

TABLE OF CONTENTS

	Page
Abstract	i-iii
Declaration by the candidate	
Certificates	
Acknowledgement	vii-viii
Table of contents	ix-xiv
List of figures	xv-xviii
List of tables	xix
List of symbols and abbreviations	xx-xiv
Chapter 1: Introduction and review of literature	1-21
1.1 Hemostasis	1
1.1.1 Primary hemostasis	1
1.1.2 Secondary hemostasis	2
1.1.3 Fibrinolysis	4
1.2 Thrombosis: prevention and treatment	5
1.3 Evolution of hematophagy	7
1.4 Ticks and their economic importance	8
1.5 Tick salivary gland: goldmine of anti-hemostatic proteins	10
1.5.1 Inhibitors of blood coagulation	11
1.5.1.1 Thrombin inhibitors	11
1.5.1.2 Factor Xa inhibitors	12
1.5.1.3 Tissue factor pathway inhibitors	12
1.5.1.4 Contact system proteins inhibitors	13
1.5.2 Inhibitors of platelet aggregation and adhesion	14
1.5.3 Fibrin(ogen)olytic agents	14
1.5.4 Immunomodulatory components	15
1.6 Tick sialome	15
1.7 <i>Haemaphysalis bispinosa</i>	20
Chapter 2: Collection and identification of cattle-ticks	22-47
Graphical abstract	22

2.1	Introduction	22
2.2	Materials and methods	23
2.2.1	Materials	23
2.2.2	Source of ticks	23
2.2.3	Scanning Electron Microscopy (SEM)	23
2.2.4	Morphological identification of ticks	24
2.2.5	DNA extraction from ticks	24
2.2.6	DNA amplification by Polymerase Chain Reaction (PCR)	24
2.2.7	Gel extraction of PCR products	25
2.2.8	Sequencing of DNA	25
2.2.9	Sequence analysis	26
2.2.10	PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of ITS2	26
2.3	Results	27
2.3.1	Morphological analysis of cattle-ticks	27
2.3.2	DNA extraction from ticks	31
2.3.3	Amplification and sequencing of ITS2 and 16S rDNA	31
2.3.4	Phylogenetic analysis	36
2.3.5	PCR-RFLP analysis of ITS2	42
2.4	Discussions	43
Chapter 3: Extraction and characterization of salivary gland extract		48-58
	Graphical abstract	48
3.1	Introduction	48
3.2	Material and methods	49
3.2.1	Materials	49
3.3.2	Isolation of salivary glands (SG)	49
3.3.3	Preparation of Salivary Gland Extract (SGE)	49
3.3.4	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)	50
3.3.5	Silver staining of SDS-PAGE gel	50
3.3.6	Preparation of platelet poor plasma from citrate goat plasma	50
3.3.7	Coagulation assays	51

3.3.8	Hemolytic activity assay	51
3.3.9	PLA ₂ activity assay	51
3.3	Results	52
3.3.1	Isolation of salivary gland and preparation of Salivary Gland Extract	52
3.3.2	SDS-PAGE gel analysis of Salivary Gland Extract	52
3.3.3	Recalcification time of SGE	53
3.3.4	Activated partial thromboplastin time (APTT) of SGE	53
3.3.5	Prothrombin time of SGE	54
3.3.6	Hemolytic activity of SGE	54
3.3.7	PLA ₂ activity of SGE	55
3.4	Discussions	55
Chapter 4: Isolation, cloning and over-expression of anti-thrombin protein from <i>H. bispinosa</i>		59-86
	Graphical abstract	59
4.1	Introduction	59
4.2	Materials and methods	60
4.2.1	Materials	60
4.2.2	Isolation of salivary glands	60
4.2.3	Isolation of total RNA from salivary gland	61
4.2.4	Salivary gland cDNA synthesis	61
4.2.5	Amplification of gene coding for thrombin inhibitors by PCR	61
4.2.6	Sequencing and analysis of the thrombin inhibitor genes	62
4.2.7	Preparation of <i>Escherichia coli</i> BL21(DE3)pLysS competent cells	62
4.2.8	Isolation of plasmids from bacterial cells	63
4.2.9	Transformation of plasmids into competent cells	63
4.2.10	Cloning of cDNA coding for haemathrins	63
4.2.11	Screening of clones by colony PCR	64
4.2.12	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)	64
4.2.13	Tricine–SDS–Polyacrylamide Gel Electrophoresis	64
4.2.14	Expression of recombinant haemathrins	65

