly [7]. This scenario is further worsened by the anticoagulant and haemorrhagic components like phospholipase A_2 (PLA_2) enzymes, L-amino acid oxidase (LAAO) and metalloproteases leading to hypovolemic shock and even death [8].

PLA_2 enzymes are one of the major protein families in Russell's viper proteome with various isoforms [9, 10]. Although the members of this family share a highly conserved structural and catalytic scaffold, plethora of pharmacological activities are reported among the isoforms apart from prey digestion [11–13]. The subtle differences in their activities is mainly attributed to the presence of various pharmacological sites (responsible for non-enzymatic activity via protein-protein interaction) which are distinct from the catalytic site (responsible for enzymatic activity via protein-phospholipid interaction) [10]. The presence of minor amino acid variations in the exposed regions of these isoforms one of the vital causes of diverse pharmacological sites [14]. The ability of the snake venom PLA_2 enzymes to inhibit blood coagulation was first reported by Boffa & Boffa in *Vipera berus* venom [15]. Henceforth, many anticoagulant PLA_2 enzymes have been reported from snake venom by several researchers. These enzymes are classified into strong, weak and non-anticoagulant based on the concentration required to delay clot formation and amino acid residues in the predicted anticoagulant region (54^{th} to 77^{th} residues) [16–18]. These enzymes act either by hydrolyzing the procoagulant phospholipids or target the coagulation factors for its activity. CM-I and CM-II from *Naja nigricollis* and Vipoxin from *Vipera ammodytes ammodytes* hydrolyze the phospholipids required for the formation of the extrinsic and intrinsic tenase complex [19–21]. CM-IV from *Naja nigricollis*, AtxA from *Vipera ammodytes ammodytes*, CBc from *Crotalus durissus terrificus* and MtxII from *Bothrops asper*, target FXa of the prothrombinase complex and impede the conversion of prothrombin to thrombin [22–25]. While Nn-PLA_2 from *Naja naja* and Nk-PLA_2-β from *Naja kaouthia* target thrombin directly to exhibit anticoagulant effect [26, 27]. On the other hand, VRV-PL-IIIb from *Daboia russelii*, CHA-E6b & CHA-E6a from *Crotalus horridus* and Tj-PLA_2 from *Trimeresurus jerdoni* inhibit ADP and collagen induced platelet aggregation [28–30].

In the present study we have isolated and characterized a strong anticoagulant PLA$_2$ enzyme, from the venom of Indian *Daboia russelii russelii*. Enzymatically it hydrolyses the phospholipids required for the formation of the tenase complexes and non-enzymatically it targets factor X (FX) and activated factor X (FXa) for exhibiting its anticoagulant activity. Hence, this purified anticoagulant PLA$_2$ enzyme was named as daboxin P (*Daboia russelii russelii* FX inhibitor PLA$_2$ enzyme).

Materials and Methods

Crude venom and chemicals/reagents

Crude venom of *Daboia r. russelii* (1 gm) was purchased from Irula Snake Catchers Society, Tamil Nadu, India. Few individuals (1–3: 4 individuals) of each snake species are caught from the forest and captivated for nearly 3–4 weeks before venom extraction. During this period venom is milked from the snakes for 4–5 times before releasing back to the forest. None of the captivated snakes are harmed or killed. Secretory phospholipase A_2 (sPLA$_2$) assay kit was obtained from Cayman Chemical Company (MI, USA). 4-vinyl pyridine, hydroxylamine hydrochloride, Cyanogen bromide activated Sepharose 4B, DB and bovine plasma fibrinogen were purchased from Sigma-Aldrich (MO, USA). BNPS-skatole [2-(2-Nitrophenylsulfenyl)-3-methyl-3-bromoindolenine)] was purchased from Bioworld (OH, USA). The Edman sequencing reagents were purchased from Applied Biosystems chemicals (Foster city, CA). The rest of the reagents and chemicals were of analytical grade and obtained from Merck Millipore (MA, USA) or Sigma (MO, USA).

Enzymes and chromogenic substrates

Serine proteases factor XIa (FXIa), factor IXa (FIXa), factor VIIa (FVIIa), factor X (FX), factor Xa (FXa) and Russell's viper venom X-activator (RVV-X) were obtained from Haematologic Technologies Inc. (Vermont, USA). Factor XIIa (FXIIa) was procured from Merck Calbiochem (Darmstadt, Germany), factor VIII (FVIII) from Creative Biomart (NY, USA), phospholipid blend from Avanti Polar Lipids Inc. (Alabama, USA) and tissue factor Innovin from Siemens (Marburg, Germany). The chromogenic substrates namely spectrozyme (MeSO$_2$-D-CHG-Gly-Arg-pNA.AcOH) was purchased from Sekisui Diagnostics (MA, USA), rest of the substrates like S-2366 (pyroGlu-Pro-Arg-pNA•HCl), S-2302 (H-D-Pro-Phe-Arg-pNA•2HCl), S-2222 (Bz-Ile-Glu(γ-OR)-Gly-Arg-pNA•HCl), S-2765 (Z-D-Arg-Gly-Arg-pNA•2HCl), S-2238 (H-D-Phe-Pip-Arg-pNA•2HCl) and S-2288 (H-D-Ile-Pro-Arg-pNA•2HCl) were procured from Chromogenix (NJ, USA).

Cell culture

Human embryonic kidney (HEK) 293 and Michigan Cancer Foundation (MCF)-7 cell lines were purchased from National Centre for Cell Science (Pune, India). Dulbecco modified Eagle's media (DMEM) was obtained from Himedia (Mumbai, India). 3-(1,3-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue from Sigma-Aldrich (Mo, USA), fetal bovine serum (FBS), streptomycin-penicillin (Strep-Pen), trypsin/EDTA solution and dimethyl sulfoxide (DMSO) were procured from Thermo Fischer (MA, USA).

Columns

Hiload^{TM} 16/600 Superdex 75 prep grade and Hiprep CM FF 16/10 columns were purchased from GE Healthcare life Sciences (Bucks, UK). Jupiter C_{18} column was procured from Phenomenex (CA, USA) and Hypersil Gold C_{18} column was obtained from Thermo Scientific (MA, USA).

Purification of the major anticoagulant protein

Crude *Daboia r. russelii* venom was fractionated on Hiload™ 16/600 Superdex 75 prep grade column (1x120 ml) pre-equilibrated with 50 mM of Tris-Cl pH 7.4 using Äkta Purifier HPLC system, GE Healthcare (Uppsala, Sweden). Fractionation was carried out at a flow rate of 1 ml/min under isocratic conditions with the same buffer. Each eluted fraction was assayed for PLA_2 and anticoagulant activity. P6 with highest anticoagulant and PLA_2 activity was further subjected to cation exchange chromatography on Hiprep CM FF 16/10 (1.6x10 cm) using Äkta Purifier HPLC system. Elution was carried out at a flow rate of 2.25 ml/min with a linear gradient of 50 mM Tris-Cl, pH 7.4 containing 0.8 M NaCl. Ion exchange fraction, CM-II with highest PLA_2 and anticoagulant activity was subjected to Rp-HPLC using Jupiter C_{18} column (3 μ, 4.6 x 250 mm, 300 Å). The column was pre equilibrated with milli q containing 0.1% trifluoro acetic acid (TFA) and fractionated using 80% acetonitrile (MeCN) containing 0.1%TFA on Äkta Purifier HPLC system. The homogeneity of the purified protein (5 μg of the protein treated with 0.45 μl of β-mercaptoethanol) was validated on SDS-PAGE and stained using Pierce™ silver staining kit, Life technologies, Thermo Fischer Scientific (MA, USA) [...]. This Rp-HPLC purified protein, named as daboxin P was used for characterization in the following experiments.

Biophysical characterization

Determination of molecular mass. Molecular mass of daboxin P was determined by electrospray ionization mass spectrometry (ESI-MS) using LCQ fleet Ion Trap, Thermo Scientific (MA, USA). Ion spray voltage was set at 4.4 kV. For nebulization nitrogen gas was used. Solvent (50% MeCN containing 0.1% formic acid) was delivered into the system at a flow rate of 50 μl/min by Accela 600 pump. Spectra were recorded in positive mode with mass to charge (m/z) ranging from 800 to 2000. Analysis and deconvolution of the spectra was performed by Promas for Xcaliber.

Primary structure determination. N-terminal sequencing: N-terminal sequencing of the native purified protein was performed by automated Edman degradation process using Protein sequencer PPSQ 31A Shimadzu Asia Pacific, (Singapore). Briefly, 89 μg (6345.56 pmol) of the purified protein (dissolved in 100 μl of milli q water) was dried on PVDF membrane and loaded onto the sequencer. Sequencing was performed for 30 cycles or till the chromatogram was readable.

Pyridylethylation: Prior to chemical cleavage, daboxin P was reduced and alkylated according to the method developed by Joseph and colleagues [...]. Briefly, 1 mg of daboxin P was dissolved in 920 μl of denaturing buffer (6 M guanidine hydrochloride and 0.1 M Tris-Cl pH 8.5) and treated with β-mercaptoethanol (30 μl). N_2 gas was purged over the reaction mixture to expel any dissolved oxygen and incubated at 37°C for 150 min. 50 μl of 4-vinyl pyridine (containing 100 ppm hydroquinone) was added and further incubated at 37°C for 150 min. The pyridylethylated sample was desalted by Rp-HPLC on Jupiter C_{18} column (4.6 x 250 mm, 3 μm, 300 Å) with a linear gradient of 80% MeCN containing 0.1% TFA at a flow rate of 0.8 ml/min. Elution was monitored at 215 and 280 nm.

Chemical cleavage: The chemical cleavage of pyridylethylated protein by BNPS-skatole and hydroxylamine hydrochloride were carried out according to the protocol developed by Crimmins and co-workers and Milner and colleagues respectively with minor modifications [...]. For cleavage with BNPS-skatole, 900 μg of the pyridylethylated protein was dissolved in 0.1% of TFA containing 6 M guanidine hydrochloride (pH 5.0). To this 1.5 mg/ml of BNPS-skatole (dissolved in 100% acetic acid) was added and incubated at 37°C for 24 h. The reaction was stopped by addition of equal volume of milli q water. Centrifugation was carried out at 12,000 rpm for 20 min and supernatant was collected.

For cleavage with hydroxylamine hydrochloride, 1 mg of pyridylethylated protein was dissolved in denaturing buffer (6 M guanidine hydrochloride, 0.1 M Tris-Cl, pH 9.0, 2 M hydroxylamine hydrochloride and 0.2 M K_2CO_3) and incubated at 45°C for 4 h. After the cleavage experiments, the samples were subjected to Jupiter C_{18} column using a linear gradient of 5–50% of 80% MeCN containing 0.1% TFA. The mass of the fractions obtained were checked using ESI-MS (Accela LCQ fleet ion trap, Thermo scientific, U.S.A) for the cleaved and uncleaved peptide fragments. The cleaved fractions were lyophilized and reconstituted in 100 μl of milli q water and loaded onto PVDF membrane for Edman degradation using Automated Protein sequencer PPSQ 31A, Shimadzu.

Enzymatic cleavage/ Tandem mass spectrometry: Daboxin P (50 μg) was treated with 50 mM of ammonium bicarbonate, 1% Protease Max and 0.5 M dithiothreitol at 56°C for 20 min. For alkylation, 550 mM of iodoacetamide was added and incubated in dark for 15 min. Following this, trypsin (1 μg/μl in 50 mM acetic acid) in the presence of 1% Protease Max was added to the reaction and incubated at 37°C for 3 h. Reaction was stopped by 100% TFA. Centrifugation was carried out at 12,000 rpm for 10 min and the supernatant was subjected to Tandem mass spectrometry as described previously [ ]. The MS/MS spectra obtained were analyzed using Proteome Discoverer 3.1 with Sequest program.

Sequence alignment and phylogenetic analysis. PLA$_2$ enzymes having maximum sequence similarity with daboxin P were retrieved by blastp analysis from the NCBI database and aligned using multiple sequence alignment software DNAMAN 4.13.5.1 (Lynnon BioSoft). The phylogenetic relationship among the PLA$_2$ enzymes exhibiting anticoagulant activity were determined by the Mega 5.05 software using neighbor joining method with a bootstrap of 1000 replicates [ ]. The evolutionary distance among the sequences was calculated with p-distance.

Secondary structure determination. The secondary structure was analyzed by circular dichroism (CD) spectroscopy using Jasco Spectropolarimeter J-810 (Tokyo, Japan). Briefly, 200 μl of 0.4 mg/ml of daboxin P (dissolved in milli q water) was subjected to the spectropolarimeter. The spectrum of the native protein was recorded at far-UV scan from 260–190 nm at a speed of 50 nm/min in quartz cuvette (0.1cm path length) at room temperature.

The effect of different pH (phosphate buffered saline at pH 3.0, 7.4 & 12) and temperature (from 25°C to 100°C) on structural conformation of daboxin P (0.4 mg/ml) was determined under the same experimental conditions as described above. Three scans were recorded for each experimental condition and subtracted from blank to obtain the final spectra of the protein.

Biochemical and biological characterization

PLA$_2$ activity. The PLA$_2$ activity of daboxin P was assayed using the sPLA$_2$ assay kit (Cayman, MI, USA) according to the manufacturer's protocol using diheptanoylthio phosphatidylcholine as substrate. The specific activity was expressed in micromoles of phosphatidylcholine hydrolyzed per min per mg of enzyme. Bee venom PLA$_2$ enzyme (0.001 μg/μl) was considered as the positive control for the experiment. The results are mean ± standard deviation (SD) of three independent experiments.

Alkylation. The alkylation of His48 of daboxin P was performed as described by Maduwage and co-workers [ ]. Briefly, 150 μg of daboxin P was treated with 15 μl of p-Bromophenacyl Bromide (pBpB) (100 μg dissolved in 30 μl of 100% ethanol). Equal amount of the protein was treated with 15 μl of ethanol as the vehicle control. The reaction mixtures were incubated at room temperature for 20 h and subsequently dialyzed in 20 mM of Tris-Cl, pH 7.4 at 4°C for 3 times. The dialyzed sample was lyophilized in a pre-conditioned lyophilizer (12N-C, Ilshin tech, South Korea) at -80°C. The lyophilized sample was reconstituted in appropriate buffer to carry out the desired assays. The extent of alkylation was verified by analyzing it using ESI-MS.

Cytotoxicity study. Cell culture: The HEK-293 and MCF-7 cell lines were grown in DMEM media enriched with 10% FBS and 1% antibiotic (Strep-Pen). The cells were grown on 96 well plate to a confluence of 70–80% at 37°C for 2–3 days in CO_2 (5%) incubator (Eppendorf, Hamburg, Germany). Cell viability was assessed by trypan blue stain and the cells were quantified on a haemocytometer.

The cytotoxic activity of the crude venom of *Daboia r. russelii* and daboxin P were determined by the MTT (3-(1,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based colorimetric method [ ]. Briefly, in two independent set of experiments, the HEK-293 and MCF-7 cells were plated on 96 well plate at a concentration of $1x10^5$ cells/well and incubated for 48 h at 37°C in CO_2 (5%) incubator. Subsequently, the cells were incubated with different concentration of crude *Daboia r. russelii* venom and daboxin P for 24 h at 37°C in CO_2 (5%) incubator. Cells treated with 0.9% NaCl was considered as the vehicle control. After incubation, the changes in morphological pattern (if any) of the treated cells were observed in an Inverted microscope (Axio Vert A1, Zeiss, Jena, Germany) at 10X magnification. 20 μl of MTT (5 mg/ml) was added and incubated at 37°C for 2 h. Following this, the formazan crystals formed by the viable cells were dissolved by 150 μl of MTT solution. The amount of formazan formed by the viable cells was quantified at 590 nm using MultiSkan Go spectrophotometer (Thermo Scientific, MA, USA). The percentage of cell viability was calculated by considering the cells without the venom treatment as 100% viable. The results are mean ± SD of three independent experiments.

***In vitro* anticoagulant activities.** The anticoagulant experiments were carried out with platelet poor human plasma (PPP) (whole blood centrifuged at 3000 rpm for 20 min at 16°C) and clot formation was monitored using Tulip Coastat 1 coagulo-analyser (Alto Santa Cruz, Goa, India). For each assay, clotting time of plasma with Tris-Cl buffer (20 mM, pH 7.4) was taken as the normal clotting time. The results are mean ± SD of three independent experiments.

Recalcification time (RT): Different concentrations of daboxin P (0.001, 0.01, 0.1, 1 μM) were pre-incubated with 150 μl of plasma at 37°C for 2 min [ ]. Formation of plasma clot was initiated by addition of 100 μl of 50 mM $CaCl_2$.

Activated partial thromboplastin time (APTT): APTT was determined using Liquicelin (APTT reagent) according to the manufacturer's instructions. Briefly, different concentrations of the purified protein (0.01, 0.1, 1 μM) were pre-incubated with human plasma (50 μl) and APTT reagent (50 μl) for 3 min at 37°C. Plasma clot was formed by addition of 25 mM $CaCl_2$ (50 μl).

Prothrombin time (PT): PT was measured using Uniplastin (PT reagent) according to the instructions of manufacturer. In brief, different concentrations of daboxin P were pre-incubated with 50 μl of human plasma at 37°C for 2 min. PT reagent (50 μl) was added to initiate the clot formation.

Stypven time: Briefly, in a reaction volume of 300 μl different concentrations of daboxin P were pre-incubated with human plasma (75 μl) for 3 min at 37°C [ ]. To this 75 μl of RVV-X (10 ng/ml) was added and incubated for 2 min at the same temperature. Clotting was initiated by addition of 25 mM of $CaCl_2$.

