

# Contents

<b>Abstract</b>	<b>i-iii</b>
<b>Declaration by the candidate</b>	<b>iv</b>
<b>Certificate of the Supervisor</b>	<b>v</b>
<b>Acknowledgement</b>	<b>vi-vii</b>
<b>Table of contents</b>	<b>viii-xiii</b>
<b>List of tables</b>	<b>xiv</b>
<b>List of figures</b>	<b>xv-xviii</b>
<b>List of abbreviations</b>	<b>xix-xxii</b>
<b>Chapter 1: General Introduction and Review of literature</b>	<b>1-51</b>
<b>1. Introduction</b>	<b>1-1</b>
1.1 Snake venom	1-13
1.1.1 Snakes	1-2
1.1.2 Venomous and non-venomous snakes	2-4
1.1.3 Venom gland	4-5
1.1.4 Composition of snake venom	6-10
1.1.5 Snakebite problem	10-13
1.2 Venom variation	13-18
1.2.1 Venom variation at the interspecies level	13-13
1.2.2 Venom variation at the intraspecies level	14-14
1.2.3 Venom variation due to ontogenetic changes	14-14
1.2.4 Venom variation due to sexual dimorphism	15-15
1.2.5 Venom variation due to geographical locations	15-16
1.2.6 Venom variation due to diet	16-16
1.2.7 Venom variation due to seasonal changes	16-17
1.2.8 Venom variation and limitations of antivenom	17-18
1.3 Overview of the haemostatic system	18-24
1.3.1 Primary Haemostasis	19-19
1.3.2 Secondary Haemostasis	19-19
1.3.2.1 Extrinsic pathway	19-20
1.3.2.2 Intrinsic pathway	20-20
1.3.2.3 Common pathway	20-20
1.3.2.4 Fibrinolysis	20-21
1.3.3 Blood coagulation factor X	21-23
1.3.3.1 Direct and Indirect inhibitors of FXa	23-24
1.4 Snake venom toxin families affecting haemostatic system	24-32
1.4.1 Procoagulant toxin families	25-28
1.4.2 Anticoagulant toxin families	28-32
1.5 Snake venom PLA <sub>2</sub> enzyme	32-45
1.5.1 Phospholipase A <sub>2</sub> enzymes in general	32-34
1.5.2 Classification of PLA <sub>2</sub> enzymes	34-36
1.5.3 Evolution of the PLA <sub>2</sub> gene	36-37
1.5.4 Structure of the PLA <sub>2</sub> enzyme	37-38
1.5.5 Catalytic mechanism of sPLA <sub>2</sub> enzymes	38-39
1.5.6 Snake venom PLA <sub>2</sub> enzyme	39-40
1.5.7 Biological activities of snake venom PLA <sub>2</sub> enzymes	40-45

1.5.7.1 Neurotoxicity	40-40
1.5.7.2 Myotoxicity	41-41
1.5.7.3 Cardiotoxicity	41-41
1.5.7.4 Antimicrobial activity	42-42
1.5.7.5 Edema inducing	42-43
1.5.7.6 Platelet aggregation initiation or inhibition	43-43
1.5.7.7 Anticoagulation	43-45
1.6 Indian <i>Daboia russelii</i> venom proteomics	45-50
1.6.1 <i>Daboia russelii</i>	45-47
1.6.2 Research so far on Indian <i>Daboia russelii</i> venom	47-50
1.7 Gap in the study	50-50
1.8 Aims and objectives of the study	51-51
<b>Chapter 2: Analysis of crude <i>Daboia russelii</i> venoms from different geographical locations of India</b>	<b>52-71</b>
<b>2.1 Introduction</b>	52-53
<b>2.2 Materials</b>	53-54
2.2.1 Snake venoms	53-54
2.2.2 Chemicals and reagents	54-54
2.2.3 Columns	54-54
<b>2.3 Methods</b>	55-58
2.3.1 Protein estimation	55-55
2.3.2 Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis	55-55
2.3.3 Size exclusion chromatography	55-56
2.3.4 Reversed phase-High Performance Liquid Chromatography	56-56
2.3.5 Phospholipase A <sub>2</sub> activity	56-56
2.3.6 Coagulation assay	56-57
2.3.6.1 Preparation of blood plasma	56-57
2.3.6.2 Recalcification time	57-57
2.3.7 Neutralization study by polyvalent antivenom	57-57
2.3.8 Immunodepletion of venom proteins	57-58
2.3.9 Statistical analysis	58-58
<b>2.4 Results</b>	59-66
2.4.1 Quantitative and qualitative analysis of crude venoms	59-62
2.4.1.1 Protein content	59-59
2.4.1.2 SDS-PAGE analysis	60-60
2.4.1.3 Chromatographic analysis	60-62
2.4.2 Biochemical assays	62-63
2.4.2.1 PLA <sub>2</sub> activity	62-63
2.4.2.2 Recalcification time	62-63
2.4.3 Neutralization study	64-65
2.4.4 Immunodepletion study	65-66
<b>2.5 Discussion</b>	66-71

