

TABLE OF CONTENTS

Contents	Page No.
Abstract	i-vii
Declaration	
Certificate	
Acknowledgements	viii-ix
Table of Contents	x-xix
List of Tables	xx-xxii
List of Figures	xxiii-xxix
List of Symbols and Abbreviations	xxx-xxxv

CHAPTER-I

INTRODUCTION

1-72

1.1 Introduction.....	1
1.2 Taxonomic classification and geographical distribution of snakes.....	2
1.3 Snakebite: A tropical health hazard.....	3
1.3.1 Global burden of snakebite.....	4
1.3.2 Indian scenario of snakebite.....	4
1.4 The Indian Spectacled Cobra (<i>Naja naja</i>).....	7
1.4.1 Taxonomic classification.....	7
1.4.2 Distinctive features and description.....	8
1.4.3 Habitat and geographical distribution.....	8
1.4.4 Venom apparatus of <i>N. naja</i>	9
1.4.5 Pathophysiology of cobra envenomation.....	10
1.5 A brief account on <i>N. naja</i> venom.....	11
1.5.1 Composition of <i>N. naja</i> venom.....	11
1.5.2 Differential composition of Indian cobra (<i>Naja naja</i>) venom: A biochemical approach.....	13
1.5.3 Deciphering the composition of Indian cobra (<i>Naja naja</i>) venom: A proteomic approach.....	14
1.6 The blood coagulation cascade to understand the anticoagulant mechanism of <i>N. naja</i> venom proteins.....	15
1.7 Snake venom phospholipase A ₂ enzymes.....	17
1.7.1 Classification of snake venom PLA ₂ enzymes.....	18
1.7.1.1 Group I PLA ₂ enzymes.....	18
1.7.1.2 Group II PLA ₂ enzymes.....	19
1.7.2 Structure of PLA ₂ enzymes.....	19
1.7.3 Catalytic property of snake venom PLA ₂ enzymes.....	21
1.7.4 Pharmacological effects of snake venom PLA ₂ enzymes.....	23
1.7.4.1 Myotoxic effect of PLA ₂ enzymes.....	23
1.7.4.2 Anticoagulant effect of PLA ₂ enzymes.....	25
1.7.4.2.1 Types of anticoagulant PLA ₂	25
1.7.4.2.2 Mechanism of anticoagulant action.....	25
1.7.5 Pharmacological site and target specificity of snake venom PLA ₂	26
1.7.5.1 Pharmacological site for myotoxic PLA ₂	26
1.7.5.2 Pharmacological site for anticoagulant PLA ₂	27
1.7.6 Evolution of snake venom PLA ₂ enzymes.....	27

TABLE OF CONTENTS

Contents	Page No.
1.8 Snake venom toxin synergism and PLA ₂ complexes.....	28
1.8.1 Snake venom toxin synergism.....	28
1.8.2 Protein complexes of snake venom PLA ₂ enzymes.....	30
1.8.2.1 Covalent complexes of snake venom PLA ₂	30
1.8.2.2 Non-covalent complexes of snake venom PLA ₂	31
1.9 Thrombosis associated cardiovascular disorders and their treatments.....	32
1.9.1 Thrombosis leads to cardiovascular diseases.....	32
1.9.2 Treatment for CVDs and limitations of commercial drugs.....	33
1.10 Therapeutic application of snake venom anticoagulant proteins.....	35
1.11 Peptides as drugs: Emergence and advantages.....	37
1.12 Gap in the study.....	39
1.13 Aims and objectives of the present study.....	39
Bibliography.....	40