Thrombin time: To determine the thrombin time, different amounts of the protein (1, 3, 5 μg) were incubated with human plasma (50 μl) for 3 min at 37°C [ ]. To this 50 μl of thrombin (10 u/ml) was added to initiate clot formation.

Fibrinogenolytic activity: Fibrinogenolytic activity was determined according to the method developed by Ouyang C and Teng CM [ ]. In brief, different amounts of daboxin P were pre-incubated with 2 mg/ml fibrinogen (dissolved in 50 mM Tris-Cl, pH 7.5, 0.15 M NaCl) for 24 h at 37°C. For positive control, equal amount of fibrinogen was incubated with thrombin (3 μl of

10 units/ml) while for negative control fibrinogen with 50 mM Tris-Cl, pH 7.5 was incubated under the same experimental conditions. The formation of fibrinogen degradation products were analyzed on 12.5% glycine SDS-PAGE stained with Coomassie Brilliant Blue R-250.

***In-vivo* anticoagulant activity.** The *in-vivo* anticoagulant activity of daboxin P was determined by the $FeCl_3$ induced carotid artery thrombosis model with minor modifications [10]. Briefly, C57Bl/6 male mice (9–11 weeks old, 24–28 g) were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg) by intraperitoneal injection (i.p.). Daboxin P (10 mg/kg) was injected into the mice (n = 6) via its tail vein. Following this, the right carotid artery was exposed using blunt dissection and a doppler flow probe (Model MA0.5VB, Transonic Systems Inc., Ithaca, NY, USA) connected to a perivascular flow module (TS420, Transonic Systems Inc., Ithaca, NY, USA) was then attached to the carotid artery to monitor blood flow. A 2x2 mm piece of filter paper (soaked in 10% $FeCl_3$ solution) was placed on the surface of the carotid artery for 3 min to initiate the thrombus formation. Saline treated mice (n = 5) were considered as the negative control for the experiment. Time-to-occlusion (TTO) is defined as the time taken for the blood flow to reach zero after the application of $FeCl_3$. Maximum measurement time was considered for 60 min after the application of $FeCl_3$. TTO was recorded as 60 min, if no occlusion occurred by this time. The animal experiments were approved by Institutional Animal Care and Use Committee, National University of Singapore.

Serine protease specificity. The effect of daboxin P on the amidolytic activity of serine proteases were determined using specific chromogenic substrates. The activated serine proteases involved in the extrinsic and intrinsic tenase complex namely, FXIIa (60 nM), FXIa (0.125 nM), FIXa (333 nM), FXa (0.43 nM), FVIIa (10 nM), sTF (30 nM) were reconstituted in the activation buffer containing 50 mM Tris-Cl pH 7.4, 1 mg/ml of BSA, 140 mM NaCl and 5 mM $CaCl_2$ [ ]. The respective chromogenic substrates, S-2302 (1 mM), S-2366 (1 mM), spectrozyme (0.4 mM), S-2765 (0.65 mM) and S-2288 (500 μM) were reconstituted in milli q water to the desired working concentration. Briefly, to a reaction volume of 200 μl, each of the serine proteases was pre-incubated with different concentration of daboxin P (0.01, 0.1 and 1 μM) for 5 min. Subsequently, respective chromogenic substrates were added to trigger the amidolytic reaction. For each assay, the rate of p-nitroaniline formation upon hydrolysis of the substrate by the specific enzyme was quantified at 405 nm using UV-Vis MultiSkan GO spectrophotometer. The hydrolysis of substrate in the absence of daboxin P was considered as 100%.

Reconstitution of the tenase complexes. Extrinsic tenase complex (ETC) with and without phospholipids: The extrinsic tenase complex was reconstituted under *in-vitro* conditions in the presence of FVIIa (5 nM) and Innovin (10X) (tissue factor, thromboplastin and Ca^{++} ions) with 67 μM of phospholipid vesicles (DOPC:DOPS 7:3) in the activation buffer. The reaction mixture was incubated at 37°C for 15 min [ ]. Similarly, the complex was formed without phospholipids with 20 nM of FVIIa and soluble tissue factor, sTF (60 nM) and incubated for 15 min at 37°C. To both the experiments, different concentrations of daboxin P (0.01, 0.1, 1 and 3 μM) were added and incubated for 15 min. Following this, the reactions were incubated with FX (30 nM) for 15 min. Both the experiments were stopped by addition of quenching buffer (50 mM Tris-Cl pH 7.4, 1 mg/ml of BSA, 140 mM NaCl and 50 mM EDTA). The amount of FXa generated was determined by addition of 500 μM of S-2222 and the rate of hydrolysis was quantified at 405 nm using UV-Vis MultiSkan GO spectrophotometer.

Intrinsic tenase complex with and without phospholipids (ITC): Reconstitution of the intrinsic tenase complex under *in-vitro* conditions was carried out in the presence of phospholipids (67 μM) in activation buffer with FVIIIa, FIXa, FX and Ca^{++} ions at 37°C [ ]. Briefly, 3 nM of FVIII was incubated with 500 pM of thrombin for 10 min to activate FVIII. Thrombin was then deactivated by 10 nM of variegin, a thrombin inhibitor from *Amblyomma variegatum* [ ]. After incubation of the reaction mixture with 1 nM of FIXa for 10 min different

concentrations of daboxin P (0.01, 0.1, 1 and 3 μM) were added and incubated for 15 min. Following this, 25 nM of FX was added and incubated for 15 min. Similarly, the complex was formed devoid of the phospholipid blend [ ] with FVIII (40 nM) and thrombin (4 nM) and incubated for 10 min. Thrombin was deactivated with 40 nM of variegin. To this 10 nM of FIXa was added and incubated for 10 min. Then, different concentrations of daboxin P (0.01, 0.1, 1 and 3 μM) were incubated for 15 min. Finally, 1 μM of FX was added and incubated for 15 min. The reactions in both the experiments were terminated by addition of the quenching buffer. The amount of FXa generated was quantified by the hydrolysis of the chromogenic substrate S-2222 (500 μM) at 405 nm using UV-Vis MultiSkan GO spectrophotometer.

For determining the inhibitory concentration (IC50) of daboxin P on extrinsic and intrinsic tenase complex, different concentration of daboxin P were pre-incubated with each of the reconstituted complexes described above in the presence of phospholipids in the activation buffer.

Prothrombinase complex: Prothrombinase complex was reconstituted in the presence of phospholipid (6% μM) with FXa, FVa, and Ca^{+2} ions under *in vitro* conditions at 37°C [ ]. Briefly, 10 pM of FXa was pre-incubated with 1 nM of FVa for 15 min. To this, different concentrations of daboxin P (0.01, 0.1, 1 and 3 μM) were added and incubated for 15 min. Thereafter, prothrombin (12.5 nM) was added and the reaction mixture was incubated for another 15 min. In another set of experiment, FXa (10 pM) was pre-incubated with different concentrations of daboxin P followed by incubation with FVa (1 nM) and prothrombin (12.5 nM) for 15 min each. For both the experiments, the reactions were stopped with quenching buffer. The amount of thrombin generated by the complex was determined by the rate of hydrolysis of the chromogenic substrate S-2238 (250 μM) at 405 nm using UV-Vis MultiSkan GO spectrophotometer.

Fluorescence emission spectroscopy. Interaction of daboxin P with the coagulation factor FX and FXa were analyzed using fluorescence emission spectrophotometer (LS 55, Perkin Elmer, Palo Alto, CA). In brief, 1 μM of daboxin P (dissolved in 20 mM Tris-Cl, pH 7.4) was incubated with either 0.05 μM of FX or 0.1 μM of FXa (reconstituted in the same buffer) for different time intervals (10 min & 20 min) at room temperature in the presence and absence of 5 mM $CaCl_2$. The change in the fluorescence emission spectra of the individual components and the mixtures were measured over a wavelength of 200 to 500 nm with an excitation wavelength of 280 nm at room temperature using a quartz cuvette (1 cm path length).

Affinity chromatography. The association of daboxin P with FX and FXa were evaluated by affinity column chromatography using CNBr activated sepharose κ 4B (Sigma, Aldrich). Briefly, 40 μg of daboxin P was covalently linked to 0.1 g of CNBr activated matrix (swollen overnight in ice cold 1 mM HCl) in the presence of coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3. Subsequently, the unbound protein molecules of daboxin P were washed away with the same buffer. To avoid non-specific binding, the immobilized matrix was treated with 0.2 M glycine for 2 h with gentle shaking at room temperature. The blocking solution was removed by an alternative wash with acidic (0.1 M acetate buffer, pH 4.0) and basic buffer (coupling buffer) for 5 times. FX or FXa (8 μg) were added to the daboxin P immobilized matrix and incubated for 1 h at room temperature with mild shaking. The unbound FX or FXa molecules were washed away with the same buffer. Elution of FX or FXa was carried out with 20 mM HCl containing increasing molar concentrations of NaCl (0.5, 1, 1.5 and 3 M). The flow through and eluents were analyzed on 12.5% glycine SDS-PAGE under non-reducing condition and visualized after staining with Pierce™ silver staining kit.

Modelling and molecular docking studies. Three dimensional (3D) molecular modelling: For the *in silico* 3D modelling of daboxin P, online server I-TASSER was employed [ ]. This server predicts the secondary conformation of a given protein by profile-profile alignment

(PPA) threading techniques. The model with maximum C-score and TM score was selected for generation of ribbon model using DS ViewerPro 5.0 (Accelrys Inc.) software. Superimposition of the predicted model with the crystal structure of ammodytoxin A, AtxA (Pdb 3G8G), was carried out using DS ViewerPro 5.0 software.

Molecular docking: The equilibrated model of daboxin P was docked with FXa (PDB: 2BOH) using the online web server, PatchDock [ ]. This server uses the geometry based docking algorithm to find optimum candidate solutions and the RMSD (root mean square deviation) clustering to remove redundant solutions [ ]. Each solution was given score based on the geometric fit as well as atomic desolvation energy [ ]. In the present analysis, a default RMSD value of 4 Å was considered for clustering solutions. The ribbon model of the docked complex of daboxin P and FXa was generated using DS ViewerPro 5.0 (Accelrys Inc.).

Identification of interface residues and hot spot residues: The daboxin P-FXa docked model with maximum surface contact area and minimum free energy generated by PatchDock server was selected for evaluating the interface residues using PDBsum server [ ]. The interface residues of a protein are those residues whose contact distances from the interacting protein partner are less than 6 Å [ ].

Contact map analysis: The interaction between the chains of FXa and daboxin P were also analyzed using CMA (Contact Map Analysis), one of the servers of the online software SPACE (Structure Prediction and Analysis based on Complementarity with Environment) [ ]. The server analyses the contact area between the residues of two protein chains or within a single chain of a given PDB file based on shape and chemical complementarity. It displays residue to residue contacts for a pair of amino acid residues involved in the interaction in the form of contact map. Contact maps provide more concrete demonstration of protein structure than its 3D atomic coordinates. For investigating the residue-residue contacts between daboxin P and FXa, a contact area threshold above 8 Å^2 has been considered for the present analysis.

Results

Purification of daboxin P, an anticoagulant PLA_2 enzyme

Fractionation of crude venom of *Daboia r. russelii* on size exclusion chromatography has resolved it into 8 prominent peaks (P1 to P8) ( ). Each individual peaks were screened for PLA_2 and anticoagulant activity. Peaks, P1 to P5 were found to be devoid of PLA_2 activity but exhibited procoagulant effect on human plasma while peaks P6 to P8 showed PLA_2 activity and anticoagulant effect on human plasma ( ). Peak P6 which exhibited highest anticoagulant (>600 s) and PLA_2 activity (93.69 μmol/min) was subjected to cation exchange chromatography. It resolved P6 into one major (CM-II) and two minor peaks (CM-I & CM-III) ( ). The major peak CM-II which showed highest anticoagulant (>600 s) and PLA_2 activity (77.82 μmol/min) ( ), was further fractionated using Rp-HPLC ( ) which resolved into one minor (Rp-1) and one major (Rp-2) peaks ( ). The homogeneity of the major peak, Rp-2 with highest PLA_2 activity and anticoagulant activity was assessed by SDS-PAGE under reduced state and named as daboxin P ( ). The molecular mass of the protein was determined by ESI-MS which showed the presence of 6 mass to charge (m/z) ratio peaks ranging from +8 to +13 charges corresponding to a deconvoluted molecular mass of 13597.62 ± 1.28 Da ( ).

Biophysical characterization

Sequencing. The first 30 residues were determined by N-terminal sequencing ( ). The cleavage of pyridylethylated daboxin P with BNPS-skatole yielded two peptide fragments of molecular mass 11.7 kDa and 3.6 kDa (data not shown) while cleavage with hydroxylamine

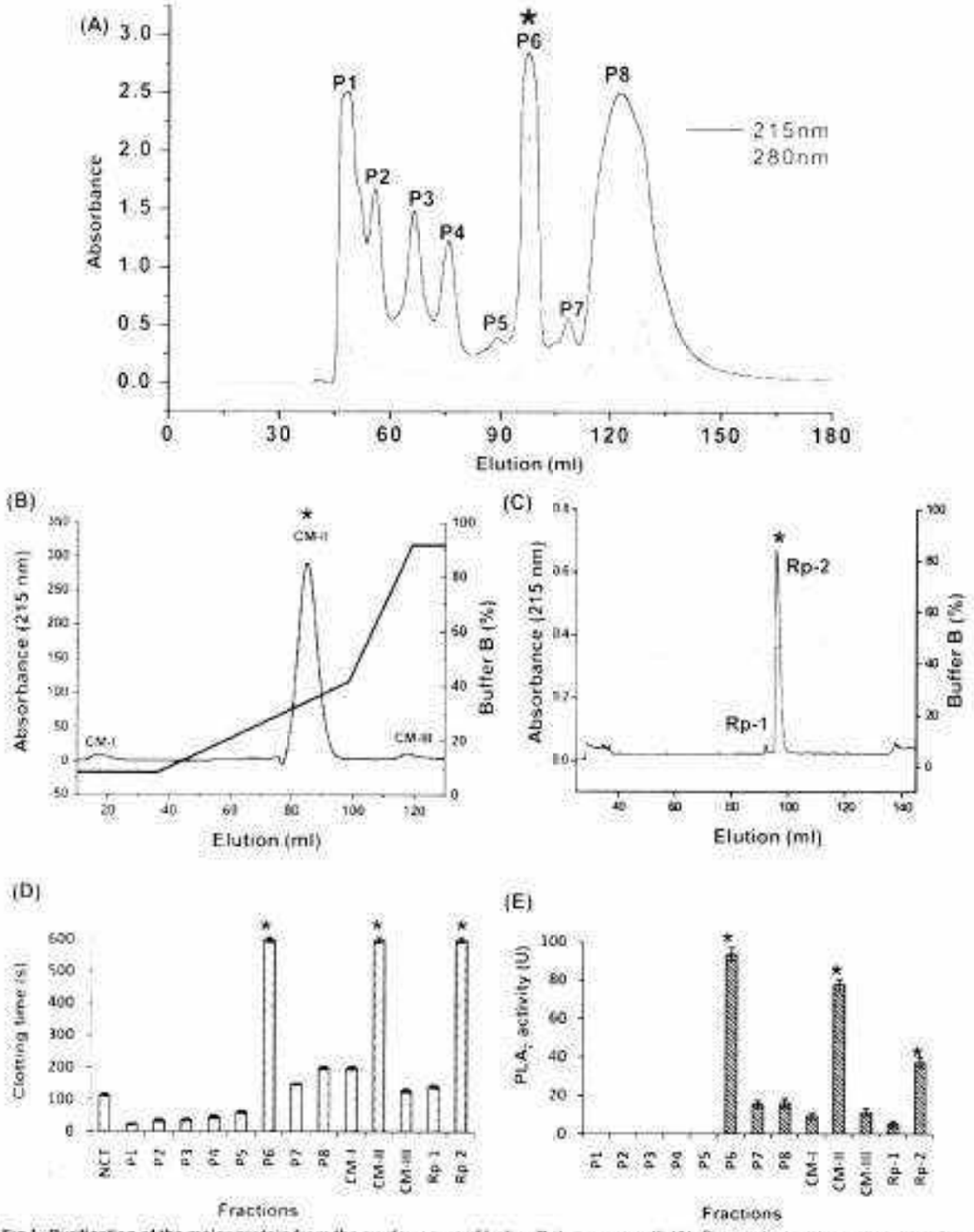

Fig 1. Purification of the major protein from the crude venom of Indian *Daboia r. russelii*. (A): Size exclusion chromatography of crude *D. r. russelii* venom. 20 mg of crude venom was dissolved in 50 mM of Tris -Cl pH 7.4 and fractionated on Hiload 16/600 superdex 75 preparative grade column pre-equilibrated with the same buffer. Fractions were eluted at a flow rate of 1 ml/min and monitored at 215 & 280 nm. For each, 1 ml fractions were collected and peaks were pooled (P1 to P8). **(B):** Ion exchange chromatography profile of P6. The gel filtration peak, P6 was loaded onto CM FF 16/10, a weak cation

exchanger column pre-equilibrated with 50 mM of Tris-Cl pH 7.4. Fractionation was carried out at a flow rate of 2.25 ml/min and eluted with a linear gradient of the same buffer containing 0.8 M NaCl and monitored at 215 nm. **(C):** Rp-HPLC profile of CM-II: ion exchange fraction CM-II was loaded on Jupiter C_{18} column pre-equilibrated with buffer A (0.1% TFA). Fractionation was carried out at a flow rate of 0.8 ml/min with a linear gradient of buffer B (80% MeCN -0.1%- TFA) and monitored at 215 nm. **(D):** Recalcification time of the fractions obtained from the chromatographic steps. Clotting time of plasma in presence of Tris-Cl buffer (20 mM, pH 7.4) was considered as normal clotting time (NCT). 1 μg of each fraction was incubated with plasma for 2 min followed by addition of 50 mM $CaCl_2$ to initiate clot formation which was monitored using Tulip Coastal-1 coagulo analyser. **(E):** PLA_2 activity of the chromatographic fractions using $sPLA_2$ assay kit. 0.01 μg of each fraction was used for screening PLA_2 activity using dihexanoylthio-phosphatidylcholine as the substrate. The amount of substrate hydrolyzed was quantified at 414 nm for 10 min at room temperature. * indicates the peak of interest in each purification step.