<b>Chapter 3: Proteomics of <i>Daboia russelii</i> (Irula) venom and identification of a major protein</b>	<b>72-120</b>
<b>3.1 Introduction</b>	72-73
<b>3.2 Materials</b>	73-75
3.2.1 Venom	73-73
3.2.2 Chemicals and Reagents	73-74
3.2.3 Column	74-74
3.2.4 Animals	74-75
<b>3.3. Methods</b>	75-82
3.3.1 Protein estimation	75-75
3.3.2 Gel filtration chromatography of crude venom of <i>Daboia russelii</i>	75-75
3.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis	75-75
3.3.4 Tandem mass spectrometry of the gel filtration peaks	75-77
3.3.5 <i>In-vitro</i> coagulation assays	77-78
3.3.5.1 Preparation of platelet poor plasma and recalcification assay	77-77
3.3.5.2 Prothrombin time (PT)	78-78
3.3.5.3 Activated partial thromboplastin time (APTT)	78-78
3.3.6 Fibrinogenolytic activity	78-78
3.3.7 Haemolytic assay	78-79
3.3.7.1 Preparation of the erythrocyte suspension	78-78
3.3.7.2 Direct and indirect haemolytic activity	79-79
3.3.8 Phospholipase A <sub>2</sub> activity	79-80
3.3.9 Proteolytic activity	80-81
3.3.10 Edema inducing activity	81-81
3.3.11 Haemorrhagic activity	81-82
3.3.12 Cytotoxicity assay	82-82
<b>3.4 Results</b>	83-112
3.4.1 Protein estimation	83-83
3.4.2 Gel filtration chromatography	83-84
3.4.3 SDS-PAGE analysis	84-85
3.4.4 Tandem mass spectrometry	85-101
3.4.5 <i>In-vitro</i> anticoagulant assays	101-103
3.4.5.1 Recalcification time	101-102
3.4.5.2 Prothrombin time	101-102
3.4.5.3 Activated partial thromboplastin time	103-103
3.4.6 Fibrinogenolytic activity	103-104
3.4.7 Haemolytic assays	104-105
3.4.7.1 Direct haemolytic assay	104-104
3.4.7.2 Indirect haemolytic assay	105-105
3.4.8 PLA <sub>2</sub> activity	105-106
3.4.9 Proteolytic activity	105-106
3.4.10 Edema inducing activity	107-107
3.4.11 Haemorrhagic activity	107-108
3.4.12 Cytotoxicity	108-110
3.4.13 Recalcification time of the gel filtration fractions of the crude <i>Daboia russelii</i> venom	111-111