CHAPTER II

REVIEW OF LITERATURE 73-107

2.1 A brief review on anticoagulant proteins from cobra venom.....	73
2.1.1 Anticoagulant phospholipase A ₂ enzymes.....	73
2.1.1.1 Enzymatic mechanism of anticoagulant action of cobra venom PLA ₂ enzymes.....	74
2.1.1.2 Non-enzymatic mechanism of anticoagulant action of cobra venom PLA ₂ enzymes.....	74
2.1.1.2.1 Cobra venom PLA ₂ thrombin inhibitors.....	75
2.1.1.2.2 Cobra venom PLA ₂ factor Xa inhibitors.....	76
2.1.2 Anticoagulant metallo-proteinases of cobra venom.....	77
2.1.3 Other anticoagulant and antiplatelet proteins of cobra venom.....	80
2.2 A brief appraisal on peptide therapeutics developed from snake venom for treatment of cardiovascular diseases.....	81
2.3 A brief account on cytotoxic PLA ₂ s and their complexes in cobra venom.....	83
2.4 A brief account on cobra venom myotoxic PLA ₂ s.....	87
2.5 Understanding the mechanism of snake venom PLA ₂ -induced myotoxicity.....	88
Bibliography.....	92

CHAPTER III

MATERIALS AND METHODS 108-156

3.1 Materials.....	108
3.1.1 Venom and antivenom.....	108
3.1.2 Chromatographic matrices, cell lines, and cell culture reagents.....	108
3.1.3 Coagulation factors and fine chemicals.....	109
3.1.4 Analytical grade chemicals and kits.....	109
3.1.5 Raising polyclonal antibodies against purified PLA ₂ isolated from <i>N. naja</i> venom.....	109
3.1.6 Animals and housing conditions.....	110

TABLE OF CONTENTS

Contents	Page No.
3.2 Methods.....	110
3.2.1 Purification of an anticoagulant protein and its complex from eastern India <i>N. naja</i> venom.....	110
3.2.1.1 Cation-exchange chromatography of <i>N. naja</i> venom.....	110
3.2.1.2 Size-exclusion chromatography of the most anticoagulant fraction...	111
3.2.2 Determination of homogeneity, purity, and molecular weight of the anticoagulant phospholipase A ₂	111
3.2.2.1 Reversed-phase HPLC of the anticoagulant PLA ₂	111
3.2.2.2 Analysis of purity and molecular mass of the anticoagulant PLA ₂ by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).....	111
3.2.2.3 Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-ToF-MS) analysis of the anticoagulant PLA ₂	113
3.2.3 Biophysical characterization.....	113
3.2.3.1 Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.....	113
3.2.3.1.1 In-solution and in-gel trypsin digestion of proteins.....	113
3.2.3.1.2 LC-MS/MS analysis of the tryptic peptides and identification of protein.....	114
3.2.3.2 Determination of secondary structure.....	115
3.2.4 Determination of venom PLA ₂ cognate complex(es) and identification of its components	115
3.2.4.1 Two-dimensional SDS-PAGE (2D SDS-PAGE) of <i>N. naja</i> venom..	115
3.2.4.2 Immunoblotting of 2D SDS-PAGE separated EI NnV.....	116
3.2.4.3 Identification of components of PLA ₂ -cognate complex.....	117
3.2.4.3.1 RP-HPLC of PLA ₂ -cognate complex.....	117
3.2.4.3.2 In-sol trypsin digestion of Nn(N)CM2 and its RP-HPLC fractions followed by LC-MS/MS.....	117
3.2.4.3.3 In-gel trypsin digestion of anti-PLA ₂ antibody detected 2D SDS-PAGE spots followed by LC-MS/MS.....	117
3.2.4.3.4 Determination of relative abundance and stoichiometry of venom proteins in PLA ₂ cognate complex.....	118
3.2.4.4 One dimensional (1D) SDS-PAGE to confirm complex formation by NnV PLA ₂	119
3.2.5 Biochemical characterizations.....	119
3.2.5.1 Estimation of protein content.....	119
3.2.5.2 Phospholipase A ₂ activity assay.....	119
3.2.5.2.1 Turbidometric method.....	119
3.2.5.2.2 Secretory PLA ₂ assay kit.....	120
3.2.5.3 Substrate specificity and enzyme kinetics.....	120
3.2.5.4 Effect of pH and temperature on enzyme activity.....	120
3.2.5.5 Effect of chemical inhibitors and heparin on enzymatic and anticoagulant activities.....	121
3.2.5.6 Neutralization by commercial antivenom.....	121