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hydrochloride resulted into two peptide fragments of molecular mass 6.6 kDa and 8.6 kDa (data not shown). Sequencing of these peptides by Automated Edman degradation revealed the rest of the amino acid residues ( ). On the other hand from tandem mass spectrometry, eleven peptide fragments of daboxin P were obtained (Table A in). These peptide fragments obtained by chemical and enzymatic cleavage were assembled and overlapped to decipher the complete sequence of daboxin P ( ). The homology of the protein sequence was analyzed by multiple sequence alignment with PLA_2 enzymes using online blastp algorithm as represented in .

Sequence analysis. The calculated molecular mass of daboxin P was found to be 13596.70 Da which is in agreement with the observed mass of 13597.62 ± 1.28 Da from ESI-MS. It has 121 amino acid with 14 cysteine residues which should correspond to 7 disulphide bonds, a characteristic feature of group IIA viper PLA_2 enzymes [ ]. It has a theoretical pI of 8.35 calculated by online software ExPASy ProtParam [ ]. At the active site, histidine residue was observed followed by an aspartate residue, a common trait for Ca^{++} dependent catalytic PLA_2 enzymes [ ]. At the predicted anticoagulant region (53NLPDCNNKSKRYRYKK68)

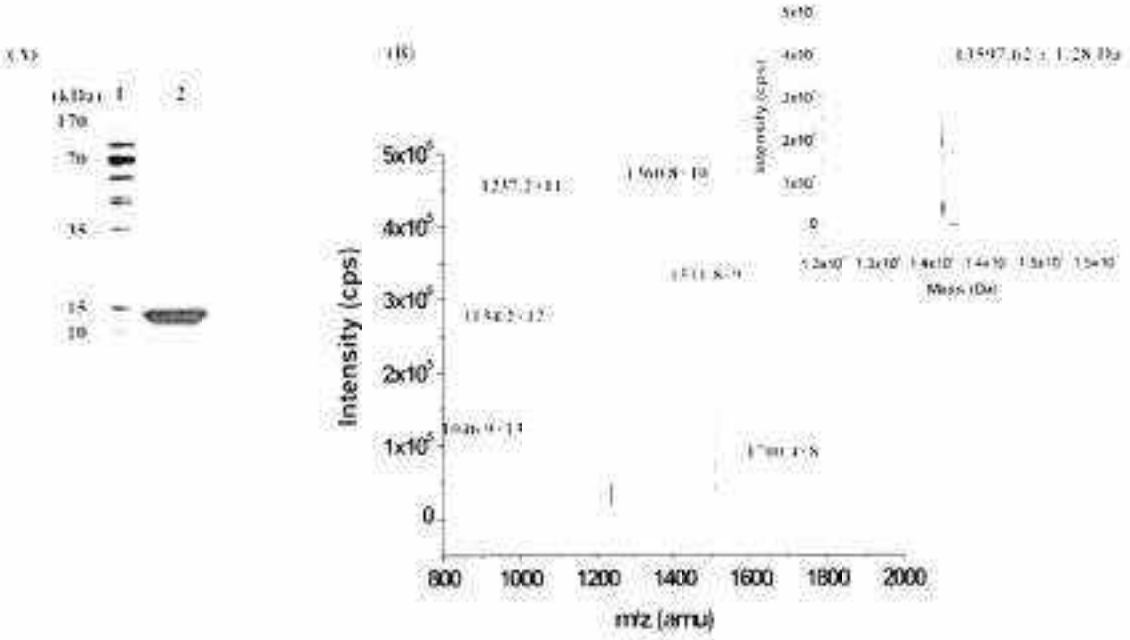

Fig 2. Homogeneity of the purified protein, daboxin P. (A) 12.5% glycine SDS-PAGE profile of the purified protein after silver staining. **Lane 1:** PageRuler™ pre-stained protein marker (170–10 kDa). **Lane 2:** daboxin P after treatment with β-mercaptoethanol. **(B):** ESI-MS spectra. The spectra show a series of multiple charged ions corresponding to a homogenous peptide. **Inset:** Reconstructed mass of daboxin P (cps: counts per second, amu: atomic mass unit).

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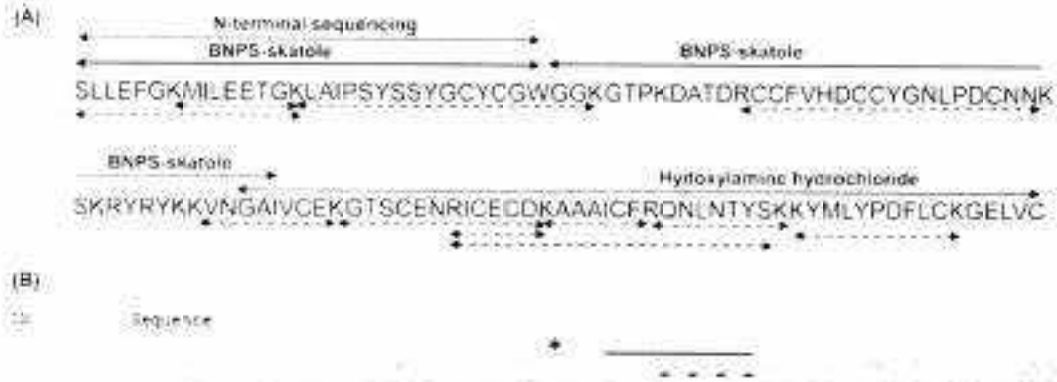

Fig 3. Primary structure of daboxin P. (A): Amino acid sequence was deciphered by Edman degradation sequencing and ESI-LC MS/MS. The peptide sequences obtained from N-terminal sequencing and chemical cleavage by BNPS-skatole and hydroxylamine hydrochloride are shown with two headed solid arrows whereas peptide sequences obtained after ESI-LC MS/MS of the tryptic digested fragments are indicated with two headed doted arrows. **(B):** Multiple sequence alignment of daboxin P with the PLA_2 enzymes from different subspecies of *Daboia russelii* (**24638087**: *D. r. russelii*, **408407675**: *D. r. siamensis*, **31615955**: *D. r. pulchella*, **49259309**: *D. r. russelii*, **31615954**: *D.r.pulchella*, **109157490**: *D. r. pulchella*, **48425253**: *D. r. pulchella*, **298351762**: *D. r. russelii*, **81174981**: *D. r. russelii*). The conserved cys residues are highlighted in grey and the amino acid substitutions in daboxin P are underlined. * indicates the His residue at the active site. The predicted anticoagulant region is highlighted with a solid black line.

doi: 10.1371/journal.pone.0153770.g003

positively charged amino acid residues (Lys and Arg) were observed which propose it to be a strong anticoagulant PLA_2 enzyme.

Homology search by blastp shows its similarity to VRV-PL-VIIIa (96%) isolated from *Daboia r. russelii* at position 59th (Asn), 62nd (Lys), 65th (Arg), 68th (lys) and 120th (Val) ([illegible]).

Phylogenetic relationship. Daboxin P showed close phylogenetic relation with the reported anticoagulant PLA_2 enzymes which are basic, like varitoxin which is a strong anticoagulant enzyme reported from *Vipera russelii* and FXa binding enzymes like DPLA2 from *Daboia russelii pulchella*, AtxA and AtxC from *V. a. ammodytes* with a bootstrap value of 99 [illegible] (Figure A in [illegible]).

Secondary structure determination. The far UV CD spectrum has shown negative minima at 222 nm and 208 nm and positive maxima at 190 nm, typical for α-helical pattern of PLA_2 enzymes [illegible]. The percentage of secondary structure was determined by online software K2D3 which calculated 12.73% of α helix and 12.36% of β sheet in daboxin P [illegible].

The CD spectrum at acidic pH (3.0) and neutral pH (7.4) showed stable structural conformation. However, the structure was distorted completely at alkaline pH (12.0) with residual α-helix of 5.51% & β sheet of 23.25% [illegible]. On the other hand, its secondary structure showed a Tm (melting temperature) value of 71.59 ± 0.4°C when scanned over a range of temperature [illegible].

Functional characterization

Catalytic activity. Daboxin P exhibited catalytic activity on diheptanoylthio-phosphatidylcholine (PC) in a dose dependent manner [illegible]. The Michaelis-Menten plot with different substrate concentration showed a hyperbolic curve for enzyme activity and the Km (6.6

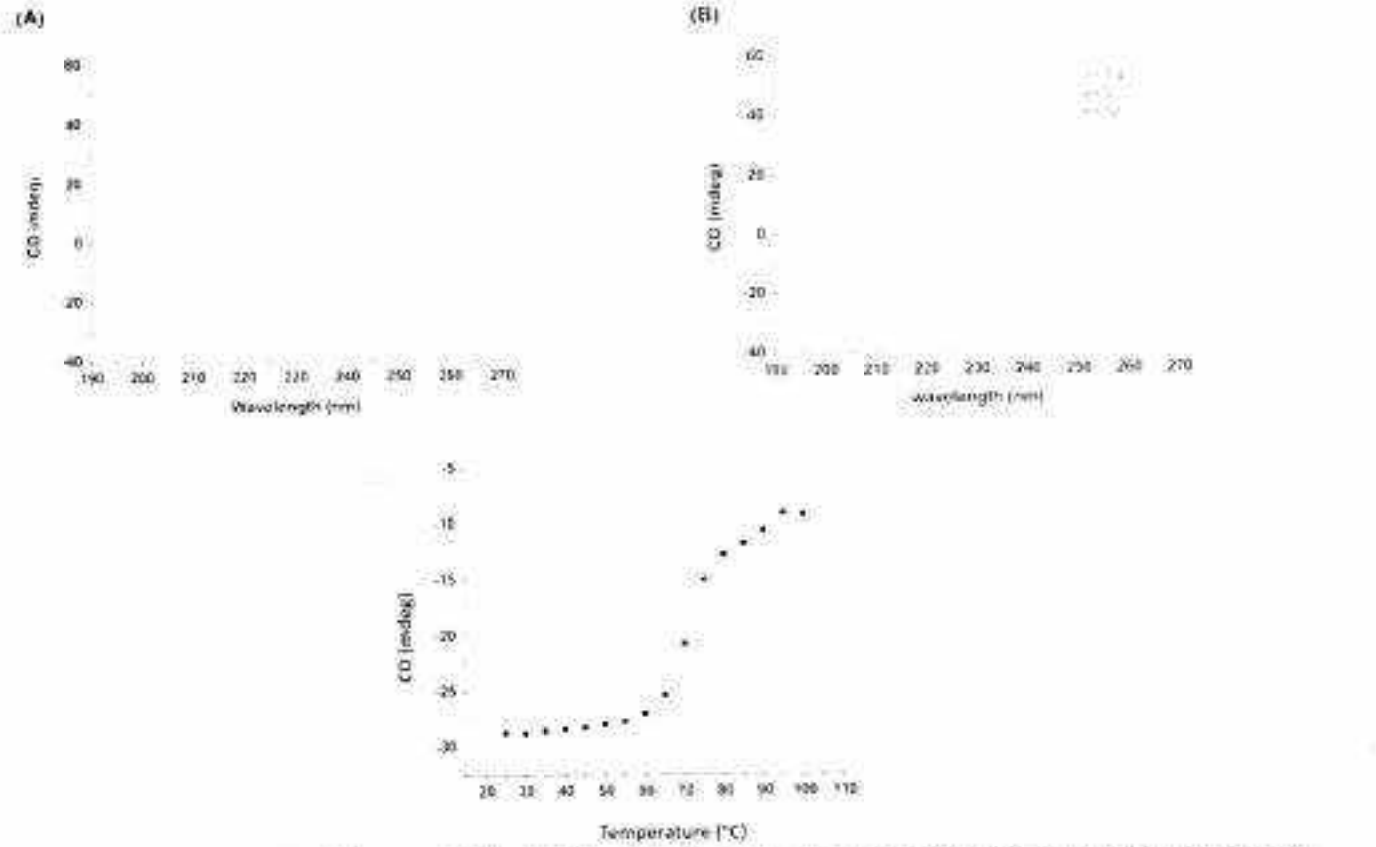

Fig 4. Far-UV circular dichroism (CD) spectra of daboxin P (0.4 mg/ml). (A): in milli q water at 25 C. **(B):** at different pH (3.0, 7.4 & 12) at 25 C. **(C)** Melting curve of daboxin P (dissolved in milli q water) at 222 nm considering temperature as a function. The curve was plotted using sigmoidal curve fit and Tm value was determined by Boltzman equation using Origin (OriginLab).

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mM) and Vmax (1.14 nmol/(min·mg)] were determined using Lineweaver Burk plot [ ]. Alkylation with pBpB has led to the complete loss of its enzymatic activity signifying the crucial role of histidine residue in catalysis [ ].

Cytotoxicity. The crude venom of *Daboia r. russelii* has shown prominent cytotoxic effect on HEK 293 and MCF 7 cells in a dose dependent manner [ ]. At a concentration of 2.8 and 3 μg/ml of crude venom, only 13.09% of HEK 293and 14.97% of MCF 7 cells respectively ( ) remained viable while the same concentrations of daboxin P did not show any cytotoxic effect, suggesting that the cytotoxic effect of the crude venom is not due to this protein ( ).

***In vitro* anticoagulant activity.** Daboxin P exhibited anticoagulant effect on the various coagulation assays in a dose dependent manner [ ]. At a concentration of 0.01 μM, it prolonged the recalcification time beyond 600 s indicating it to be a strong anticoagulant enzyme [ ]. 1 μM of the protein delayed activated partial thromboplastin time up to 130 s but did not exhibit prominent effect on prothrombin time [ ]. Daboxin P exhibited anticoagulant effect on stypven time in a dose dependent manner displaying its inhibitory effect on FX [ ]. Nevertheless, it did not show any inhibitory effect on thrombin time and was devoid of fibrinogenolytic activity (Figure B i & ii in). This suggests that daboxin P might target a component/coagulation factor upstream of the common pathway.

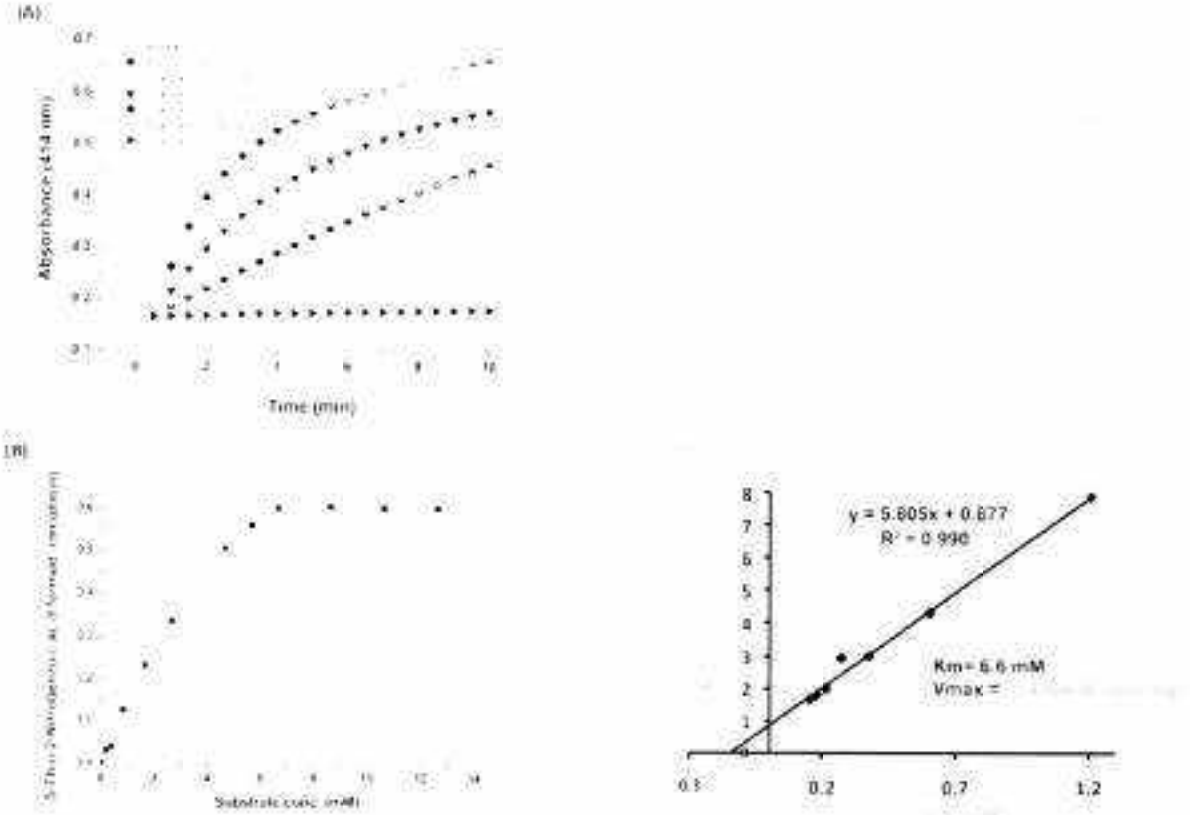

Fig 5. Phospholipase A_2 activity of daboxin P. **(A)** Progress curve of diheptanoyl thio-PC cleavage by daboxin P, bee venom PLA_2 enzyme and histidine modified daboxin P* at 414 nm. **(B)** Michaelis-Menten's curve for $sPLA_2$ assay. **(C)** The Lineweaver-Burk plot of daboxin P for determination of Km and Vmax.

doi:10.1371/journal.pone.0153770.g005

***In-vivo* anticoagulant activity.** Daboxin P exhibited anticoagulant effect in the carotid artery of mouse treated with $FeCl_3$. Mice administered with saline had a time-to occlusion within 10 min. However, daboxin P administration resulted in ~4-fold increase (35.18 ± 21.58 min) in the time to occlusion in comparison to the saline treated mice (8.29 ± 2.61 min) (Fig 6). This result was in accordance with the *in-vitro* anticoagulant assays on human plasma and highlighted the anticoagulant property of daboxin P under *in-vivo* condition.