	(rHaemathrins)	
4.2.15	Mass culture of recombinant protein for His-tag purification	65
4.2.16	His-tag purification of rHaemathrins	66
4.2.17	Dialysis of partially purified rHaemathrins	66
4.2.18	Peptide mass fingerprinting of rHaemathrins	66
4.2.19	Cleavage of rHaemathrins by enterokinase (EK)	67
4.2.20	ESI-MS analysis of recombinant haemathrin 1 and 2	67
4.2.21	Circular Dichroism (CD) measurements of haemathrins	67
4.3	Results	68
4.3.1	Isolation of total RNA from <i>H. bispinosa</i> salivary gland	68
4.3.2	cDNA synthesis from salivary gland total RNA	68
4.3.3	Amplification of thrombin inhibitors from <i>H. bispinosa</i>	69
4.3.4	Sequencing and analysis of haemathrin 1 and 2	69
4.3.5	Nucleotide sequence alignment of haemathrin 1 and 2	71
4.3.6	Amino acid sequence alignment of haemathrin 1 and 2	72
4.3.7	Cloning of cDNA coding for haemathrins	73
4.3.8	Expression of recombinant haemathrins (rHaemathrins)	74
4.3.9	Expression of rHaemathrin at different temperatures	75
4.3.10	Expression of rHaemathrin 2 with different IPTG concentrations	76
4.3.11	Expression of rHaemathrins for different time intervals	76
4.3.12	Mass culture of recombinant protein for His-tag purification	77
4.3.13	His-tag purification of rHaemathrins	78
4.3.14	Dialysis of partially purified rHaemathrins	79
4.3.15	Peptide mass fingerprinting of rHaemathrins	79
4.3.16	Cleavage of rHaemathrins by enterokinase (EK)	80
4.3.17	ESI-MS analysis of recombinant haemathrin 1 and 2	82
4.3.18	Circular Dichroism (CD) measurements of rHaemathrins	83

4.4	Discussions	84
Chapter 5:	Biochemical, pharmacological and biophysical characterization of recombinant haemathrins	87-115
	Graphical abstract	87
5.1	Introduction	87
5.2	Materials and methods	88
5.2.1	Materials	88
5.2.2	Blood coagulation assay	88
5.2.3	Fibrinogen clotting time	88
5.2.4	Selectivity of rHaemathrins against serine protease	89
5.2.5	Dose-dependent thrombin inhibition	89
5.2.6	Time-dependent thrombin inhibition	89
5.2.7	Chromatographic analysis of rHaemathrins treated with thrombin	90
5.2.8	Structure prediction and docking	90
5.2.9	Hemolytic activity assay	91
5.2.10	Anti-microbial activity assay	91
5.2.11	Cell cytotoxicity assay	91
5.3	Results	92
5.3.1	Blood coagulation assay	92
5.3.2	Selectivity of haemathrins against serine protease	93
5.3.3	Fibrinogen clotting time	96
5.3.4	Thrombin inhibitory activity	97
5.3.5	Time-dependent thrombin inhibition	99
5.3.6	Chromatographic analysis of rHaemathrins treated with thrombin	100
5.3.7	Inhibitory activity of peptidic fragments	103
5.3.8	Computational docking of rHaemathrin to α -thrombin	104
5.3.9	Hemolytic activity assay	108
5.3.10	Anti-microbial sensitivity test	108
5.3.11	Cell cytotoxicity assay	110
5.4	Discussions	110

Chapter 6: Conclusion and future prospects	116-118
6.1 Conclusion	116
6.2 Future prospects	118
6.2.1 Site directed mutagenesis and thrombin inhibition	118
6.2.2 Structure determination of haemathrin and co-crystallization with thrombin	118
6.2.3 Design of synthetic peptide (thrombin inhibitor)	118
References	119-137
List of publications	138
Appendix I	139
Appendix II	140
Appendix III Reprint of publication	