TABLE OF CONTENTS

Contents	Page No.
3.2.6 <i>In silico</i> interaction of PLA ₂ with coagulation factor(s) docking analysis and peptide designing.....	122
3.2.6.1 Structure prediction of the purified PLA ₂	122
3.2.6.2 Docking analysis with thrombin and factor Xa.....	122
3.2.6.3 Designing of anticoagulant peptides and their synthesis.....	122
3.2.6.4 Physico-chemical characterization and <i>in silico</i> antigenicity determination of the synthetic peptides.....	122
3.2.6.5 Structure prediction of the 7-mer anticoagulant peptide.....	123
3.2.6.6 Docking of 7-mer peptide with thrombin and FXa.....	123
3.2.7 Preparation of peptide solutions.....	123
3.2.8 <i>In vitro</i> effect of NnV PLA ₂ and synthetic peptide on coagulation factors (thrombin, factor Xa, and prothrombin).....	123
3.2.8.1 Effect on thrombin.....	123
3.2.8.1.1 Effect on amidolytic activity of thrombin.....	123
3.2.8.1.2 Effect on fibrinogen clotting activity of thrombin.....	124
3.2.8.1.3 Kinetics of thrombin inhibition.....	124
3.2.8.1.4 Effect of inhibitors on thrombin inhibition.....	125
3.2.8.1.4.1 Effect of heparin and/or antithrombin on inhibition of amidolytic activity of thrombin by PLA ₂ and/or peptide.....	125
3.2.8.1.4.2 Effect of heparin on inhibition of fibrinogen clotting activity of thrombin by PLA ₂	126
3.2.8.1.4.3 Effect of <i>p</i> -BPB on inhibition of amidolytic activity of thrombin by PLA ₂	126
3.2.8.2 Effect on factor Xa.....	127
3.2.8.2.1 Effect on amidolytic activity of FXa.....	127
3.2.8.2.2 Effect on prothrombin activation by FXa.....	127
3.2.8.2.3 Kinetics of FXa inhibition.....	128
3.2.8.3 Effect of 7-mer peptide on prothrombin: An amidolytic assay to determine thrombin formation.....	128
3.2.9 Protein-protein interaction studies.....	129
3.2.9.1 Spectrofluorometry assay of interaction of purified PLA ₂ / peptide with phospholipid and coagulation factors.....	129
3.2.9.1.1 Interaction with PC.....	129
3.2.9.1.2 Interaction with thrombin and FXa.....	129
3.2.9.2 Surface plasmon resonance to determine the binding with thrombin.	130
3.2.10 Pharmacological characterization.....	130
3.2.10.1 Whole blood clotting assay.....	130
3.2.10.2 Plasma clotting assays.....	130
3.2.10.2.1 Screening of anticoagulant activity of peptides.....	130
3.2.10.2.2 Plasma re-calcification assay.....	131
3.2.10.2.3 Effect of time on re-calcification of PPP.....	131
3.2.10.2.4 Prothrombin time (PT) assay.....	131
3.2.10.2.5 Activated partial thromboplastin time (APTT) assay.....	132
3.2.10.2.6 Thrombin time (TT) assay.....	132