3.4.14 PLA <sub>2</sub> activity of the gel filtration fractions of the crude <i>Daboia russelii</i> venom	111-112
<b>3.5 Discussion</b>	112-120
<b>Chapter 4: Purification of a major protein from the venom of <i>Daboia russelii</i> (Irula) and its biophysical characterization.</b>	<b>121-148</b>
<b>4.1 Introduction</b>	121-122
<b>4.2 Materials</b>	122-123
4.2.1 Chemicals and Reagents	122-122
4.2.2 Column	122-123
<b>4.3 Methods</b>	123-130
4.3.1 Purification of the major anticoagulant PLA <sub>2</sub> enzyme	123-124
4.3.1.1 Gel filtration chromatography	123-123
4.3.1.2 Ion exchange chromatography	123-123
4.3.1.3 Reversed phase high performance liquid Chromatography	123-124
4.3.2 Biophysical characterization	124-130
4.3.2.1 Molecular mass determination	124-124
4.3.2.2 Primary structure determination	125-129
4.3.2.2.1 N-terminal sequencing	125-126
4.3.2.2.2 Pyridylethylation	126-127
4.3.2.2.3 BNPS-skatole cleavage	127-128
4.3.2.2.4 Hydroxylamine hydrochloride cleavage	128-129
4.3.2.3 Tandem mass spectrometry	129-129
4.3.2.4 Multiple sequence alignment	129-129
4.3.2.5 Phylogenetic analysis	129-129
4.3.2.6 Secondary structure determination	129-130
<b>4.4 Results</b>	130-144
4.4.1 Purification of daboxin P	130-134
4.4.1.1 Gel filtration chromatography	130-131
4.4.1.2 Ion exchange chromatography	131-132
4.4.1.3 Rp-HPLC	132-134
4.4.2 Biophysical characterization	134-144
4.4.2.1 Molecular mass of daboxin P	134-134
4.4.2.2 Primary sequence of daboxin P	134-138
4.4.2.2.1 N-terminal sequencing	134-135
4.4.2.2.2 Pyridylethylation	135-135
4.4.2.2.3 Cleavage with BNPS-skatole	135-136
4.4.2.2.4 Cleavage with hydroxylamine hydrochloride	137-138
4.4.2.3 Tandem mass spectrometry	138-139
4.4.2.4 Sequence analysis of daboxin P	139-140
4.4.2.5 Phylogenetic relationship of daboxin P	141-142
4.4.2.6 Secondary structure of daboxin P.	142-144
<b>4.5 Discussion</b>	144-148

<b>Chapter 5: Biochemical and biological characterization of the purified protein.</b>	<b>149-173</b>
<b>5.1 Introduction</b>	149-150
<b>5.2 Materials</b>	150-151
5.2.1 Chemicals and reagents	150-150
5.2.2 Animals	150-151
<b>5.3 Methods</b>	151-156
5.3.1 PLA <sub>2</sub> activity & determination of Km and Vmax	151-151
5.3.2 Alkylation of histidine residue of daboxin P	151-152
5.3.3 Proteolytic activity of daboxin P	152-152
5.3.4 Direct and indirect haemolytic activity of daboxin P	152-152
5.3.5 <i>In vitro</i> -anticoagulant activities of daboxin P	152-153
5.3.5.1 Stypven time of daboxin P	153-153
5.3.5.2 Thrombin time of daboxin P	153-153
5.3.6 Fibrinogenolytic activity of daboxin P	153-153
5.3.7 <i>In-vivo</i> anticoagulant activity of daboxin P	153-154
5.3.8 Antibacterial activity of daboxin P	154-154
5.3.9 Cytotoxicity study of daboxin P	154-154
5.3.10 Inhibition study of daboxin P	154-155
5.3.11 Western blotting	155-156
<b>5.4 Results</b>	156-169
5.4.1 PLA <sub>2</sub> activity of daboxin P	156-156
5.4.2 Alkylation of histidine residue of daboxin P	157-157
5.4.3 Proteolytic activity of daboxin P	157-158
5.4.4 Direct haemolytic activity of daboxin P	158-159
5.4.5 Indirect haemolytic activity of daboxin P	159-159
5.4.6 <i>In-vitro</i> anticoagulant activity of daboxin P	160-162
5.4.6.1 Recalcification time of daboxin P	160-160
5.4.6.2 Prothrombin time of daboxin P	160-161
5.4.6.3 Activated partial thromboplastin time of daboxin P	161-161
5.4.6.4 Stypven time of daboxin P	161-162
5.4.6.5 Thrombin time of daboxin P	162-162
5.4.6.6 Fibrinogenolytic activity of daboxin P	163-163
5.4.7 <i>In-vivo</i> anticoagulant activity of daboxin P	163-164
5.4.8 Antibacterial activity of daboxin P	164-165
5.4.9 Cytotoxicity of daboxin P	165-167
5.4.10 Neutralization study	167-168
5.4.11 Western blotting	169-169
<b>5.5 Discussion</b>	169-173
<b>Chapter 6: <i>In-silico</i> structural elucidation and mechanism of the purified protein</b>	<b>174-200</b>
<b>6.1 Introduction</b>	174-175
<b>6.2 Materials</b>	175-175
<b>6.3 Method</b>	176-183
6.3.1 Three dimensional (3D) molecular modelling of daboxin P.	176-177
6.3.2 Screening for the inhibitory effect of daboxin P on the amidolytic activity of the serine proteases.	177-178