Serine protease specificity. Screening for inhibitory effect of daboxin P on the amidolytic activity of various serine proteases involved in the extrinsic (FVIIa), intrinsic (FXIIa, FXIa, FIXa) and common (FXa) pathway revealed that it did not inhibit any of these serine proteases when assayed using respective synthetic chromogenic substrates (Fig 7A). However, it exerted inhibitory effect on both the extrinsic (ETC) (IC50 = 0.43 nM) and intrinsic (ITC) (IC50 = 39.20 nM) tenase complexes causing hindrance in the activation of FX to FXa (Fig 7B). The residual activity of ETC and ITC was observed to be 9.05% ± 1.9 and 2.8% ± 1.6 respectively upon treatment with 3 μM of daboxin P (Fig 7B & 7C). Further, when the tenase complexes were reconstituted without phospholipid, the residual activity of ETC and ITC was found to be 25% ± 2.7 and 20.05% ± 2.3 respectively. Moreover, when the tenase complexes were treated with alkylated daboxin P (3 μM), only 23% ± 1.8 (ETC) and 19.03% ± 1.8 (ITC) of residual activity remained which is comparable to the residual activity of both the complexes reconstituted without phospholipids (Fig 7B & 7C). Interestingly, the thrombin formation by

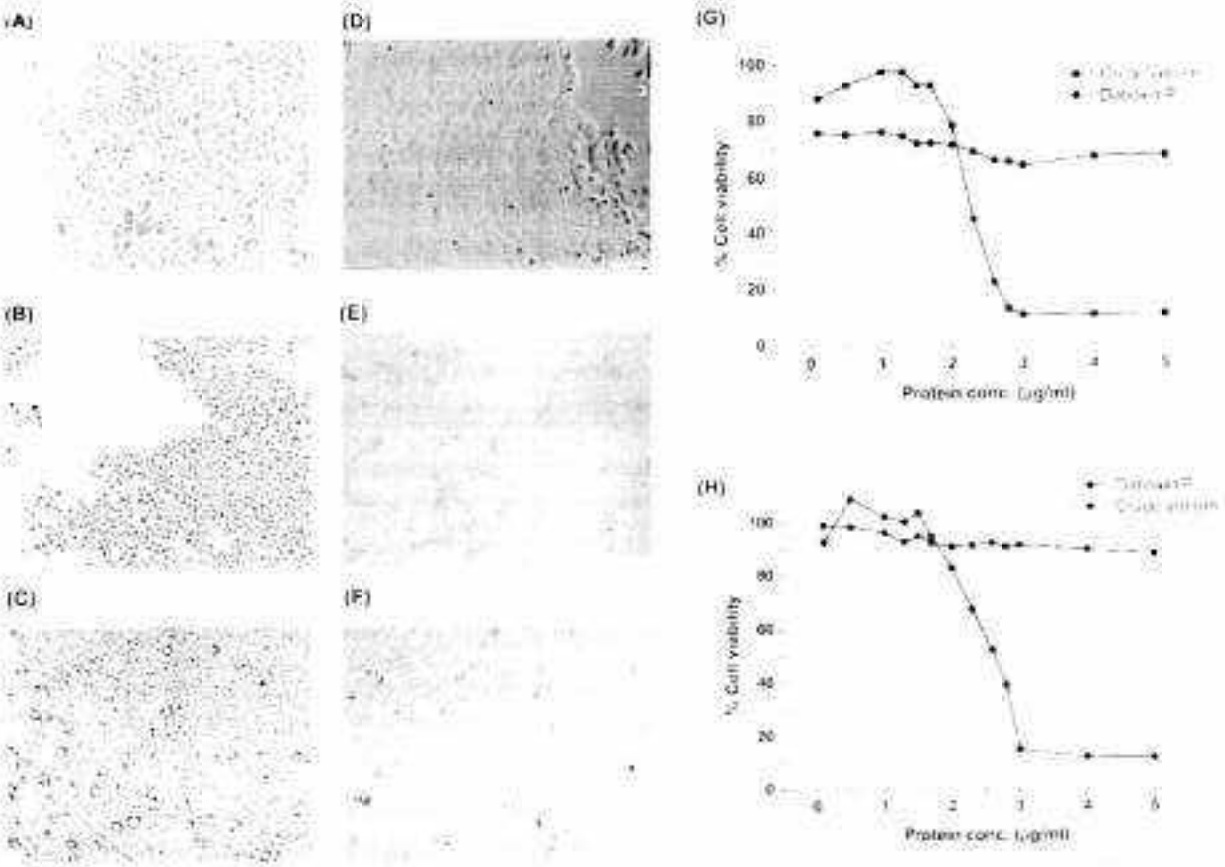

Fig 6. Cytotoxic effect of crude *Daboia r. russelii* venom and daboxin P. Microscopic images were photographed at 10X magnification under inverted microscope (Axio Vert A1., Zeiss) after treatment with venom samples for 24 h **(A)**: HEK-293 cells treated with 0.9% NaCl were considered as negative control **(B)**: HEK-293 cells treated with crude *Daboia r. russelii* venom (5 μg/ml) **(C)**: HEK-293 cells treated with daboxin P (5 μg/ml) **(D)**: MCF-7 cells treated with 0.9% NaCl were considered as negative control **(E)**: MCF-7 cells treated with crude *Daboia r. russelii* venom (5 μg/ml) **(F)**: MCF-7 cells treated with daboxin P (5 μg/ml). Percentage cell viability **(G)**: HEK-293; **(H)**: MCF-7 after treatment with crude venom and daboxin P using MTT based colorimetric assay. Percentage cell viability was calculated by considering the cells without venom treatment as 100% viable. The results are mean ± SD of three independent experiments.

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the prothrombinase complex was unaffected when daboxin P was added after the formation of the FXa–FVa complex. However, only 11.28% of residual activity remained when it was pre-incubated with FXa followed by addition of FVa for the formation of prothrombinase complex ( ).

Fluorescence emission spectroscopy. The fluorescence emission spectrum of FX and FXa was quenched by daboxin P with increasing incubation time ( ). The presence or absence of Ca^{+2} ions did not show any effect on the quenching spectra for both FX and FXa when treated with daboxin P (data not shown).

Affinity column chromatography. The electrophoretic profile of the flow through for both FX and FXa did not show any protein bands suggesting the binding of these proteins to the daboxin P immobilized CNBr matrix. Nevertheless, the eluent at 1.0 M NaCl showed prominent protein bands for both the serine proteases suggesting the disturbance of the protein-protein interaction at high salt concentration ( ).

Molecular modelling and protein-protein docking. The predicted 3D model of daboxin P has shown structural similarity to the crystal structure of AnxA (PDB: 3G8C) with a RMSD

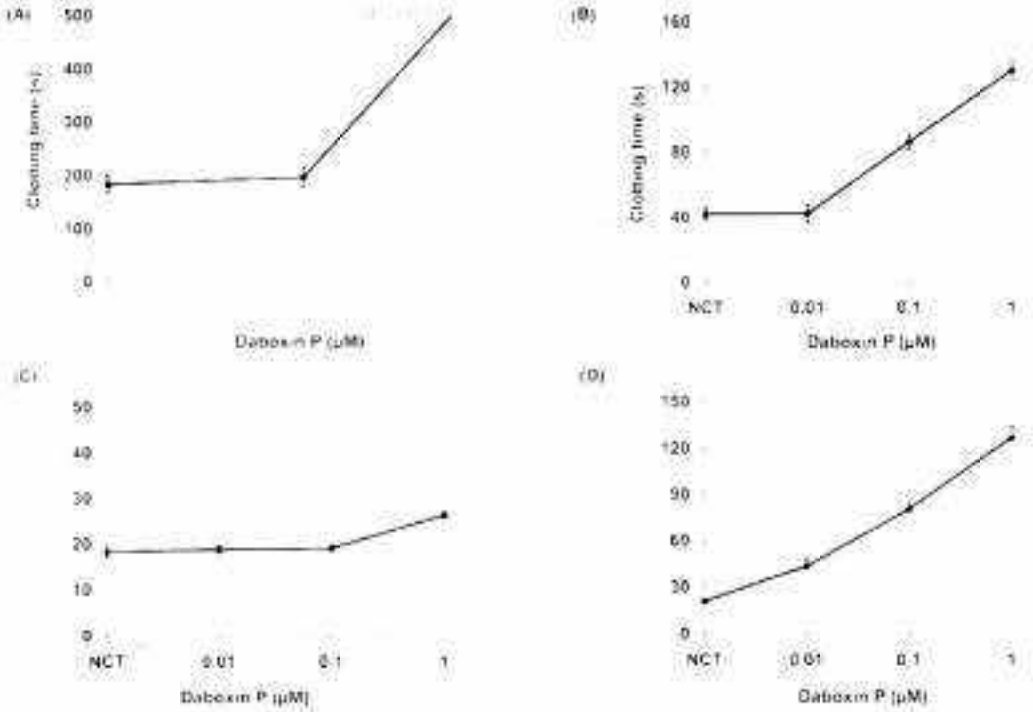

Fig 7. Anticoagulant activities of daboxin P on platelet poor human plasma (PPP). (A): Recalcification time, different concentrations of daboxin P (0.001, 0.01, 0.1) were pre-incubated with 150 μl of plasma at 37°C for 2 min. 100 μl of 50 mM $CaCl_2$ was added to initiate clot formation. **(B):** Activated partial thromboplastin time, daboxin P (0.01, 0.1 & 1 μM) was pre-incubated with 50 μl of plasma and 50 μl APTT reagent (Liquicelin) for 3 min at 37°C. 50 μl of 25 mM CaCl$_2$ was added to form clot. **(C):** Prothrombin time, different concentrations of daboxin P (0.01, 0.1 & 1 μM) were pre-incubated with 50 μl of plasma at 37°C for 2 min. 50 μl of PT reagent (Uniplastin) was added to initiate the clot formation. **(D):** Stypven time, daboxin P (0.01, 0.1 & 1 μM) was pre-incubated with 75 μl plasma for 3 min at 37°C. 75 μl RVV-X (10 ng/ml) was added and incubated for 2 min. 25 mM of $CaCl_2$ was added to initiate clot formation. For all the experiments, the clot formation was monitored using Tulip Coastal 1 coagulo analyser and the time taken for clot formation in the presence of Tris-Cl buffer (20 mM, pH 7.4) was considered as normal clotting time (NCT). The results are mean ± SD of three independent experiments.

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value of 0.9 Å which validates the predicted model (Figure C i in S1 File). The conserved critical regions and amino acid residues in daboxin P are shown in Figure C ii in S1 File.

The best protein-protein docked model of daboxin P and FXa chosen in terms of minimum free energy and maximum surface contact area, was obtained from PatchDock web server [ ]. Daboxin P interacts with the heavy chain (B-Chain) of FXa with a geometric shape complementarity score of 1223.4. Geometric scoring refers to good molecular shape complementarity between the docked chains due to optimal fit with wide interface areas and lesser steric clashes [ ]. The approximate interface area of the complex was found to be 1837 $Å^2$ with atomic contact energy (ACE) of 472.62 kcal/mol.

The interface and possible interacting residues across the interface of the complex were predicted by PDBsum. The total number of interface residues in protein-protein complex was found to be 31 and the interface area for each chain involved in the interaction was observed to be more than ~500 $Å^2$. The docked complex was stabilized by molecular interactions like hydrogen bonding and non- bonded contacts. The interface amino acid residues of FXa (chain B) and daboxin P (chain C) involved in the interaction are shown in Fig 12 inset and the interface plot statistics is summarized in Table B in S1 File.

The docked complex shows interaction of daboxin P with the heavy chain (Chain B) of FXa at eight critical regions some of which are reported to be crucial for FVa binding [ ]. The daboxin P residues Trp30, Gly31, Gly32 of the Ca^{+2} binding loop; Asp48, Tyr51, Gly52 of helix

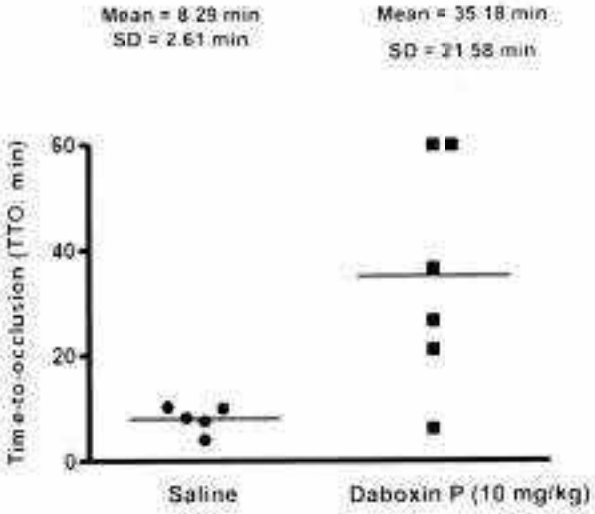

Fig 8. Effect of daboxin P on the time to occlusion (TTO) in $FeCl_3$ induced carotid artery thrombosis in mice. C57BL/6 male mice anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg) (i.p) were injected (i.p.) with daboxin P (10 mg/kg) in tail vein. Saline treated mice were considered as negative control. Each data point represents the time-to-occlusion (TTO) of a single mouse. Maximum experimental time was considered for 60 min after $FeCl_3$ induction.

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C; Asn58 of the anticoagulant region and Phe113 of C terminal region interact with residues Thr132, Arg165, Lys169, Asn166, Leu170, Tyr225 and Arg125 of heavy chain of FXa (

Contact map analysis shows fourteen residues of daboxin P share a contact area greater than 8 Å^2 with sixteen residues of the heavy chain of FXa while only four residues of daboxin P are found to share a contact area greater than the threshold limit (8 Å^2) with three residues of the light chain of FXa. This suggests the interaction of daboxin P with the light and heavy chain of FXa ( ) (Table C in).