TABLE OF CONTENTS

Contents	Page No.
3.2.10.2.7 Determination of fatty acid release from plasma.....	133
3.2.10.3 Platelet modulating activity.....	133
3.2.10.3.1 Isolation of platelet rich plasma and washed platelets.....	133
3.2.10.3.2 Effect on PRP or washed platelets.....	133
3.2.10.3.3 Effect of phospholipids on PLA ₂ induced platelet deaggregation.....	134
3.2.10.3.4 Effect of heparin, <i>p</i> -BPB, and antivenom on platelet modulating property.....	134
3.2.10.3.5 Effect on collagen-induced platelet aggregation.....	135
3.2.10.3.6 Effect on thrombin-induced platelet aggregation.....	135
3.2.10.3.7 Platelet binding assay.....	135
3.2.10.4 Direct and indirect hemolytic activity assay.....	136
3.2.10.5 Antibacterial activity assay.....	136
3.2.10.6 <i>In vitro</i> cell-cytotoxicity assay.....	137
3.2.10.6.1 Effect on platelet viability.....	137
3.2.10.6.2 Effect on cancerous and normal mammalian cell lines.....	137
3.2.10.6.3 Flow cytometry analysis to determine the cell cycle kinetics.	138
3.2.10.6.4 Effect on rat skeletal muscle cells or myoblasts.....	138
3.2.10.6.4.1 Culturing and partial differentiation of rat myoblasts...	138
3.2.10.6.4.2 Bright field imaging and MTT-based myoblasts viability assay.....	138
3.2.10.6.4.3 Effect on myoblast morphology.....	139
3.2.10.6.4.4 Assessment of serum CK and LDH.....	139
3.2.10.6.5 Neutralization of PLA ₂ cognate complex-induced cytotoxicity by polyvalent antivenom and anti-PLA ₂ antibody.....	139
3.2.10.6.6 Inhibition of PLA ₂ cognate complex-induced cytotoxicity by chemical modification of His47 residue.....	140
3.2.11 Binding and internalization of purified PLA ₂ to myoblasts.....	140
3.2.11.1 Determination of binding of purified EI NnV PLA ₂ and Nn(N)CM2 to L6 myoblasts by ELISA.....	140
3.2.11.2 Immunofluorescence assay to determine the time-dependent binding followed by internalization of purified PLA ₂ in L6 myoblasts.....	141
3.2.11.3 Confocal microscopy to determine internalization of fluorescein isothiocyanate (FITC)-conjugated purified PLA ₂ in myoblasts.....	142
3.2.12 Binding of purified PLA ₂ and its cognate complex [Nn(N)CM2] to myoblast membrane proteins.....	142
3.2.12.1 Isolation of myoblast membrane proteins.....	142
3.2.12.2 Immunological assays to determine binding of purified PLA ₂ to L6 myoblast membrane proteins (L6MP).....	143
3.2.12.2.1 ELISA to determine binding to L6MP.....	143
3.2.12.2.2 Immunoblot analysis of L6MP binding to purified PLA ₂ and Nn(N)CM2.....	143

TABLE OF CONTENTS

Contents	Page No.
3.2.12.2.3 LC-MS/MS to identify the NnPLA ₂ -I binding L6MP.....	144
3.2.12.3 Isolation and purification of PLA ₂ -binding L6MP.....	144
3.2.12.3.1 Affinity purification of PLA ₂ binding L6MP.....	144
3.2.12.3.2 RP-HPLC and identification of PLA ₂ binding L6MP.....	145
3.2.13 <i>In silico</i> analysis to determine interaction of PLA ₂ and its cognate complex [Nn(N)CM2 fraction] with coagulation factors and vimentin.....	145
3.2.13.1 Structure prediction of interacting proteins/peptides.....	145
3.2.13.2 Docking analysis with vimentin and predicting free-energy of interaction.....	146
3.2.14 Validation of <i>in silico</i> docking results.....	146
3.2.14.1 ELISA to determine binding of purified PLA ₂ to vimentin.....	146
3.2.14.2 Spectrofluorometry analysis to determine dose- and time-dependent binding of purified PLA ₂ and its cognate complex [Nn(N)CM2] to vimentin.....	146
3.2.15 Assessment of <i>in vivo</i> toxicity and therapeutic potential of the purified PLA ₂ and 7-mer anticoagulant custom peptide.....	147
3.2.15.1 Toxicity assessment.....	147
3.2.15.2 Histological study of major organs.....	148
3.2.15.3 Assessment of <i>in vivo</i> anticoagulant activity.....	148
3.2.15.4 Assessment of <i>in vivo</i> antithrombotic potential.....	148
Bibliography.....	149

CHAPTER IV

RESULTS AND DISCUSSION: Characterization of an acidic phospholipase A₂ purified from Indian cobra (*Naja naja*) venom, and assessment of *in vivo* toxicity, and anticoagulant activity of this anticoagulant PLA₂ in a rodent model