6.3.3 Effect of daboxin P on extrinsic tenase complex.	178-178
6.3.4 Effect of daboxin P on intrinsic tenase complex.	179-179
6.3.5 Determination of the IC50 of daboxin P for the extrinsic and intrinsic tenase complex.	179-179
6.3.6 Effect of daboxin P on prothrombinase complex.	180-180
6.3.7 Fluorescence emission spectroscopy of FX/FXa and daboxin P.	180-180
6.3.8 CNBr activated sepharose affinity chromatography	180-181
6.3.9 Molecular docking of daboxin P with FXa.	181-183
<b>6.4 Results</b>	183-194
6.4.1 <i>In-silico</i> 3D molecular modelling of daboxin P.	183-185
6.4.2 Screening for serine protease specificity of daboxin P	186-186
6.4.3 Effect of daboxin P on extrinsic tenase complex	186-188
6.4.4 Effect of daboxin P on intrinsic tenase complex	188-189
6.4.5 Effect of daboxin P on prothrombinase complex	190-190
6.4.6 Fluorescence emission spectroscopy	190-191
6.4.7 Affinity column chromatography	191-192
6.4.8 Molecular docking of daboxin P and FXa	192-194
<b>6.5 Discussion</b>	194-200
<b>Chapter 7: Conclusion and Future Prospects</b>	<b>201-205</b>
7.1 Conclusion	201-203
7.2 Future prospects of the current study	203-205
<b>Bibliography</b>	<b>206-236</b>
<b>Appendix I: Alignment of peptide fragments obtained from tandem mass spectrometry</b>	<b>xxiii</b>
<b>Appendix II: List of Publications</b>	<b>xxiv</b>
<b>Appendix III: List of Conferences and Seminars attended</b>	<b>xxv</b>
<b>Appendix IV: List of Papers/Posters presented in National and International seminar/conferences</b>	<b>xxvi</b>
<b>Appendix V: Permissions and Approval from Ethical committee</b>	<b>xxvii</b>

## List of Tables

<b>Chapter 1:</b>	<b>General Introduction and Review of literature</b>	<b>1-51</b>
Table 1.1:	List of venomous and non-venomous	3-4
Table 1.2:	List of enzymatic protein families of snake venom	7-8
Table 1.3:	List of non-enzymatic protein families of snake venom	8-9
Table 1.4:	List of venomous snakes in India	11
Table 1.5:	Scientific classification of <i>Daboia russelii</i>	46
Table 1.6:	List of proteins isolated and characterized from Indian <i>Daboia russelii</i> venom.	48-50
<b>Chapter 2:</b>	<b>Analysis of crude <i>Daboia russelii</i> venoms from different geographical locations of India</b>	<b>52-71</b>
Table 2.1:	Protein content of crude <i>Daboia russelii</i> venoms from different geographical locations.	59
<b>Chapter 3:</b>	<b>Proteomics of <i>Daboia russelii</i> (Irula) venom and identification of a major protein</b>	<b>72-120</b>
Table 3.1:	Protein estimation of the gel filtration peaks of crude <i>Daboia russelii</i> venom.	84
Table 3.2:	List of peptide fragments obtained by MS/MS in each gel filtration peaks of crude <i>Daboia russelii</i> venom.	87-99
Table 3.3:	Isoforms of protein families identified in the crude <i>Daboia russelii</i> venom.	100
<b>Chapter 4:</b>	<b>Purification of a major protein from the venom of <i>Daboia russelii</i> (Irula) and its biophysical characterization.</b>	<b>121-148</b>
Table 4.1:	Amino acid residues of daboxin P deduced after N-terminal sequencing.	135
Table 4.2:	Amino acid residues of daboxin P deduced after cleavage with BNPS-skatole.	136
Table 4.3:	Amino acid residues of daboxin P deduced after cleavage with hydroxylamine hydrochloride.	138
Table 4.4:	Peptide fragments of daboxin P obtained after tandem mass spectrometry.	138
Table 4.5:	Peptide fragments of daboxin P obtained after Edman degradation sequencing and tandem mass spectrometry.	139
<b>Chapter 6:</b>	<b><i>In-silico</i> structural elucidation and mechanism of the purified protein.</b>	<b>174-200</b>
Table 6.1:	Amino acid residues of daboxin P and FXa involved in the interaction based on PDBsum analysis.	193
Table 6.2:	Interface statistics of the docked complex of daboxin P and FXa.	193