Discussion

Snake venom is a plethora of pharmacologically active components with plausible therapeutic relevance in the treatment of coagulopathic abnormalities, hypotension and as diagnostic tools. Anticoagulant proteins are one such components of the venom mostly sought for the treatment of thrombosis and thromboembolism. Daboxin P, a non toxic PLA_2 enzyme purified from the venom of Indian *Daboia r. russelii* exhibits strong anticoagulant activity under *in vitro* and *in vivo* conditions. Dissection of the coagulation cascade reveals its effect upstream of the common pathway. However, it did not exhibit inhibitory effect on the amidolytic activity of the tested serine proteases. Nonetheless, daboxin P inhibited the activation of FX to FXa by both the tenase complexes in the presence and absence of phospholipids suggesting its enzymatic and non-enzymatic mode of action. Apart from phospholipids and calcium ions, FX is common in the tenase complexes hence daboxin P might be interacting with FX to exhibit its non enzymatic mechanism of anticoagulant activity. Fluorescence emission spectroscopy supports this hypothesis where we observed the quenching of emission spectra of FX upon incubation with daboxin P for different time intervals. Apart from inhibiting the tenase complexes, daboxin P also inhibited the thrombin formation by the prothrombinase complex when pre incubated with FXa in presence of phospholipids and Ca^{2+} ions followed by addition of FVa. Thus, daboxin P might interact with FXa to a region other than the active site or inhibit the complex formation by creating a steric hindrance. The quenched fluorescence emission spectra

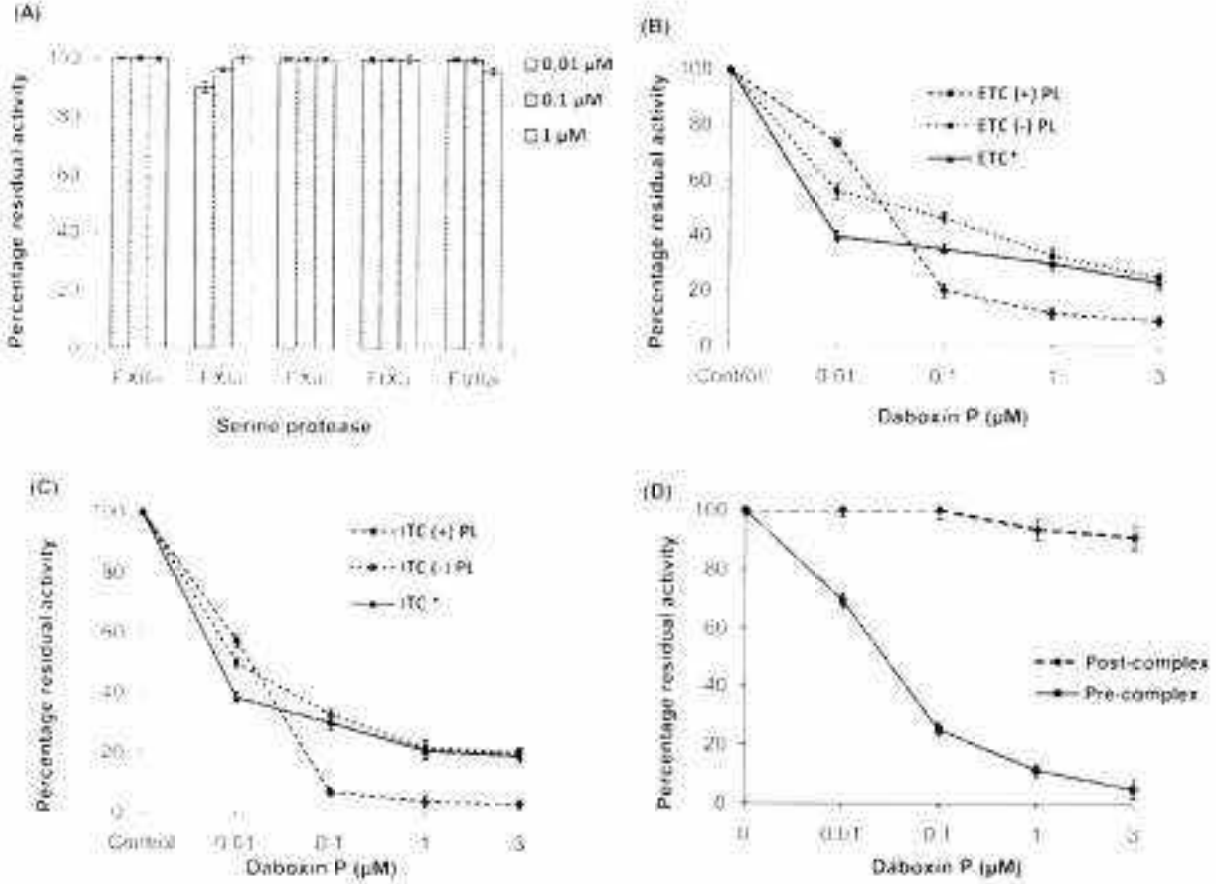

Fig 9. Percentage residual amidolytic activity of various serine proteases and complexes pre-incubated with daboxin P. (A): Residual activity of FXIIa, FXIa, FXa, FIXa and FVIIa; **(B):** Activity of extrinsic tenase complex (ETC) **(C):** intrinsic tenase complex (ITC), in the presence or absence of phospholipid and alkylated daboxin P (indicated by *). **(D):** Residual activity of prothrombinase complex. Daboxin P was either pre-incubated with FXa followed by addition of FVa (pre-complex) or after reconstitution of FXa-FVa complex (post-complex). The rate of hydrolysis of respective chromogenic substrates for all the assays was measured at 405 nm using Multiskan Go spectrophotometer. Activity of the serine protease/complex without daboxin P was considered as 100%. The results are mean ± SD of three independent experiments.

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of FXa upon incubation with daboxin P suggest the probable interaction of daboxin P with FXa for exhibiting its anticoagulant activity non-enzymatically. This interaction is further supported by the affinity chromatography studies where FX and FXa were coupled to daboxin P immobilized CNBr resin and eluted at 1 M NaCl.

CM-IV, a PLA_2 enzyme isolated from the venom of *Naja nigricollis* exhibits anticoagulant activity by inhibiting both the extrinsic tenase complex and prothrombinase complex [6, 7]. It interacts with FVIIa and FXa through its anticoagulant region which shares partial sequence similarity to a region of tissue factor and partly to the light chain of FVa respectively [6, 7]. However, the anticoagulant region of daboxin P does not share any such sequence similarity with FVa or TF, suggesting a different mechanism of action for daboxin P to exhibit its anticoagulant effect. Ammodytoxin A (AtxA), a PLA_2 enzyme from the venom of *Vipera ammodytes ammodytes* inhibits prothrombinase complex by binding to FVa binding site on FXa [8, 9]. Sequence alignment of daboxin P with AtxA shows ~76% sequence similarity with minor substitutions at helix A, helix B, Ca^{2+} binding loop, β-wing, helix D and C-terminal region (Figure D in S1 File). Such variations in the amino acid sequence of the PLA_2 enzymes are well

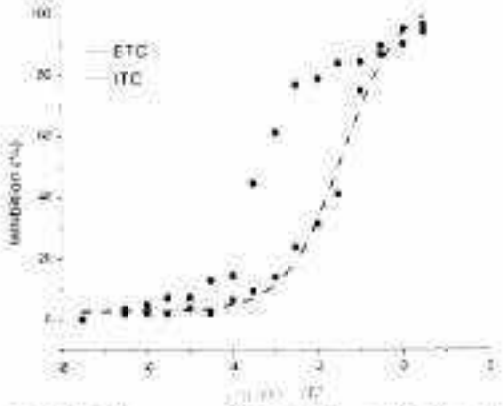

Fig 10. Inhibition curve (IC50) of daboxin P for the extrinsic and intrinsic tenase complex. Different concentrations of daboxin P ($1x10^{-5}$, $1x10^{-5}$, $1x10^{-4}$, $1x10^{-3}$, $1x10^{-2}$, $1x10^{-1}$, 1, $3x10^{-5}$, $3x10^{-5}$, $3x10^{-4}$, $3x10^{-3}$, $3x10^{-2}$ and 3 μM) were pre-incubated with reconstituted tenase complexes as described above. The IC_{50} was calculated by fitting the points by non-linear curve fit using Origin (OriginLab, Northampton, MA).

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documented in literature which are known to confer wide range of pharmacological specificity to these proteins towards various physiological targets [ ].

Based on molecular docking analysis, Faure and co-workers reported the interaction of the heavy chain of FXa with the Ca^{++} binding loop, helix C, β-wing and the C-terminal region of

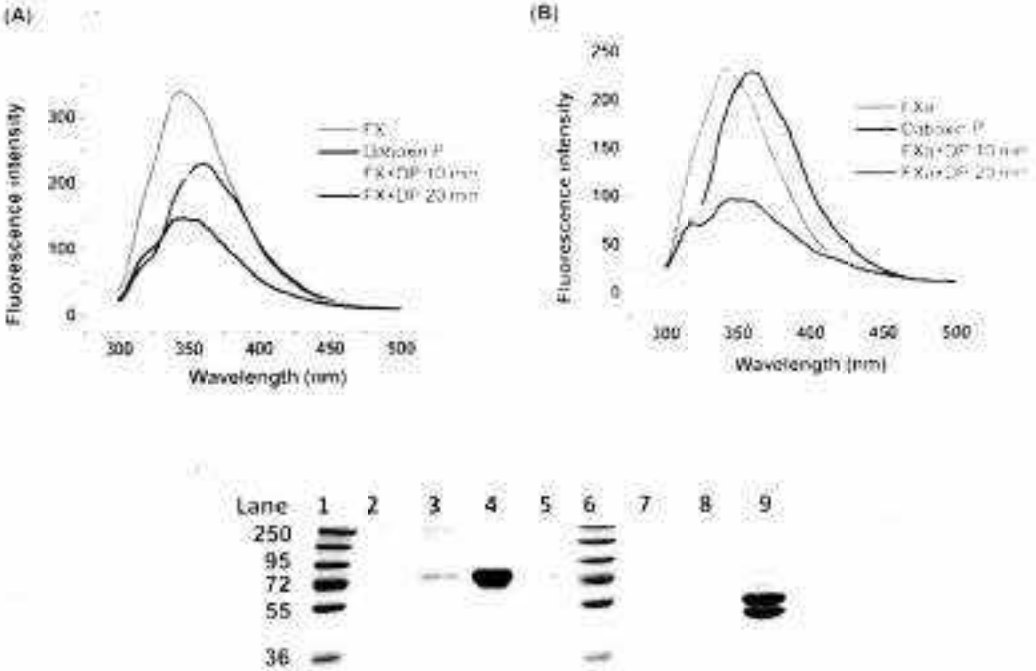

Fig 11. Interaction of daboxin P with FX and FXa. (A): Fluorescence emission spectra of daboxin P, FX, and the complex (daboxin P + FX). **(B)** Fluorescence emission spectra of daboxin P, FXa, and the complex (daboxin P + FXa). 1 μM of daboxin P (DP) was pre-incubated with either 0.05 μM of FX or 0.1 μM of FXa for different time intervals (10 min & 20 min) at room temperature. The emission spectra of the individual proteins and the complexes were measured from 200 to 500 nm with an excitation wavelength of 280 nm using quartz cuvette (1 cm path length). **(C):** Electrophoretic profile of the flow through and elute obtained after affinity column chromatography. **Lane 1**: PageRuler™ Plus pre-stained protein ladder (250–10 kDa). **Lane 2**: flow through (FX). **Lane 3:** FX after elution with 1.0 M NaCl. **Lane 4**: control (FX). Lane 5: blank. **Lane 6:** PageRuler™ Plus pre-stained protein ladder (250–10 kDa). **Lane 7**: flow through (FXa). **Lane 8:** FXa after elution with 1.0 M NaCl. **Lane 9:** control (FXa).

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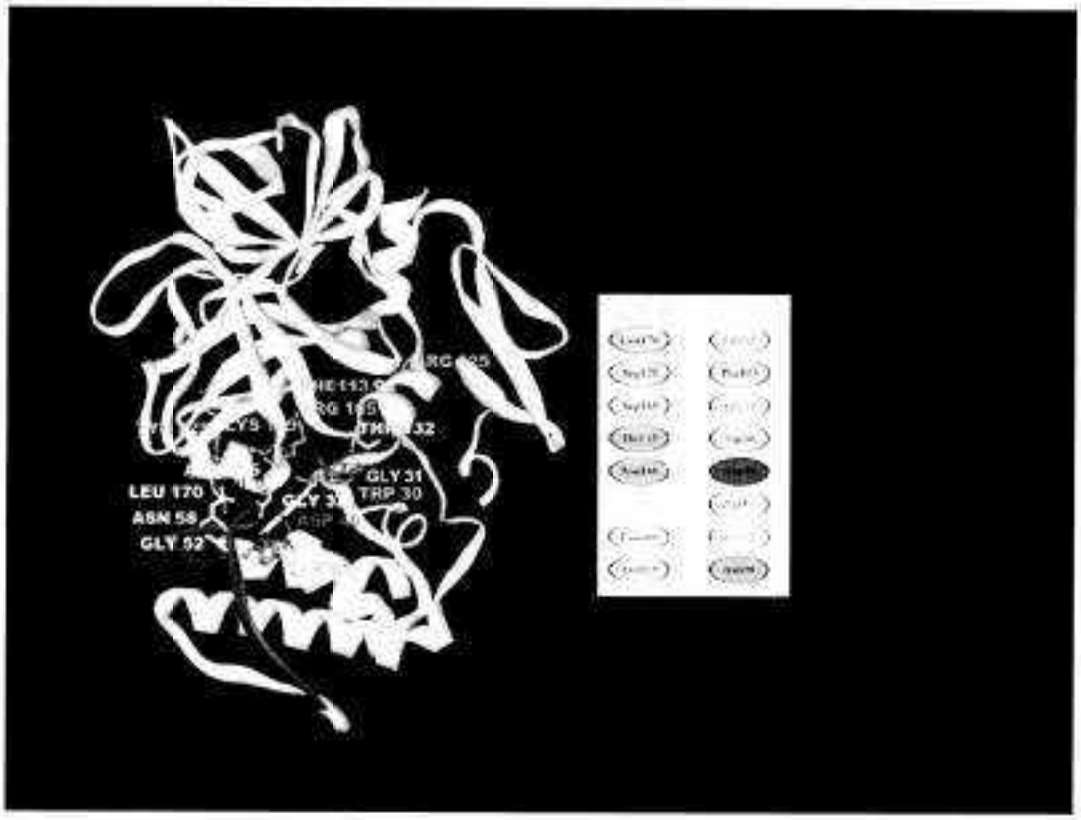

Fig 12. 3D ribbon model of the docked complex of FXa and daboxin P. The interface surface residues involved in the interaction were predicted by PDBsum. The Ca^{+2} binding loop (Trp30, Gly31, Gly32); helix C (Asp48, Tyr51, Gly52); anticoagulant region (Asn58) and C-terminal region (Phe113) of daboxin P interact with the heavy chain of FXa (Thr132, Arg165, Lys169, Asn166, Leu170, Tyr225 and Arg125) are represented in scaled ball and stick. **Inset:** Diagram illustrating the interaction of the seven residues of chain B (heavy chain of FXa) with eight residues of chain C (daboxin P) as predicted by PDBsum server. Orange line denotes non-bonded contacts and blue line denote hydrogen bond

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AtxA, while light chain of FXa is involved in interaction with helix A, B and the β-wing [,]. On the other hand, the present *in silico* molecular docking analysis shows the interaction of the Ca^{+2} binding loop (Trp30, Gly31, Gly32); helix C (Asp48, Tyr 51 and Gly52), anticoagulant region (Asn58) and C- terminal region (Phe113) of daboxin P with the heavy chain of FXa (Thr132, Arg165, Lys169, Asn166, Leu170, Tyr225 and Arg125 of FXa) but not with its light chain. The residues of helix A (Leu3, Met7, Leu10), helix B (Asn16, Pro17, Leu18, Thr19) and the anticoagulant region (Arg68) of AtxA are reported to be involved in the interaction with the light chain of FXa [ ]. However, most of these crucial residues were found to be substituted in daboxin P (Lys7, Leu16, Ala17, Ile18, Pro19, Lys68) due to which daboxin P might not have shown any interaction with the light chain of FXa in the docking study. Nevertheless,

Table 1. Critical residues of daboxin P and FXa involved in interaction based on PDBsum analysis.

Daboxin P	FXa
Trp30, Gly31, Gly32 (Ca^{+2} binding loop)	Thr132, Arg165, Lys169 (Heavy chain)
Asp48, Tyr51, Gly52 (Helix C)	Asn166,Lys169,Leu170 (Heavy chain)
Asn58 (Anticoagulant region)	Tyr225(Heavy chain)
Phe113 (C-terminal region)	Arg125(Heavy chain)

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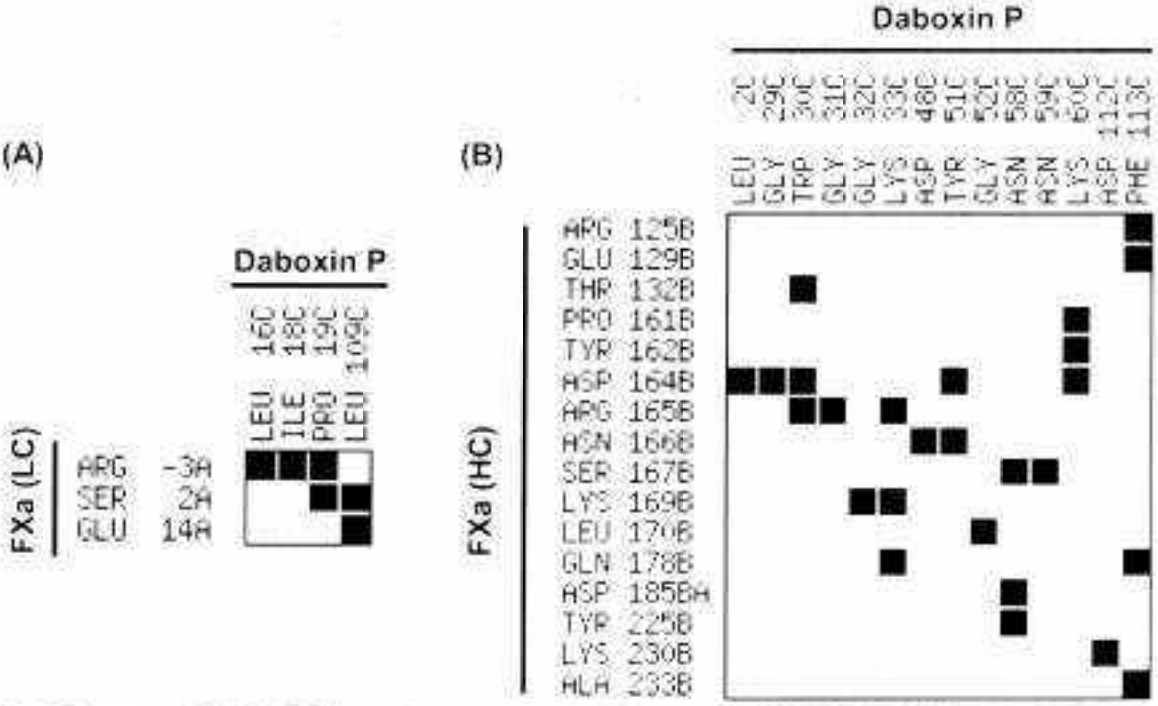

Fig 13. Contact map of daboxin P-FXa complex generated using online server Contact Map Analysis. (A): residue to residue contact of light chain of FXa and daboxin P **(B):** residue to residue contact of heavy chain of FXa and daboxin P. The residue to residue contact area of the interacting amino acid residues for the chains has been considered above 8 A° for the design of the contact map.