	157-205
4.1 Brief Introduction.....	157
4.2 Results.....	158
4.2.1 Isolation and purification of an anticoagulant PLA ₂ from <i>N. naja</i> venom...	158
4.2.1.1 Cation-exchange chromatography of <i>N. naja</i> venom.....	158
4.2.1.2 Gel filtration chromatography of NnCM1.....	159
4.2.2 RP-HPLC of NnCM1GF5.....	161
4.2.3 Determination of molecular weight of NnPLA ₂ -I.....	161
4.2.4 LC-MS/MS to identify the purified protein.....	163
4.2.5 Circular dichroism (CD) spectroscopy for secondary structure determination of NnPLA ₂ -I.....	163
4.2.6 Biochemical characterization.....	164
4.2.6.1 Dose-dependent phospholipase A ₂ activity of NnPLA ₂ -I.....	164
4.2.6.2 Substrate specificity of NnPLA ₂ -I.....	165
4.2.6.3. Kinetics of PC hydrolysis by NnPLA ₂ -I.....	165
4.2.6.4 Effect of temperature and pH on catalytic activity of NnPLA ₂ -I.....	166
4.2.7 Pharmacological characterization.....	167
4.2.7.1 Anticoagulant activity of NnPLA ₂ -I.....	167

TABLE OF CONTENTS

Contents	Page No.
4.2.7.1.1 Effect of NnPLA ₂ -I on re-calcification time of plasma and its comparison with commercial anticoagulants.....	167
4.2.7.1.2 Effect of NnPLA ₂ -I on PT and APTT of PPP.....	167
4.2.7.2 Effect of NnPLA ₂ -I on coagulation factors.....	169
4.2.7.2.1 Effect of NnPLA ₂ -I on fibrinogen clotting time of thrombin...	169
4.2.7.2.2 Effect of NnPLA ₂ -I on amidolytic activity of thrombin.....	171
4.2.7.2.3 Kinetics of thrombin inhibition by NnPLA ₂ -I.....	172
4.2.7.2.4 Effect on amidolytic and prothrombin activation property of factor Xa.....	173
4.2.7.3 Effect on other serine proteases.....	175
4.2.7.4 Platelet modulating activity of NnPLA ₂ -I.....	175
4.2.7.4.1 Effect of NnPLA ₂ -I on platelet rich plasma (PRP) and washed platelets.....	175
4.2.7.4.2 Effect of NnPLA ₂ -I on collagen- and thrombin-induced platelet aggregation.....	176
4.2.7.4.3 Neutralization and inhibition of antiplatelet and platelet binding property of NnPLA ₂ -I.....	176
4.2.7.5 Hemolytic activity, cell cytotoxicity, and antibacterial activity.....	178
4.2.8 Inhibition and neutralization of catalytic and anticoagulant activities of NnPLA ₂ -I.....	179
4.2.8.1 Effect of chemical inhibitors and heparin on the activities of NnPLA ₂ -I.....	179
4.2.8.2 Effect of heparin on thrombin inhibition by NnPLA ₂ -I.....	180
4.2.8.3 Neutralization of catalytic and anticoagulant activities of NnPLA ₂ -I by commercial antivenoms.....	182
4.2.9 Interaction with phospholipids and coagulation factor(s).....	183
4.2.9.1 <i>In silico</i> structure prediction and interaction with thrombin.....	183
4.2.9.1.1 Structure prediction of NnPLA ₂ -I.....	183
4.2.9.1.2 Docking of NnPLA ₂ -I with human thrombin.....	184
4.2.9.2 Spectrofluorometry assay of interaction of NnPLA ₂ -I with PC.....	185
4.2.9.3 Spectrofluorometry assay of interaction of NnPLA ₂ -I with FXa and thrombin	185
4.2.10 Assessment of <i>in vivo</i> toxicity and therapeutic potential of NnPLA ₂ -I.....	187
4.2.10.1 Assessment of lethality and behavioral parameters of rats.....	187
4.2.10.2 Effect on serological parameters of blood.....	188
4.2.10.3 Effect on blood parameters.....	188
4.2.10.4 Effect on histological parameters.....	188
4.2.10.5 Assessment of <i>in vivo</i> anticoagulant property of NnPLA ₂ -I.....	191
4.3 Discussion.....	193
Bibliography.....	198