## List of Figures

<b>Chapter 1:</b>	<b>General Introduction and Review of literature</b>	<b>1-51</b>
Figure 1.1:	Different types of venom apparatus in venomous snakes.	5
Figure 1.2:	Clinical pathologies of snakebite in general.	10
Figure 1.3:	Haemostatic system during vascular injury.	21
Figure 1.4:	Snake venom procoagulant proteins affecting the haemostatic system.	27
Figure 1.5:	Snake venom anticoagulant proteins affecting the haemostatic system.	31
Figure 1.6:	Phospholipid hydrolysis by phospholipase enzymes.	32
Figure 1.7:	Hydrolysis of glycerophospholipid by PLA <sub>2</sub> enzymes.	33
Figure 1.8:	Types of PLA <sub>2</sub> enzymes in the living system.	34
Figure 1.9:	<i>Daboia russelii</i> snake and its global distribution.	45
<b>Chapter 2:</b>	<b>Analysis of crude <i>Daboia russelii</i> venoms from different geographical locations of India</b>	<b>52-71</b>
Figure 2.1:	Steps involved in 2 <sup>nd</sup> generation antivenomics study.	58
Figure 2.2:	Map of India showing geographical locations of four crude <i>Daboia. russelii</i> venoms.	59
Figure 2.3:	12.5% Glycine SDS-PAGE profile of crude <i>Daboia russelii</i> venoms under reduced condition.	60
Figure 2.4:	Gel filtration chromatography profile of crude venoms of <i>Daboia russelii</i> on Shodex column.	61
Figure 2.5:	Rp-HPLC profile of crude <i>Daboia russelii</i> venoms on Jupiter C <sub>18</sub> column.	62
Figure 2.6:	PLA <sub>2</sub> activity of crude <i>Daboia russelii</i> venoms using turbidometric method.	63
Figure 2.7:	Recalcification time of crude <i>Daboia russelii</i> venoms.	63
Figure 2.8:	Percentage residual PLA <sub>2</sub> activity of crude <i>Daboia russelii</i> venoms pre-incubated with polyvalent antivenom.	64
Figure 2.9:	Neutralization of recalcification time of crude <i>Daboia russelii</i> venoms pre-incubated with polyvalent antivenom.	65
Figure 2.10:	Rp-HPLC profiles of the non-retained fractions of crude <i>Daboia russelii</i> venoms on Jupiter C <sub>18</sub> column.	66
<b>Chapter 3:</b>	<b>Proteomics of <i>Daboia russelii</i> (Irula) venom and identification of a major protein.</b>	<b>72-120</b>
Figure 3.1:	Steps involved in tandem mass spectrometry.	77
Figure 3.2:	Carbamidomethylation of cysteine by iodoacetamide.	77
Figure 3.3:	Diheptanoyl Thio-PC hydrolysis by PLA <sub>2</sub> enzymes.	80
Figure 3.4:	Size exclusion chromatography profile of crude <i>Daboia russelii</i> venom on Superdex 75 column.	83
Figure 3.5:	Electrophoretic profile of crude <i>Daboia russelii</i> venom and its gel filtration fractions under reduced conditions.	85
Figure 3.6:	Isoforms of snake venom protein families identified in	