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contact map analysis shows interaction of the light chain of FXa with helix B (Leu16, Ile18, Pro19) and C terminal region (Leu109) of daboxin P while the heavy chain of FXa (Arg125B, Glu129B, Thr132B, Pro161B, Tyr162B, Asp164B, Arg165B, Asn166B, Ser167B, Lys169B, Leu170B, Gln178B, Asp185B, Tyr225B, Lys230B, Ala233B) is found to interact with helix A (Leu2), Ca^{2+} binding loop (Gly29, Trp30, Gly31, Gly32, Lys33), helix C (Asp48, Tyr51, Gly52), anticoagulant region (Asn58, Asn59, Lys60), and the C terminal region (Asp112, Phe113) of daboxin P ( ).

It has been reported that Arg165, Lys169 and Lys230 of FXa form the core for FVa binding site while Arg125 and Glu129 are crucial for the complex formation [ ]. Hence, binding of daboxin P to FXa might hinder the association of FXa and FVa for the formation of the pro thrombinase complex in presence or absence of other co factors. Norledge B.V. and colleagues reported the involvement of Asp4 and Asp7 of EGF II of light chain and Asp185a, Lys186 and Lys134 of heavy chain of FXa for binding to TF/FVIIa complex based on site directed mutagenesis and molecular docking [ ]. With docking analysis it has been observed that daboxin P binds to FXa (discussed above) in close vicinity to FVIIa-TF binding region on FX. Thus, interaction of daboxin P with those proximal regions might create a steric hindrance in light chain and heavy chain regions of FX and inhibit the binding of FVIIa-TF or FIXa-FVIIIa. The residues involved in this interaction could not be confirmed by docking analysis as the crystal structure of FX is not available. Based on the biochemical, biophysical and docking analysis, it is proposed that daboxin P might be exhibiting its anticoagulant activity by interacting with FX and FXa. However, site directed mutagenesis of the critical amino acids residues needs to be carried out to confirm the interaction of daboxin P with FX and FXa.

Thus, present study evaluates daboxin P as a probable natural inhibitor of FX and FXa from snake venom. FXa is one of the most pivotal components of the physiological system which plays critical role not only in haemostasis but also in intracellular signal transduction leading to pathophysiological events like fibrosis, cancer and tissue modelling restoration [-]. Hence, thriving for natural FX/FXa inhibitors will eventually lead to manipulation/management of various pathophysiological aspects of human system.

Supporting Information

S1 File. Table A. Peptide fragments of daboxin P obtained from tandem mass spectrometry. MH+ stands for mass/charge (*m/z*) of the peptide (protonated molecular ions), Z represents the number of charges a peptide carries after ionization. **Score** implies the sum of all peptide cross correlation (Xcorr) values. **Figure A. The Phylogenetic relationship of daboxin P with the reported anticoagulant PLA_2 enzymes from snake venom.** The phylogenetic tree was constructed by neighbour joining (NJ) tree using Mega 5 software with a bootstrap value of 1000. Anticoagulant PLA_2 enzymes with complete sequence were obtained from Pubmed Protein Database ( ) and aligned using ClustalW. The sequences used in the study were CBc (*Crotalus durissus terrificus*), CB crotoxin (*Crotalus durissis collilineatus*), CBb (*Crotalus durissus terrificus*), PLA2 F17 (*Crotalus durissus terrificus*), CBa2 (*Crotalus durissus terrificus*), Cdc 9 (*Crotalus durissus cumanensis*), Cdc 10 (*Crotalus durissus cumanensis*), bH3 PLA2 (*Bothrops leucurus*), Bothropstoxin (*Bothrops jararacussu*), DPLA2 (*Daboia russelii russelii*), vurtoxin (*Vipera renardi*), AtxA (*Vipera ammodytes ammodytes*), AtxC (*Vipera ammodytes ammodytes*), bAhp (*Gloydius halys*), BP III (*Protobothrops flavoviridis*), II BP (*Protobothrops flavoviridis*), Ts K49b (*Trimeresurus stejnegeri*), MTxII (*Bothrops asper*), PLA2 homology (*Bothrops atrox*), BthA I PLA2 (*Bothrops jararacussu*), Vur PL2B (*Vipera renardi*), EC 1 PLA2 (*Echis carinatus*), PLa2Vb (*Vipera berus berus*), Cb11 (*Pseudocerastes fieldi*), HDP 1P (*Vipera nikolskii*), HD 2P (*Vipera nikolskii*), Caudoxin (*Bitis caudalis*), APLA2 2 (*Ophiophagus hannah*); CM III/CM IV (*Naja nigricollis*); CM III (*Naja massambica*); CM II (*Naja massambica*) and CM I (*Naja massambica*). **Figure B. Effect of daboxin P on thrombin time and fibrinogen. (i):** Different amount of daboxin P (1, 3, 5 μg) were pre incubated with 50 μl platelet poor plasma for 3 min at 37°C. 50 μl of thrombin (10 u/ml) was added to initiate clot formation and monitored on Tulip Coastat-1 coagulo analyser. Clotting time in presence of Tris buffer (20 mM, pH 7.4) was taken as normal clotting time (NCT). The results are mean ± SD of three independent experiments. **(ii):** Assessment of fibrinogenolytic activity on SDS PAGE, different amount of daboxin P (0.01, 0.1, 1, 5 μg) were pre incubated with 300 μl of 2 mg/ml fibrinogen for 24 h at 37°C. Fibrinogen with thrombin (3 μl of 10 units/ml) was considered as the positive control and fibrinogen with buffer (50 mM Tris-Cl, pH 7.5) was taken as the negative control. Fibrinogen degradation products were analyzed on 12.5% glycine SDS PAGE, stained with CBB R 250. (C: Fibrinogen, Thr: Thrombin, Rvv: crude Russell's viper venom). **Figure C. Three dimensional (3D) molecular modelling (i):** The 3D ribbon structure of daboxin P (in red, blue, green & white) was superimposed with X ray crystallographic structure of AtxA (PDB 3G8G) (in yellow). **(ii):** 3D ribbon model of daboxin P as predicted by I TASSER server. The Ca^{+2} binding loop (YGCYCGCGG in torquise blue), anticoagulant region (53NLPDCNNKSKRYRYKK68) (in yellow) and the active site His residue (in stick) are highlighted. **Table B. Summary of interface plot statistics of the docked model of daboxin P and FXa. Table C. List of displayed residue to residue contact area of the light (A) and heavy chain (B) of FXa with daboxin P (C). Figure D. Sequence alignment of daboxin P with ammodytoxin A.** The amino acid residues varying in daboxin P with respect to ammodytoxin A are underlined. The predicted anticoagulant region in both the

PLA$_2$ enzymes is highlighted in red.
(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: MS RD. Performed the experiments: MS NS MM VSKM JKI. Analyzed the data: MS RM RD. Contributed reagents/materials/analysis tools: RD. Wrote the paper: MS RD.

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Administrative

No. BT/43/NE/TBP/2010
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY
(NER DIVISION)
****

Block-2, 7th Floor
CGO Complex, Lodhi Road
New Delhi-110003
Dated 14/3/2011

ORDER

Sanction of the President is hereby accorded under Rule 18 of the Delegation of Financial Powers Rules, 1978 for the implementation of the project under 'DBT's Twinning programme for the NE' titled "Search for novel treatments for snake venom poisoning : Composition analysis of Naja naja and Daboia russelli venoms and complete characterization of their major toxins" by Dr. Robin Doley, Department of Molecular Biology and Biotechnology, Tezpur University and Prof. D. Velmurugan, University of Madras, Chennai, and Prof. B. S. Vishwanath, Deparment of Studies in Biochemistry, University of Mysore, Mysore, at a total cost of ₹ 104.90 lakhs (Rupees One Crore Four Lakhs and Ninety Thousand only) for a period of three years, on the terms and conditions detailed as under:

2.0 The Project:

2.1 **Project Title:** Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venoms and **complete characterization of their major toxins**

2.2 **Project Investigators**

2.2.1 **Principal Investigator:** **Dr. Robin Doley,**
Assistant Professor
Department of Molecular Biology and Biotechnology,
Tezpur University,
Tezpur-784028, District Sonitpur
Assam, India

2.2.2 **Principal Investigator: (Collaborator I)** **Prof. D. Velmurugan**
Professor & Head,
Centre of Advanced Study in Crystallography and Biophysics, University of Madras,
Guindy Campus, Chennai-600025

2.2.3 **Principal Investigator: (Collaborator II)** **Prof. B. S. Vishwanath**
Professor
Deparment of Studies in Biochemistry,
University of Mysore, Manasagangotri,
Mysore- 570006

2.3 **Objectives:**

- Determination of venom composition
- Isolation and purification of major toxins
- Biochemical and pharmacological characterization of the toxin
- Determination of amino acid sequences of toxins:
- Determination of three dimensional structures

2.4 **Time Schedule:**

The duration of the project is **three years** from the date of issue of sanction order.

2.5 **Fellowship Conditions:**

JRF emoluments shall be ₹ 16,000/- + HRA for the 1st two years and ₹ 18,000/- + HRA for the 3rd year for SRF and applicable, only if JRF/SRF is NET/GATE/BET/BINC qualified otherwise it shall be ₹ 12,000/- +HRA for the 1st two years for JRF and ₹ 14,000/- + HRA for the 3rd year for SRF.

2.6 Equipment and Manpower Details:

2.6.1 Tezpur University, Tezpur, Assam

(a) Equipments: (i) Nanodrop with PC and printer (ii) Gradient PCR (iii) Static incubator (iv) Shaker incubator (v) Tissue homogeniser (vi) Micropipette (vii) HPLC Columns (viii) Refrigerated Centrifuge (ix) Bench top centrifuge (Non refrigerarted) (x) UV VIS Spectorphotometer (xi) Spinix- Vortex Shaker (xii) Protein and DNA electrophoresis, semi dry transfer system + power supply (xiv) Magnetic Stirrer Hot Plate

(b) Manpower:

(Rupees in lakhs)

Position	Ist Year	IInd year	IIIrd year	Total
JRF (1) @ ₹ 16,000 for the 1st two years & for the 3rd year SRF @ ₹ 18,000/- respectively +10% HRA	2.11	2.11	2.38	6.60
1 Project assistant @ ₹ 8000 /month	0.96	0.96	0.96	2.88
TOTAL	3.07	3.07	3.34	9.48

2.6.2 University of Madras

(a) Equipments: Nil

(b) Manpower:

(Rupees in lakhs)

Position	Ist Year	IInd year	IIIrd year	Total
1 Project assistant @ ₹ 8000/- pm	0.96	0.96	0.96	2.88

2.6.3 University of Mysore

(a) Equipments: (i) HPLC system (ii) UV VIS Spectrophotmeter

(b) Manpower:

(Rupees in lakhs)

Position	Ist Year	IInd year	IIIrd year	Total
1 Project Assistant @ ₹ 8000/- pm	0.96	0.96	0.96	2.88

2.7 **Budget Estimate:**

The total cost of the project ₹ 104.90 lakhs (Rupees One Crore Four lakhs and Ninety **thousand only)** as per budget summary given below:

2.7.1 Tezpur University, Tezpur, Assam

(Rupees in lakhs)

Heads	Ist Year	IInd year	IIIrd year	Total
Non Recurring (Equipments and accessories)	31.61	0.00	0.00	31.61
Recurring				
1. Manpower	3.07	3.07	3.34	9.48
2. Consumables	13.00	6.00	4.00	23.00
3. Travel	0.75	0.75	0.75	2.25
4. Contingency	0.50	0.50	0.50	1.50
Overhead charges	1.00	0.50	0.50	2.00
Non Recurring + Recurring	**49.93**	**10.82**	**9.09**	**69.84**

2.7.2 University of Madras, Chennai.

(Rupees in lakhs)

Heads	Ist Year	IInd year	IIIrd year	Total
Non Recurring (Equipments and accessories)	0.00	0.00	0.00	**0.00**
Recurring				
1. Manpower	0.96	0.96	0.96	**2.88**
2. Consumables	1.50	1.50	1.50	**4.50**
3.Travel	0.50	0.50	0.50	**1.50**
4. Contingency	0.30	0.30	0.30	**0.90**
5.Overhead charges	0.40	0.40	0.30	**1.00**
Non Recurring + Recurring	**3.66**	**3.66**	**3.56**	**10.78**

2.7.2 University of Mysore, Mysore

(Rupees in lakhs)

Heads	Ist Year	IInd year	IIIrd year	Total
Non Recurring (Equipments and accessories)	16.00	0.00	0.00	**16.00**
Recurring				
1. Manpower	0.96	0.96	0.96	**2.88**
2. Consumables	1.00	0.50	0.50	**2.00**
3.Travel	0.50	0.50	0.50	**1.50**
4. Contingency	0.30	0.30	0.30	**0.90**
5.Overhead charges	0.50	0.25	0.25	**1.00**
Non Recurring + Recurring	19.26	2.51	2.51	24.28

(i) Grand Total for 3 years (09.84+10.78+24.28) = ₹ 104.90 Lakhs
(ii) Total for 1st year release (49.93+3.66+19.26) = ₹ 72.85 Lakhs

2.8 The expenditure is debit able to:

Demand No. 85	Department of Biotechnology
"3425"	Other Scientific Research (Major Head)
29	Research and Development
29.04	Medical Biotechnology
29.04.31	Grants-in-aid 2010-11(Plan)

2.9 Other Terms & Conditions:

2.9.1 The other terms and conditions governing this sanction are attached at **Annexure I.**

2.9.2 A Memorandum of Agreement (MoA) will be signed between the department and the grantee institute on a ₹ 100/- stamp paper in the format given at **Annexure II** and the subsequent releases will be made only after signing of MoA by the grantee institute and its acceptance by DBT. All pages need to be signed by the PI and the forwarding authority and the MoA returned to DBT within 30 days of issue of this letter.

2.10. This issues under the powers delegated to this Department and with the concurrence of IFD, DBT vide their Dy No. 102/I.F.D./SAN/7782/2010-2011 Dated 04-03-11.

2.10.1 This sanction order has been noted at serial No. 304, 305, 306 in the Register of Grants.

2.10.2 The accounts of grantee institution shall be open to inspection by sanctioning auditory / audit.

(Dr. T. Madhan Mohan)
Adviser

To

The Pay & Accounts Officer,
Department of Science & Technology
New Delhi - 110016

Copy to

1. The Principal Director of Audit (Scientific Department), AGCR Building, New Delhi - 110 002
2. Cash Section, DBT (2 Copies)
3. Sanction Folder.
4. IFD, DBT
5. The Registrar, Tezpur University, Tezpur- 784028, District- Sonitpur, Assam
6. The Registrar, University of Madras, Chepauk, Chennai- 600005.
7. The Registrar, University of Mysore, Vishwavidyalaya Karyasoudha Crawford Hall, Mysore- 570006.
8. Dr. Robin Doley, Assistant Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, District Sonitpur, Assam, India
9. Prof. D. Velmurugan, Professor & Head, Centre of Advanced Study in Crystallography and Biophysics, University of Madras, Guindy Campus, Chennai-600025
10. Prof. B. S. Vishwanath, Department of Studies in Biochemistry, University of Mysore, Manasagangotri, Mysore- 570006
11. Concern file
12. U.S. (IFD) to re-appropriate the budget from the NER budget

(Dr. T. Madhan Mohan)
Adviser

No. BT/43/NE/TBP/2010
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY
(NER DIVISION)
****

Block-2, 7th Floor
CGO Complex, Lodhi Road
New Delhi-110003
Dated 14/3/2011

ORDER

In continuation of this department's sanction order of even number dated 14/3/2011, sanction of the President of India is hereby accorded under Rule 18 of the Delegation of Financial Powers Rules, 1978 for the release of First year grant of **₹ 49.93 lakhs (Rupees Forty Nine lakhs and Ninety Three Thousand only)** to The Registrar, Tezpur University, Tezpur for the implementation of the project under 'DBT's **Twinning programme for the NE**' titled "**Search for novel treatments for snake venom poisoning : Composition analysis of *Naja naja* and *Daboia russelli* venoms and complete characterization of their major toxins**" by Dr. Robin Doley, Department of Molecular Biology and Biotechnology, Tezpur University and Prof. D. Velmurugan, University of Madras, Guindy Campus, Chennai and Prof. B. S. Vishwanath, Deparment of Studies in Biochemistry, Manasagangotri, Mysore, as per the details given below:

(Rupees in Lakhs)

Heads	Grant as per 4th year
Non Recurring (Equipment's and accessories)	31.61
Recurring	
1. Manpower	3.07
2. Consumables	13.00
3.Travel	0.75
4.Contingency	0.50
5.Overhead charges	1.00
Non Recurring + Recurring	**49.93**

(Rupees Forty Nine lakhs and Ninety Three thousand only)

1. The other terms and conditions governing the financial sanction will remain unaltered.

2. The account of the guarantee institution shall be open to inspection by the sanctioning authority/audit.

3. The amount ₹ **49.93 lakhs (Rupees Forty Nine lakhs and Ninety Three thousand only)**, will be drawn by the Drawing and Disbursing Officer, Department of Biotechnology, New Delhi and distributed to "**The Registrar, Tezpur University, Tezpur- 784028, District-Sonitpur, Assam**" by an account payee Demand Draft/Cheque only.