TABLE OF CONTENTS

Contents	Page No.
CHAPTER V	
RESULTS AND DISCUSSION: Study on <i>in vitro</i> myotoxicity (cytotoxicity towards rat myoblasts) of NnPLA₂-I and its acidic cognate complex on rat myotubes and their neutralization by commercial polyvalent anti-snake venom and anti-NnPLA₂-I antibody	206-265
5.1 Brief introduction.....	206
5.2 Results.....	207
5.2.1 2D SDS-PAGE analysis of eastern India <i>N. naja</i> venom followed by western blot analysis to detect complex formation by NnPLA ₂ -I.....	207
5.2.2 Cation-exchange chromatography of eastern India NnV.....	208
5.2.3 LC-MS/MS of Nn(N)CM2 and anti-NnPLA ₂ -I antibody recognized regions of 2D SDS-PAGE of NnV.....	209
5.2.4 Determination of stoichiometry of venom proteins in the cognate complex.....	216
5.2.5 Isolation and identification of individual components of Nn(N)CM2 by RP-HPLC and LC-MS/MS analysis.....	216
5.2.6 One-dimensional SDS-PAGE analysis of Nn(N)CM2.....	218
5.2.7 Determination of molecular mass of Nn(N)CM2 by gel filtration chromatography.....	219
5.2.8 Quantitation of NnPLA ₂ -I in Nn(N)CM2 by ELISA.....	220
5.2.9 Cytotoxicity of PLA ₂ and its cognate complex against the mammalian and bacterial cells.....	221
5.2.10 Assessment of release of creatine kinase and lactate dehydrogenase from NnPLA ₂ -I cognate complex treated L6 myoblasts.....	222
5.2.11 Cytotoxicity of NnPLA ₂ -I cognate complex and its individual components on rat myoblasts.....	223
5.2.11.1 Determination of cytotoxicity by MTT assay.....	223
5.2.11.2 Determination of cytotoxicity by bright field microscopy.....	225
5.2.11.3 Determination of cytotoxicity by acridine orange / ethidium bromide staining.....	227
5.2.12 ELISA to determine binding of NnPLA ₂ -I to rat myoblasts	228
5.2.13 Time-dependent internalization of NnPLA ₂ -I in rat myoblasts.....	228
5.2.13.1 Fluorescence microscopy to determine internalization of NnPLA ₂ -I.....	228
5.2.13.2 Confocal microscopy to determine internalization of FITC-conjugated NnPLA ₂ -I.....	229
5.2.14 Binding of NnPLA ₂ -I and its cognate complex to rat myoblast membrane proteins (L6MP).....	231
5.2.14.1 ELISA showed binding of NnPLA ₂ -I and its cognate complex to L6MP.....	231
5.2.14.2 Immunoblot analysis confirmed binding of NnPLA ₂ -I and its cognate complex to L6MP.....	231
5.2.15 RP-HPLC of affinity purified L6MP.....	233
5.2.16 ELISA to determine the binding of NnPLA ₂ -I to L6RP1.....	234

TABLE OF CONTENTS

Contents	Page No.
5.2.17 Identification of NnPLA ₂ -I binding L6MP by LC-MS/MS analysis.....	234
5.2.18 Spectrofluorometry analysis to determine binding of NnPLA ₂ -I and its cognate complex to vimentin.....	237
5.2.18.1 Dose- and time-dependent binding of NnPLA ₂ -I to vimentin.....	237
5.2.18.2 Dose- and time-dependent binding of NnPLA ₂ -I cognate complex to vimentin.....	239
5.2.19 <i>In silico</i> analysis to demonstrate binding of NnPLA ₂ -I and its cognate complex to the rod region of vimentin.....	240
5.2.19.1 Docking of NnPLA ₂ -I with different chains of vimentin.....	240
5.2.19.2 Docking of CTx, LNTx, and NGF with vimentin.....	244
5.2.19.3 Docking of NnPLA ₂ -I with CTx, LNTx, and NGF to form a cognate complex.....	246
5.2.19.4 Docking of 3FTx-NnPLA ₂ -I and 3FTx-NGF-NnPLA ₂ -I complexes with vimentin.....	248
5.2.20 ELISA to determine NnPLA ₂ -I does not bind to tail region of vimentin...	251
5.3 Discussion.....	252
Bibliography.....	257