	gel filtration peaks by tandem mass spectrometry.	100
Figure 3.7:	Relative distribution of snake venom protein families in Indian <i>Daboia russelii</i> venom.	101
Figure 3.8:	Recalcification time of crude <i>Daboia russelii</i> venom.	102
Figure 3.9:	Prothrombin time of crude <i>Daboia russelii</i> venom.	102
Figure 3.10:	Activated partial thromboplastin time of crude <i>Daboia russelii</i> venom.	103
Figure 3.11:	Fibrinogenolytic activity of crude venom of <i>Daboia russelii</i> on 12.5% glycine SDS-PAGE.	104
Figure 3.12:	Direct haemolytic activity of crude <i>Daboia russelii</i> venom.	104
Figure 3.13:	Indirect haemolytic activity of crude <i>Daboia russelii</i> venom.	105
Figure 3.14:	PLA <sub>2</sub> activity of crude <i>Daboia russelii</i> venom using sPLA <sub>2</sub> kit.	106
Figure 3.15:	Proteolytic activity of crude <i>Daboia russelii</i> venom on casein.	106
Figure 3.16:	Edema inducing activity of crude <i>Daboia russelii</i> venom.	107
Figure 3.17:	Haemorrhagic activity of crude <i>Daboia russelii</i> venom.	107
Figure 3.18:	Microscopic images of HEK-293 cell lines after treatment with crude venom of <i>Daboia russelii</i> .	109
Figure 3.19:	Cytotoxic effect of crude venom of <i>Daboia russelii</i> on HEK-293 cell lines.	109
Figure 3.20:	Microscopic images of MCF-7 cell lines after treatment with crude venom of <i>Daboia russelii</i> .	110
Figure 3.21:	Cytotoxic effect of crude venom of <i>Daboia russelii</i> on MCF-7 cell lines.	110
Figure 3.22:	Recalcification time of the gel filtration peaks of crude <i>Daboia russelii</i> venom.	111
Figure 3.23:	PLA <sub>2</sub> activity of the gel filtration peaks of crude <i>Daboia russelii</i> venom using sPLA <sub>2</sub> kit.	112
<b>Chapter 4:</b>	<b>Purification of a major protein from the venom of <i>Daboia russelii</i> (Irula) and its biophysical. Characterization</b>	<b>121-148</b>
Figure 4.1:	Steps involved in protein ionization by ESI-MS.	124
Figure 4.2:	Reaction mechanism of Edman degradation.	125
Figure 4.3:	Parts of PPSQ-31A protein sequencer.	126
Figure 4.4:	Pyridylethylation reaction of cysteine residues.	126
Figure 4.5:	Reaction of protein cleavage by BNPS-Skatole.	127
Figure 4.6:	Reaction of protein cleavage by hydroxylamine hydrochloride.	128
Figure 4.7:	Principle of circular dichroism spectroscopy.	130
Figure 4.8:	Gel filtration chromatography profile of crude <i>Daboia russelii</i> venom.	131
Figure 4.9:	Ion exchange chromatography profile of P6.	132

Figure 4.10:	Recalcification time and PLA <sub>2</sub> activity of ion exchange fractions of P6.	132
Figure 4.11:	Rp-HPLC profile of CM-II and homogeneity of Rp-2.	133
Figure 4.12:	Recalcification time and PLA <sub>2</sub> activity of Rp-HPLC fractions of CM-II.	133
Figure 4.13:	Molecular mass of daboxin P.	134
Figure 4.14:	Rp-HPLC profile and molecular mass of pyridylethylated daboxin P.	135
Figure 4.15:	Rp-HPLC profile and molecular mass of the peptide fragments of daboxin P after BNPS-skatole cleavage.	136
Figure 4.16:	Rp-HPLC profile and mass of the peptide fragments of daboxin P hydroxylamine hydrochloride cleavage.	137
Figure 4.17:	Assembled peptide fragments of daboxin P.	139
Figure 4.18:	Multiple sequence alignment of daboxin P with snake venom PLA <sub>2</sub> enzymes.	140
Figure 4.19:	Phylogenetic tree of daboxin P with anticoagulant snake venom PLA <sub>2</sub> enzymes.	141
Figure 4.20:	Secondary structure of daboxin P.	143
Figure 4.21:	Secondary structure of daboxin P at different pH.	143
Figure 4.22:	Secondary structure of daboxin P at different temperature.	144
Figure 4.23:	Primary structure of daboxin P with conserved regions.	146
Figure 4.24:	Sequence alignment of daboxin P with VRV-PL-VIIIa.	147