4. The expenditure is debitable to

Demand No. 86	-	Department of Biotechnology
"3425"	-	Other Scientific Research (Major Head)
29	:	Research and Development
29.04	:	Medical Biotechnology
29.04.31	:	Grants-in-aid 2010-11(Plan)

5. This issues under the powers delegated to this Department and with the concurrence of IFD, DBT vide their Dy No. 102/I.F.D./SAN/7783/2010-2011 Dated 04-03-11.

6. This sanction order has been noted at serial No 305 in the Register of Grants.

7. Being the first release of the project, Utilization Certificate is not applicable.

(Dr T Madhan Mohan)
Adviser

To
The Pay & Accounts Officer,
Department of Science & Technology
New Delhi - 110 016.

Copy to:
1. The Principal Director of Audit (Scientific Departments), AGCR Building, New Delhi - 110 002.
2. Cash Section, DBT (2 Copies)
3. Sanction Folder.
4. IFD, DBT
5. The Registrar, Tezpur University, Tezpur- 784028, District- Sonitpur, Assam.
6. Dr. Robin Doley, Assistant Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, District Sonitpur, Assam, India.
7. Concern file.
8. U.S (IFD) to **re-appropriate the budget from the NER budget**.

(Dr T Madhan Mohan)
Adviser

BIOTECH CONSORTIUM INDIA LIMITED

BCIL

5th Floor, Anuvrat Bhawan, 210, Deen Dayal Upadhyaya Marg, New Delhi-110002
Tel : 2321 9064 - 67 **Fax** : 011-2321 9063
Email : info.bcil@nic.in, bcildelhi@vsnl.com **Website** : http://www.bcil.nic.in

BCIL/NER-BPMC/2012/1461 November 27, 2012

The Registrar,
Tezpur University,
Napaam, Sonitpur
Tezpur-784028,
Assam

Dear Sir,

Sub.: Release of grant to the implementing agency of the project entitled "Search for [illegible] treatments for snake venom poisoning: Composition analysis of Naja naja and Daboia russelli venoms and complete characterization of their major toxins" under DBT's Twinning Program for the NE

As you may be kindly aware, the Department of Biotechnology (DBT), Government of India has setup a North Eastern Region-Biotechnology Programme Management Cell (NER-BPMC) for promotion of Biotechnology in the North Eastern Region of India through Biotech Consortium India Limited (BCIL), a company promoted by the Department of Biotechnology.

We are pleased to inform you that DBT has sanctioned 2nd year grant towards implementation of the above project at your organization. Please find enclosed herewith a copy of the Release Order No. BT/43/NE/TBP/2010 dated November 12, 2012.

Please also find enclosed herewith a cheque no. 750238 dated 27.11.2012 for an amount of Rs. 8.99 lakhs as second year release towards implementation of the above project at your organization.

Kindly acknowledge the receipt.

Thanking you,

Yours faithfully

(Vinod Kumar)
Deputy Manager

CC for kind information to:

Dr. Robin Doley
Assistant Professor, Department of Molecular Biology and Biotechnology,
Tezpur University,
Napaam, Sonitpur
Tezpur-784028, Assam

Appl. No. C1-51

File No. BT/43/NE/TBP/2010
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY
(NER DIVISION)
****

Block-2, 7th Floor,
CGO Complex, Lodhi Road
New Delhi-110003
Dated 12 / 11 /2012

ORDER

In continuation of this department's sanction order of even number dated 14 / 03 /2011, sanction of the President of India is hereby accorded under Rule 18 of the Delegation of Financial Powers Rules, 1978 for the release of the **second year grant** of **₹ 8.99 lakhs (Rupees Eight lakhs and Ninety Nine thousand only)** to **The Registrar, Tezpur University, Tezpur** for the implementation of the project under **'DBT's Twinning Program for the NE'** titled **"Search for novel treatments for snake venom poisoning : Composition analysis of Naja naja and Daboia russelli venoms and complete characterization of their major toxins"** by Dr. Robin Doley, Department of Molecular Biology and Biotechnology, Tezpur University and Prof. D. Velmurugan, University of Madras, Guindy Campus, Chennai, and Prof. B. S. Vishwanath, Deparment of Studies in Biochemistry, Manasagangotri, Mysore, as per the details given below

(Rupees in lakhs)

Heads	2nd Year
Recurring	
1. Manpower	2.73
2. Consumables	4.64
3.Travel	0.73
4. Contingency	0.50
5.Overhead charges	0.39
TOTAL	**8.99**

(Rupees Eight lakhs and Ninety Nine thousand only)

2. The other terms and conditions governing the financial sanction will remain unaltered.

3. Both NER & Rest of India (RoI), Institutions scientists should work together for the objectives stated in the sanction of the project and any deviation from this would attract closure of the project at any point of time.

4. In the project review meetings, both the PI's from NER & RoI Institutions should participate & make presentation.

5. The outcomes of the project such as research papers, patents, copy rights etc. should be made jointly.

6. The NER Scientists are to be trained at the collaborating institute appropriately to empower the NER Scientists.

7. The project personal such as Research Associate, JRF/SRF, Research Assistant are also to be trained at least once in the collaborating national institute.

8. The collaborating institute scientist should visit NER institutions more frequently to guide NER scientists in design and conduct of experiments.
9. The account of the guarantee institution shall be open to inspection by the sanctioning authority/audit.

10. M/s Biotech Consortium India Ltd., Anuvrat Bhawan, 210, Deen Dayal Upadhyay Marg, New Delhi-110 002, who is administrating the North Eastern Region Biotechnology Program Management Cell (NER-BPMC) of the department, is hereby authorized to disburse the amount of **₹ 8.99 lakhs (Rupees Eight lakhs and Ninety Nine thousand only),** as referred in para 1 above, in favour of **"The Registrar, Tezpur University, Tezpur- 784028, District- Sonitpur, Assam"**, by an account payee cheque/demand draft under scheme North Eastern Region Biotechnology Program.

11. This issues under the powers delegated to the department and with the concurrence of IFD, DBT vide. **Dy No. 102/I.F.D/SAN/3086-3099/2012-2013 Dated 26/09/2012.**

12. This sanction order has been noted at **Serial No. 963** in the Register of Grants.

13. A copy of the UC-SE for the year 2011-2012 is enclosed.

14. The Competent Authority has allowed a sum of **₹ 28,45,896/-** to be carried forward to the current financial year 2012-2013.

Vaishali Panjabi

(Dr. Vaishali Panjabi)
Scientist 'C'

To,

The Pay & Accounts Officer,
Department of Biotechnology,
New Delhi - 110 016

Copy to:

1. The Principal Director of Audit (Scientific Department), AGCR Building, New Delhi - 110 002.
2. Cash Section, DBT (For information only).
3. Sanction Folder.
4. IFD, DBT
5. The Registrar, Tezpur University, Tezpur- 784028, District- Sonitpur, Assam.
6. Dr. Robin Doley, Assistant Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, District Sonitpur, Assam.
7. Concern file.
8. The Managing Director, Biotech Consortium India Limited, (NER-BPMC), 5th Floor, Anuvart Bhawan 210 Deen Dayal Upadhyay Marg, New Delhi- 110002.

Vaishali Panjabi

(Dr. Vaishali Panjabi)
Scientist 'C'

BIOTECH CONSORTIUM INDIA LIMITED

BCIL

5th Floor, Anuvrat Bhawan, 210, Deen Dayal Upadhyaya Marg, New Delhi-110002
Tel : 2321 9064 - 67 **Fax** : 011- 2321 9063
Email : info.bcil@nic.in, bcildelhi@vsnl.com **Website** : http://www.bcil.nic.in

BCIL/NER-BPMC/2014

September 8, 2014

The Registrar
Tezpur University,
Napaam, Tezpur-784028,
Assam

Dear Sir/Madam,

Sub.: DBT's Sanction Order No. BT/43/NE/TBP/2010 dated September 3, 2014 for implementation of the project "Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and Daboia russelli venoms and complete characterization of their major toxins"at your organization: Release of 3rd year grant

As you may be kindly aware, the Department of Biotechnology (DBT), Government of India, has setup a North Eastern Region-Biotechnology Programme Management Cell (NER-BPMC) for promotion of Biotechnology in the North Eastern Region of India through Biotech Consortium India Limited (BCIL), a company promoted by the Department of Biotechnology

We are pleased to inform you that DBT has sanctioned 3rd year grant towards implementation of the above project at your organization. Please find enclosed herewith a copy of the Release Order No. BT/43/NE/TBP/2010 dated September 3, 2014.

Our Accounts Division has transferred an amount of Rs. 4.50 lakhs as 3rd year release towards implementation of the above project at your organization as per the following bank details:

Account Holder Name: Registrar, Tezpur University
Name of the Bank: State Bank of India, Tezpur Branch
Bank Account Number: 30448821505
IFSC Code/MICR Code: SBIN0000195

Kindly acknowledge the receipt.

Thanking you,

Yours faithfully

(Vinod Kumar)
Deputy Manager

CC for kind information to:

1. The Vice Chancellor, Tezpur University, Napaam, Tezpur-784028, Assam
2. Dr. Robin Doley, Assistant Professor,Dept. of Molecular Biology & Biotechnology, Tezpur University, Napaam, Tezpur-784028, Assam

Appl. No. C1-51

File No. BT/43/NE/TBP/2010
GOVERNMENT OF INDIA
MINISTRY OF SCIENCE & TECHNOLOGY
DEPARTMENT OF BIOTECHNOLOGY
(NER DIVISION)
.....

Block-2, 7th Floor,
CGO Complex, Lodhi Road
New Delhi-110003
Dated: 3 / 9 /2014

ORDER

In continuation of this department's sanction order of even number dated 14/03/2011, sanction of the President of India is hereby accorded under Rule 18 of the Delegation of Financial Powers Rules, 1978 for the release of the Third year grant of **₹ 4.50 lakhs (Rupees Four lakhs and Fifty thousand only)** to **The Registrar, Tezpur University, Tezpur, Assam** for the implementation of the project under 'DBT's Twinning Program for the NE' titled "**Search for novel treatments for snake venom poisoning: Composition analysis of Naja naja and Daboia russelli venoms and complete characterization of their major toxins**" by Dr. Robin Doley, Department of Molecular Biology and Biotechnology, Tezpur University and Prof. D. Velmurugan, University of Madras, Chennai, and Prof. B. S. Vishwanath, Deparment of Studies in Biochemistry, University of Mysore, Mysore as per the details given below:

(Rupees in lakhs)

Heads	3rd Year
I. Non Recurring (Equipments & accessories)	0.00
Sub Total – I	0.00
II. Recurring	
1. Manpower	1.80
2. Consumables	2.00
3. Travel	0.30
4. Contingency	0.20
5. Overhead Charges	0.20
Sub Total- II (1-5)	4.50
GRAND TOTAL (I+II)	4.50

*The balance under NR head (₹ 0.13 lakhs) has been re-appropriated to overhead

(Rupees Four lakhs and Fifty Thousand only)

2. The other terms and conditions governing the financial sanction will remain unaltered.

3. Both NER & Rest of India (RoI) Institutions scientists should work together for the objectives stated in the sanction of the project and any deviation from this would attract closure of the project at any point of time.

4. In the project review meetings, both the PIs from NER & RoI Institutions should participate & make presentation.

5. The outcomes of the project such as research papers, patents, copy rights etc. should be made jointly.

6. The NER Scientists are to be trained at the collaborating institute appropriately to empower the NER Scientists.

7. The project personal such as Research Associate, JRF/SRF, Research Assistant are also to be trained at least once in the collaborating national institute.

8. The collaborating institute scientist should visit NER institutions more frequently to guide NER scientists in design and conduct of experiments.

9. The account of the guarantee institution shall be open to inspection by the sanctioning authority/audit.

10. M/s Biotech Consortium India Ltd., Anuvrat Bhawan, 210, Deen Dayal Upadhyay Marg, New Delhi-110 002, who is administrating the North Eastern Region Biotechnology Program Management Cell (NER-BPMC) of the department, is hereby authorized to disburse the amount of **₹ 4.50 lakhs (Rupees Four lakhs and Fifty thousand only)** as referred in para 1 above, in favour **of "The Registrar, Tezpur University, Tezpur-784028, District- Sonitpur, Assam"**, by an account payee cheque/demand draft under scheme North Eastern Region Biotechnology Program.

11. This issue under the powers delegated to the department and with the concurrence of IFD, DBT vide **Dy No. 102/I.F.D/SAN/1686-1689/2014-2015 Dated 31/07/2014**.

12. This sanction order has been noted at **Serial No.** 226 in the Register of Grants.

13. A copy of the UC-SE for the year 2013-2014 is enclosed.

14. The Competent Authority has allowed a sum of **₹ 5,123 /-** to be carried forward to the current financial year 2014-2015.

(Dr. Vaishali Panjabi)
Scientist 'D'

To,
The Pay & Accounts Officer,
Department of Biotechnology,
New Delhi - 110 003

Copy to:

1. The Principal Director of Audit (Scientific Department), AGCR Building, New Delhi - 110 002.
2. Cash Section, DBT (For information only).
3. Sanction Folder.
4. IFD, DBT
5. The Registrar, Tezpur University, Tezpur- 784028, District- Sonitpur, Assam.
6. Dr. Robin Doley, Assistant Professor, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur-784028, District-Sonitpur, Assam, India.
7. Concern file
8. The Managing Director, Biotech Consortium India Limited (NER-BPMC), 5th Floor, Anuvrat Bhawan 210 Deen Dayal Upadhyay Marg, New Delhi- 110002.

(Dr. Vaishali Panjabi)
Scientist 'D'

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. Title of the Project : **"Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venom and complete characterization of their major toxins."**
2. Sanctioned Project Cost : 69.84 Lakhs
3. Revised cost, if any : NA
4. Duration of the project : 3Years
5. Sanction Order No. & Date : No. BT/43/NE/TBP/2010, 14/03/2011
6. Date of commencement of Project : 14th March 2011
7. Extension, if any : Till 14th September 2014
8. Date of completion of project : 14/09/2014

Details of grant, expenditure and balance

S. No.	Heads	Sanctioned Cost	Year-wise Releases made (Lakhs)					Year-wise Expenditure incurred (Lakhs)					
			1st yr (2011-2012)	2nd yr (2012-2013)	3rd Yr (2013-2014)	4th Yr (2014-2015)	Total	1st yr (2011-2012)	2nd yr (2012-2013)	3rd Yr (2013-2014)	4th Yr (2014-2015)	Total	Balance
A.	Non-recurring page no.-												
	Equipments	31.61	31.61	0	0	-0.12943	31.48057	10.56136	15.64034	5.27887	0	31.48057	0
B.	Recurring												
1.	Manpower	9.48	3.07	2.73	0	1.80	7.6	1.8080	2.07355	2.03519	0.8	6.71674	0.88326
2.	Consumables	23.0	13.0	4.64	0	2.0	19.64	11.63681	6.00262	0	1.99867	19.6381	0.0019
3.	Travel	2.25	0.75	0.73	0	0.30	1.78	0.7345	0.52214	0.2654	0.25	1.77204	0.00796
4.	Contingency	1.5	0.50	0.5	0	0.20	1.2	0.49965	0.20397	0.26395	0.23205	1.19962	0.00038
5.	Overhead	2.0	1.0	0.39	0	0.2 *0.12943	1.71943	0.88784	0.195	0.25958	0.37701	1.71943	0
6	Interest earned	2011 to 2015				1.17550	1.17550	0	0	0	0	0	1.17550
	Total	38.23	18.82	8.99	0	5.80493	33.11493	15.5668	8.99728	2.82412	3.65773	31.04593	2.069
	Grand Total (A+B)	**69.84**	**49.93**	**8.99**	**0**	**5.80493**	**64.5955**	**26.12816**	**24.63762**	**8.10299**	**3.65773**	**62.5265**	**2.069**

***Rs. 0.12943 lakhs from non recurring head was re appropriated to overhead (No. BT/43/NE/TBP/2010, dated 03/09/2014) in the 4th year hence total amount for non-recurring head is Rs. 31.48057**

(PROJECT INVETIGATOR)
Dr. Robin Doley
Associate Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University
(a Central University)
Napaam, Tezpur- 784 028
Sonitpur, Assam (India)

(HEAD OF THE INSTITUTE)

29.6.15
(FINANCE OFFICER)

Utilisation Certificate

(for the financial year ending 31st March 2012)

(Rs. in Lakhs)

1. Title of the Project/Scheme: **"Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venom and complete characterization of their major toxins."**

2. Name of the Organisation: **Department of Molecular Biology and Biotechnology, Tezpur University**

3. Principal Investigator:

Dr. Robin Doley, Associate Professor
Department of Molecular Biology and Biotechnology
Tezpur University, Tezpur, Assam, India
Off: +91 3712 267008 Ext 5412
Email: doley@tezu.ernet.in

4.	Deptt. of Biotechnology sanction order No. & date of sanctioning the project:	**No. BT/43/NE/TBP/2010, 14/03/2011**
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 (*please give No. and dates of sanction orders showing the amounts paid*):	**49.93**
7.	Other receipts/interest earned, if any, on the DBT grants:	**NA**
8.	Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):	**49.93**
9.	Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed):	**26.12816**
10.	Unspent balance refunded, if any *(Please give details of cheque No. etc.):*	**NA**
11.	Balance amount available at the end of the financial year:	**23.80184**
12.	Amount allowed to be carried forward to the next financial year vide letter No. & date:	**NA**

Certified that the amount of Rs.__**26.12816 Lakhs**_mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of Rs. __**23.80184 Lakhs**_____ remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

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 utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. (Cash Book)
2. (Ledgers)
3. (Vouchers)
4. (Bank Statements)