Chapter VI

RESULTS AND DISCUSSION: Designing, characterization, and elucidation of mechanism of action of a 7-mer peptide from the anticoagulant region of NnPLA₂-I, and assessment of <i>in vivo</i> toxicity, anticoagulant and antithrombotic activity of this anticoagulant peptide in a rodent model	266-300
6.1 Brief Introduction.....	266
6.2 Results.....	267
6.2.1 Design and synthesis of peptides corresponding to thrombin-binding regions of NnPLA ₂ -I.....	267
6.2.2 Determination of physico-chemical properties of the synthesized peptides	267
6.2.3 Docking of ACR1-12 with thrombin.....	268
6.2.4 Screening of anticoagulant activity of ACR1-12.....	268
6.2.5 Anticoagulant activity of ACR9.....	269
6.2.5.1 Effect of ACR9 on re-calcification time of whole blood and PPP....	269
6.2.5.2 Effect of ACR9 on PT, APTT, and TT of PPP.....	270
6.2.6 Thrombin inhibition by ACR9.....	271
6.2.6.1 Effect on fibrinogen clotting time of thrombin.....	271
6.2.6.2 Effect on amidolytic activity of thrombin.....	272
6.2.6.3 Kinetics of thrombin inhibition by ACR9.....	274
6.2.7 Factor Xa inhibition by ACR9.....	275
6.2.7.1 Effect on prothrombin activation by FXa.....	275
6.2.7.2 Effect on amidolytic activity of FXa.....	276
6.2.7.3 Kinetics of FXa inhibition by ACR9.....	278
6.2.8 Analysis of <i>in silico</i> binding of ACR9 to thrombin and FXa.....	279
6.2.8.1 Determination of structure of ACR9.....	279
6.2.8.2 Docking of ACR9 with thrombin.....	280
6.2.8.3 Docking of ACR9 with FXa.....	281

TABLE OF CONTENTS

Contents	Page No.
6.2.9 Determination of antigenicity of ACR9.....	282
6.2.10 Spectrofluorometry interaction of ACR9 with thrombin and FXa.....	282
6.2.10.1 Interaction with thrombin.....	282
6.2.10.2 Interaction of ACR9 with FXa.....	283
6.2.11 Surface plasmon resonance (SPR) analysis to demonstrate binding of ACR9 to thrombin.....	283
6.2.12 <i>In vitro</i> cytotoxicity of ACR9.....	284
6.2.12.1 Effect of ACR9 on mammalian erythrocytes.....	284
6.2.12.2 Effect of ACR9 on mammalian cells by MTT assay.....	285
6.2.12.3 Effect of ACR9 on cell cycle by flow cytometry analysis.....	285
6.2.13 Assessment of <i>in vivo</i> toxicity of ACR9.....	286
6.2.13.1 Effect on survival and behavioral parameters.....	286
6.2.13.2 Effect on serological parameters.....	286
6.2.13.3 Effect on blood parameters.....	286
6.2.13.4 Effect on histological parameters.....	289
6.2.14 <i>In vivo</i> therapeutic property of ACR9.....	290
6.2.14.1 <i>In vivo</i> anticoagulant property of ACR9.....	290
6.2.14.2 <i>In vivo</i> antithrombotic property of ACR9.....	290
6.3 Discussion.....	293
Bibliography.....	295
 Chapter VII	
CONCLUSIONS AND FUTURE PERSPECTIVES	301-303
7.1 Conclusions and future perspectives.....	301
 Publications and Patents	304-306
 Awards received in National Conferences and Seminars/Other Awards	307