**Chapter 5: Biochemical and biological characterization of the purified protein. 149-173**

Figure 5.1:	Alkylation reaction of histidine residue by PBP.	152
Figure 5.2:	PLA <sub>2</sub> activity of daboxin P.	156
Figure 5.3:	PLA <sub>2</sub> activity of modified and unmodified daboxin P.	157
Figure 5.4:	Proteolytic activity of daboxin P.	158
Figure 5.5:	Direct haemolytic activity of daboxin P.	158
Figure 5.6:	Indirect haemolytic activity of daboxin P.	159
Figure 5.7:	Recalcification time of daboxin P.	160
Figure 5.8:	Prothrombin time of daboxin P.	160
Figure 5.9:	Activated partial thromboplastin time of daboxin P.	161
Figure 5.10:	Stypven time of daboxin P.	162
Figure 5.11:	Thrombin time of daboxin P.	162
Figure 5.12:	Fibrinogenolytic activity of daboxin P on SDS-PAGE.	163
Figure 5.13:	Time to occlusion of daboxin P in carotid artery of mice treated with FeCl <sub>3</sub> .	164
Figure 5.14:	Antibacterial activity of daboxin P.	164
Figure 5.15:	Microscopic images of HEK293 cell treated with daboxin P.	165
Figure 5.16:	Cytotoxicity of daboxin P on HEK293 cell using MTT assay.	166
Figure 5.17:	Microscopic images of the daboxin P treated MCF-7 cell lines.	166
Figure 5.18:	Cytotoxicity of daboxin P on MCF-7 cells using MTT assay.	167

	cell lines using MTT based colorimetric assay.	
Figure 5.19:	Neutralization of PLA <sub>2</sub> activity of daboxin P by polyvalent antivenom.	168
Figure 5.20:	Neutralization of recalcification time of daboxin P by polyvalent antivenom.	168
Figure 5.21:	Immunoblot of daboxin P using polyvalent antivenom.	169
<b>Chapter 6:</b>	<b><i>In-silico</i> structural elucidation and mechanism of the purified protein.</b>	<b>174-200</b>
Figure 6.1:	Steps involved in 3D modeling by I-TASSER.	176
Figure 6.2:	Schematic representation of amidolytic assay by activated serine proteases.	177
Figure 6.3:	Steps involved in molecular docking.	182
Figure 6.4:	Predicted amino acid residues of daboxin P involved in secondary structure formation by I-TASSER.	184
Figure 6.5:	Predicted amino acid residues of daboxin P involved in formation of solvent accessible regions by I-TASSER.	184
Figure 6.6:	Normalized Z-score of 10 threading templates for daboxin P used by I-TASSER.	184
Figure 6.7:	TM-score and RMSD value of 10 structural analogs of daboxin P in PDB determined by I-TASSER.	184
Figure 6.8:	3D molecular modeling of daboxin P.	185
Figure 6.9:	Sequence alignment of daboxin P with AtxA.	185
Figure 6.10:	Ribbon model of daboxin P and its solvent accessible surface generated by DS ViewerPro 5.0.	185
Figure 6.11:	Percentage residual amidolytic activity of the activated serine protease pre-incubated with daboxin P.	186
Figure 6.12:	Percentage residual activity of the extrinsic tenase complex pre-incubated with of daboxin P.	187
Figure 6.13:	IC <sub>50</sub> curve for extrinsic tenase complex.	188
Figure 6.14:	Percentage residual activity of the intrinsic tenase complex pre-incubated with daboxin P.	189
Figure 6.15:	IC <sub>50</sub> curve for the intrinsic tenase complex.	189
Figure 6.16:	Percentage residual activity of the prothrombinase complex pre-incubated with daboxin P.	190
Figure 6.17:	Fluorescence emission spectra of daboxin P, FX, FXa.	191
Figure 6.18:	SDS-PAGE profile of affinity chromatography fractions.	191
Figure 6.19:	<i>In-silico</i> molecular docking of daboxin P with FXa.	192
Figure 6.20:	Contact map analysis of daboxin P-FXa.	193
Figure 6.21:	Sequence alignment of the anticoagulant region of daboxin P and CM-IV with a region of FVa and TF using DNAMAN.	197
Figure 6.22:	Sequence alignment of daboxin P with AtxA along with conserved secondary structure.	197
Figure 6.23:	Proposed anticoagulation mechanism of daboxin P.	200