(PROJECT INVESTIGATOR)

29.06.16

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

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

Utilisation Certificate

(for the financial year ending 31st March 2013)

(Rs. in Lakhs)

1. Title of the Project/Scheme: **"Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venom and complete characterization of their major toxins."**

2. Name of the Organisation: **Department of Molecular Biology and Biotechnology, Tezpur University**

3. Principal Investigator:

Dr. Robin Doley, Associate Professor
Department of Molecular Biology and Biotechnology
Tezpur University, Tezpur, Assam, India
Off: +91 3712 267008 Ext 5412
Email: doley@tezu.ernet.in

4. Deptt. of Biotechnology sanction order No. & date of sanctioning the project: **No. BT/43/NE/TBP/2010, 14/03/2011**

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: **23.80184 (No. BT/43/NE/TBP/2010, 14/03/2011)**

6. Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid*): **8.99 (No. BT/43/NE/TBP/2010, Dt: 12/11/2012)**

7. Other receipts/interest earned, if any, on the DBT grants: **NA**

8. Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7): **32.79184**

9. Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed): **24.63762**

10. Unspent balance refunded, if any *(Please give details of cheque No. etc.):* **NA**

11. Balance amount available at the end of the financial year: **8.15422**

12. Amount allowed to be carried forward to the next financial year vide letter No. & date: **NA**

Certified that the amount of Rs.__**24.63762 Lakhs**__mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of Rs. __**8.15422 Lakhs**_____ remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

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 utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. (Cash Book)
2. (Ledgers)
3. (Vouchers)
4. (Bank Statements)

(PROJECT INVESTIGATOR)

29.6.16

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

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

Utilisation Certificate

(for the financial year ending 31st March 2014)

(Rs. in Lakhs)

1. Title of the Project/Scheme: **"Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venom and complete characterization of their major toxins."**

2. Name of the Organisation: **Department of Molecular Biology and Biotechnology, Tezpur University**

3. Principal Investigator: **Dr. Robin Doley, Associate Professor**
   Department of Molecular Biology and Biotechnology
   Tezpur University, Tezpur, Assam, India
   Off: +91 3712 267008 Ext 5412
   Email: doley@tezu.ernet.in

4. Deptt. of Biotechnology sanction order No. & date of sanctioning the project: **No. BT/43/NE/TBP/2010, 14/03/2011**

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: **8.15422 (No.BT/43/NE/TBP/2010,Dt: 12/11/2012)**

6. Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid)*: **0**

7. Other receipts/interest earned, if any, on the DBT grants: **NA**

8. Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7): **8.15422**

9. Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed): **8.10299**

10. Unspent balance refunded, if any *(Please give details of cheque No. etc.)*: **NA**

11. Balance amount available at the end of the financial year: **0.05123**

12. Amount allowed to be carried forward to the next financial year vide letter No. & date: **NA**

Certified that the amount of Rs.__ **8.10299 Lakhs**__mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of Rs. __**0.05123 Lakhs**_____ remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

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 utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. (Cash Book)
2. (Ledgers)
3. (Vouchers)
4. (Bank Statements)

29.6.15

(PROJECT INVESTIGATOR)

Dr. Malay Dolai
Associate Professor
Dept. of Molecular
Biology and Biotechnology
Tezpur University
Tezpur-784028, Assam, India

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

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

Utilisation Certificate

(for the financial year ending 31st March 2015)

(Rs. in Lakhs)

1. Title of the Project/Scheme: **"Search for novel treatments for snake venom poisoning: Composition analysis of *Naja naja* and *Daboia russelli* venom and complete characterization of their major toxins."**

2. Name of the Organisation: **Department of Molecular Biology and Biotechnology, Tezpur University**

3. Principal Investigator: **Dr. Robin Doley, Associate Professor**
   Department of Molecular Biology and Biotechnology
   Tezpur University, Tezpur, Assam, India
   Off: +91 3712 267008 Ext 5412
   Email: doley@tezu.ernet.in

4. Dept. of Biotechnology sanction order No. & date of sanctioning the project: **No. BT/43/NE/TBP/2010, 14/03/2011**

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: **0.05123 (No.BT/43/NE/TBP/2010,Dt: 12/11/2012)**

6. Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid)*: **4.50 (No. BT/43/NE/TBP/2010, 03/09/2014)**

7. Other receipts/interest earned, if any, on the DBT grants: **1.17550**

8. Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7): **5.72673**

9. Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed): **3.65773**

10. Unspent balance refunded, if any *(Please give details of cheque No. etc.):* **2.069**

11. Balance amount available at the end of the financial year: **2.069**

12. Amount allowed to be carried forward to the next financial year vide letter No. & date: **NA**

Certified that the amount of Rs.__ **3.65773 Lakhs**__mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of Rs. __**2.069 Lakhs (including the interest earned)** _____ remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____)/will be adjusted towards the grants-in-aid payable during the next year.

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 utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. (Cash Book)
2. (Ledgers)
3. (Vouchers)
4. (Bank Statements)
5.

(PROJECT INVESTIGATOR)

Dr. Robin Doley
Associate Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University
(a Central University)
Napaam, Tezpur- 784 028
Sonitpur, Assam (India)

29.4.15

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

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

Annexure A

Manpower Staffing Details (In the financial year wise manner)

14th March 2011- 31st March 2012

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY (in Rs.)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR (in Rs.)	TOTAL SALARY PAID DURING PROJECT PERIOD (In Rs.)
Mr. Diganta Das	JRF	01/07/11	NA	12,000.00	1,08,000.00	1,08,000.00
Mr. Debajit Kalita	PA	28/06/11		8,000.00	72,800.00	72,800.00
				Total	1,80,800.00	1,80,800.00

1st April 2012- 31st March 2013

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY (in Rs.)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR (in Rs.)	TOTAL SALARY PAID DURING PROJECT PERIOD (In Rs.)
Mr. Diganta Das	JRF	01/07/11	NA	12,000.00	1,32,000.00	1,32,000.00
Mr. Debajit Kalita	PA	28/06/11	30/09/12	8,000.00	48,000.00	48,000.00
Ms. Niharika Gogoi	PA	17/12/12	NA	8,000.00	27,355.00	27,355.00
				Total	2,07,355.00	2,07,355.00

1st April 2013- 31st March 2014

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY (in Rs.)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR (in Rs.)	TOTAL SALARY PAID DURING PROJECT PERIOD (In Rs.)
Mr. Diganta Das	JRF	01/07/11	31/07/13	12,000.00	48,000.00	48,000.00
Mr. Diganta Das	SRF	01/08/13	NA	14,000.00	96,000.00	96,000.00
Ms. Niharika Gogoi	PA	17/12/12	31/05/13	8,000.00	21,067.00	21,067.00
Ms. Palakshi Das	PA	07/10/13	NA	8,000.00	38,452.00	38,452.00
				Total	2,03,519.00	2,03,519.00

1st April 2014- 31st March 2015

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY (in Rs.)	TOTAL SALARY PAID DURING THE FINANCIAL YEAR (in Rs.)	TOTAL SALARY PAID DURING PROJECT PERIOD (In Rs.)
Mr. Diganta Das	SRF	01/08/13	31/07/14	14,000.00	48,000.00	48,000.00
Mr. Diganta Das	SRF (Arrear)	01/08/13	31/07/14	@2,000.00 /month	24,000.00	24,000.00
Ms. Palakshi Das	PA	07/10/13	31/03/14	8,000.00	8,000.00	8,000.00
				Total	80,000.00	80,000.00

(Signature of Principal Investigator)

(Signature of Accounts Officer) 19-6-15

(SIGNATURE OF HEAD OF THE INSTITUTE)

Annexure B

Manpower Expenditure Details (In financial year wise manner):

14th March 2011- 31st March 2012

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
JRF	1	Rs. 12,000	Rs. 2,11,000	Rs. 6,60,000	NA	NA	NA	Rs. 2,11,000	Rs. 1,08,000	Rs. 1,03,000
PA	1	Rs. 8,000	Rs. 96,000	Rs. 2,28,000	NA	NA	NA	Rs. 96,000	Rs. 72,800	Rs. 23,200

1th April 2012- 31st March 2013

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
JRF	1	Rs. 12,000	Rs. 2,11,000	Rs. 6,60,000	NA	NA	NA	Rs. 1,77,000	Rs 1,32,000	Rs. 45,000
PA	1	Rs. 8,000	Rs. 96,000	Rs. 2,28,000	NA	NA	NA	Rs. 96,000	Rs 75,355	Rs. 20,645

1th April 2013- 31st March 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
JRF	1	Rs. 12,000	Rs. 2,38,000	Rs. 6,60,000	NA	NA	NA	0	Rs. 1,44,000	
SRF	1	Rs. 14,000								
PA	1	Rs. 8,000	Rs. 96,000	Rs. 2,28,000	NA	NA	NA	0	Rs. 59,519	

1th April 2014- 31st March 2015

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	Rs. 14,000	Rs. 1,44,000	Rs. 6,60,000	NA	NA	NA	Rs. 1,44,000	Rs. 48,000	Rs. 72,000
SRF (Arrear)									Rs. 24,000	
PA	1	Rs. 8,000	Rs. 96,000	Rs. 2,28,000	NA	NA	NA	Rs. 36,000	Rs. 8,000	Rs.- 28,000

29-6-15

(Signature of Principal Investigator) **(Signature of Accounts Officer)**

Associate Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University
Tezpur-784028, Assam, India

Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
Tezpur University

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* Details of manpower salary/ fellowship revision along with due- drawn statement and arrears requested should be given separately, if applicable.

Due- Drawn Statement

14th March 2011 to 31st March 2012

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Diganta Das Junior Research Fellow	July 2011	12,000.00	12,000.00	
	August 2011	12,000.00	12,000.00	
	September 2011	12,000.00	12,000.00	
	October 2011	12,000.00	12,000.00	
	November 2011	12,000.00	12,000.00	
	December 2011	12,000.00	12,000.00	
	January 2012	12,000.00	12,000.00	
	February 2012	12,000.00	12,000.00	
	March 2012	12.000.00	12.000.00	
	Total	**1,08,000.00**	**1,08,000.00**	

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Debojit Kalita Project Assistant (Resigned on 31/09/12)	June 2011	800.00	800.00	
	July 2011	8000.00	8000.00	
	August 2011	8000.00	8000.00	
	September 2011	8000.00	8000.00	
	October 2011	8000.00	8000.00	
	November 2011	8000.00	8000.00	
	December 2011	8000.00	8000.00	
	January 2012	8000.00	8000.00	
	February 2012	8000.00	8000.00	
	March 2012	8000.00	8000.00	
	Total	**72,800.00**	**72,800.00**	

Total (JRF+PA) = 1, 08,000.00+ 72,800.00= 1, 80, 800.00

Due- Drawn Statement

1st April 2012 to 31st March 2013

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Diganta Das Junior Research Fellow	April 2012	12,000.00	12,000.00	
	May 2012	12,000.00	12,000.00	
	June 2012	12,000.00	12,000.00	
	July 2012	12,000.00	12,000.00	
	August 2012	12,000.00	12,000.00	
	September 2012	12,000.00	12,000.00	
	October 2012	12,000.00	12,000.00	
	November 2012	12,000.00	12,000.00	
	December 2012	12.000.00	12.000.00	
	January 2013	12,000.00	12,000.00	
	February 2013	12,000.00	12,000.00	
	Total	**1,32,000.00**	**1,32,000.00**	

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Debojit Kalita*	April 2012	8000.00	8000.00	
	May 2012	8000.00	8000.00	
	June 2012	8000.00	8000.00	
	July 2012	8000.00	8000.00	
	August 2012	8000.00	8000.00	
	September 2012	8000.00	8000.00	
	Total	**48,000.00**	**48,000.00**	

***Project Assistant (Resigned on 31/09/12)**

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Ms. Neeharika Gogoi, Project Assistant	December 2012	8000.00	3355.00	4645.00
	January 2013	8000.00	8000.00	
	February 2013	8000.00	8000.00	
	March 2013	8000.00	8000.00	
	Total	27,355.00	25,355.00	4645.00

Total (JRF+PA)= 1, 32, 000.00+ 48,000.00 + 27,355.00= Rs. 207,355.00

Due- Drawn Statement

1st April 2013- 31st March 2014

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Diganta Das *(Junior Research Fellow/Senior Research Fellow)*	March 2013	12,000.00	12,000.00	
	April 2013	12,000.00	12,000.00	
	May 2013	12,000.00	12,000.00	
	June 2013	12,000.00	12,000.00	
	July 2013	14,000.00	12,000.00	2,000.00
	August 2013	14,000.00	12,000.00	2,000.00
	September 2013	14,000.00	12,000.00	2,000.00
	October 2013	14,000.00	12,000.00	2,000.00
	November 2013	14,000.00	12,000.00	2,000.00
	December 2013	14.000.00	12.000.00	2,000.00
	January 2014	14,000.00	12,000.00	2,000.00
	February 2014	14,000.00	12,000.00	2,000.00
	Total	**1,58,000.00**	**1,44,000.00**	**16,000.00**

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Ms. Niharika Gogoi*, Project Assistant	April 2013	8000.00	8000.00	
	May 2013	8000.00	8000.00	
	June 2013	8000.00	5,067.00	2,933.00
	Total	**24,000.00**	**21,067.00**	**2,933.00**

*Project Assistant (Resigned on 30/06/13)

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Ms. Palakshi Das, Project Assistant	October 2013	8000.00	6452.00	1,548.00
	November 2013	8000.00	8000.00	
	December 2013	8000.00	8000.00	
	January 2014	8000.00	8000.00	
	February 2014	8000.00	8000.00	
	Total	**40,000.00**	**38,452.00**	**1,548.00**

Total (JRF+PA) = 1,44,000.00+ 21,067.00 + 38,452.00= Rs. 203,519.00

Due- Drawn Statement

1st April 2014- 31st March 2015

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Diganta Das Senior Research Fellow	March 2014	14,000.00	12,000.00	2,000.00
	April 2014	14,000.00	12,000.00	2,000.00
	May 2014	14,000.00	12,000.00	2,000.00
	June 2014	14,000.00	12,000.00	2,000.00
	Total	**56,000.00**	**48,000.00**	**8,000.00**

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Mr. Diganta Das Senior research fellow (Arrear)	July 2013 to June 2014	24,000.00	24,000.00	
	Total	**24,000.00**	**24,000.00**	

Name of the Project Staff	Month and Year	Due (Rs)	Drawn(Rs)	Difference (Rs)
Ms. Palakshi Das Project Assistant	March 2014	8,000.00	8,000.00	
	Total	**8,000.00**	**8,000.00**	

Total (SRF+Arrear+PA) = 48,000.00+ 24,000.00+ 8,000.00= Rs. 80,000.00

29.6.15

(Signature of Principal Investigator)

(Signature of Accounts Officer)

(SIGNATURE OF HEAD OF THE INSTITUTE)

* List of equipment purchased indicating the item wise costs may please be provided.

List of equipment purchased

Sl. No.	Items	Cost in Foreign Currency/INR	Amount in Rupees	Status
1	Shaker incubator with Static plate form	Rs. 2,19,656.00	2,19,656.00	Installed
2	Accessories of shaker incubators	Rs. 14,321.00	14,321.00	Installed
3	Tissue homogenizer	Rs. 1,09074.00	1,09,074.00	Installed
4	Micropipette set	Rs. 1,33,192.25	1,33,192.00	Installed
5	Bench top centrifuge	Rs. 62311.50	62311.50	Installed
6	Vortex	Rs. 17252.00	17,252.00	Installed
7	Magnetic Stirrer	Rs. 34,617.50	34,617.50	Installed
8	HPLC Columns	USD 5,724.00	3,20,430.00	Installed
9	Protein and DNA electrophoresis	USD 2,872.00	1,45,282.00	Installed
10	2 KV UPS and printer for Spectrophotometer	Rs. 89,665.00	89,665.00	Installed
11	Refrigerated Centrifuge	Euro 6,337.00	4,39,799.00	Installed
12	Spectrophotometer	Euro 9,556.00	6,83,728.00	Installed
13	Gradient PCR	Euro 4,817.00	3,50,842.00	Installed
14	Nano Drop2000 with PC	USD 8,500.00	5,27,887.00	Installed
		Total	**31,48,057.00**	

(PROJECT INVESTIGATOR)

29.6.16

(FINANCE OFFICER)

(HEAD OF THE INSTITUTE)

Appendix-A

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

1.	Name of the Sanctioning Authority:	**Department of Biotechnology, Govt. of India**
2.	Sl. No.	**306**
3.	Name of the Grantee Institution	**Tezpur University**
4.	No. & Date of sanction order	**No. BT/43/NE/TBP/2010, 14/03/2011**
5.	Amount of the sanctioned grant	**69.84 Lakhs**
6.	Brief purpose of the grant	**To work for DBT Twinning Programme for NE entitled:"Search for novel treatments for snake venom poisoning: composition analysis of *Naja naja* and *Daboia russelli* venoms and complete characterization of their major toxins"**
7.	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	**31.48057 lakhs as on 31st March 2015**
9.	Purpose for which utilised at present	**To work for DBT Twinning Programme for NE**
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 realised on disposal	**NA**
15.	Remarks	**NIL**

29-04-15

(FINANCE OFFICER)

(PROJECT INVESTIGATOR)

Dr. Robin Doley
Associate Professor
Dept. of Molecular Biology and Biotechnology
Tezpur University
(a Central University)
Napaam, Tezpur- 784 028
Sonitpur, Assam (India)

(HEAD OF THE INSTITUTE)