

**TOXINOLOGICAL, PROTEOMIC AND PHARMACOKINETIC
CHARACTERIZATION OF EQUATORIAL SPITTING COBRA
(*NAJA SUMATRANA*) VENOM**

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ABSTRACT

Naja sumatrana, the Equatorial spitting cobra, is listed as one of the medically important species in Southeast Asia and is the common spitting cobra in Peninsula Malaysia. The aims of this study are to investigate the toxinology, proteome and pharmacokinetic characteristics of *N. sumatrana* venom, which will contribute to management of cobra envenomation. The lethality and enzymatic activities of *N. sumatrana* were compared to venoms from two other regional spitting cobras: *Naja sputatrix*, *Naja siamensis* and a non-spitting cobra *Naja kaouthia*, which also occurs in Malaysia. Previously, the three spitting cobras were considered as belonging to one species, *N. sputatrix*. Results showed that the three spitting cobra venoms possess different venom composition, but all three contain basic phospholipases A₂ and high content of polypeptide cardiotoxins. The proteome of *N. sumatrana* venom was investigated using shotgun analysis, combination of multi-dimensional chromatography and 2DE. Shotgun analysis revealed the presence of 50 individual proteins in the venom, with three finger toxins (both neurotoxins and cardiotoxins) and phospholipase A₂ constituted about 38% and 36%, respectively of the types of proteins identified. The ion exchange and reverse phase HPLC revealed the presence multiple basic venom proteins including 17 identified protein toxins (8 PLA₂, 4 neurotoxins and 5 cardiotoxins) whereas the Sephadex[®] G-50-2DE revealed the presence of another 11 high molecular weight proteins. Of the 17 protein toxins identified, only 7 exist in substantial amount, including 2 phospholipases A₂, 1 short and 1 long α -neurotoxin and 3 cardiotoxins. Together these seven major venom toxins constituted 87% of total venom protein and are primarily responsible for the pathophysiological action of the venom.

The pharmacokinetics of *N. sumatrana* and *N. sputatrix* venom in rabbits were also investigated. The serum toxin levels-time profile of both venoms following intravenous administration fitted a two-compartment model of pharmacokinetics with similar

pharmacokinetic parameters. The two venoms also have comparable intramuscular bioavailability in rabbits, both approximately 40%. To further understand the pharmacokinetics of venom toxin components, the pharmacokinetics of three main *N. sumatrana* venom toxins (short chain α -neurotoxin, cardiotoxin and basic phospholipase A₂) were also investigated. When toxins were injected intramuscularly, neurotoxin and cardiotoxin reached C_{max} within 30 min, which was much faster than phospholipase A₂, and the whole venom, reflecting a very rapid absorption of neurotoxin and cardiotoxin from the site of injection to systemic circulation. It was found that neurotoxin and cardiotoxin were eliminated from systemic circulation more quickly than other venom components. The neurotoxin had an intramuscular bioavailability ($F_{i.m.} = 81.5\%$) much higher than phospholipase A₂ ($F_{i.m.} = 68.6\%$) and cardiotoxin ($F_{i.m.} = 45.6\%$). The high bioavailability and short T_{max} of neurotoxin when injected intramuscularly explained why neurotoxic effect is the dominant symptom in most cobra bites. In conclusion, a comprehensive understanding of the toxinology, proteome and pharmacokinetics of *N. sumatrana* venom and its toxins provide significant insights into the overall pathophysiological actions of the venom.

ABSTRAK

Ular tedung jenis meludah, *Naja sumatrana* telah disenaraikan sebagai salah satu species yang penting dari segi perubatan di Asia Tenggara. Ia merupakan sejenis ular tedung jenis meludah yang biasa terdapat di Semenanjung Malaysia. Projek ini bertujuan untuk mengkaji sifat-sifat toksinologi, proteom dan farmakokinetik bisa ular *N. sumatrana*. Ini dapat menyumbang kepada pengurusan pembisaan ular tedung. Aktiviti-aktiviti maut dan enzim bisa ular *N. sumatrana* telah dibandingkan dengan spesies ular tedung lain di rantau ini (termasuk Malaysia) iaitu bisa daripada ular tedung jenis meludah: *Naja sputatrix*, *Naja siamensis* dan ular tedung jenis tidak meludah: *Naja kaouthia* yang juga terdapat di Malaysia. Sebelum ini, ketiga-tiga ular tedung jenis meludah tersebut dikategorikan sebagai satu species dan dinamakan sebagai *N. sputatrix*. Keputusan menunjukkan bahawa ketiga-tiga ular tedung jenis meludah mempunyai komposisi bisa yang berlainan, tetapi ketiga-tiga ular tersebut mempunyai phospholipase A₂ bersifat alkali dan kandungan kardiotoxin polipeptida yang tinggi. Proteom bisa ular *N. sumatrana* telah dikaji dengan analisis shotgun, kombinasi kromatografi pelbagai dimensi dan 2DE. Analisis shotgun menunjukkan 50 protein individu dalam bisa ular, di mana 'three finger toxins' (neurotoksin dan kardiotoxin) serta phospholipase A₂ telah merangkumi 38% dan 36% masing-masing, daripada jenis protein yang telah dikenalpasti. Kromatografi penggantian ion dan HPLC terbalik-fasa telah menunjukkan pelbagai protein bersifat alkali termasuk 17 toksin (8 PLA₂, 4 neurotoksin dan 5 kardiotoxin) manakala Sephadex[®] G-50-2DE menunjukkan 11 protein yang mempunyai berat molekul tinggi. Daripada 17 toksin yang dikenalpasti, hanya 7 toksin hadir dalam kuantiti yang besar. Ini termasuk 2 phospholipase A₂, 1 α -neurotoksin pendek, 1 α -neurotoksin panjang dan 3 kardiotoxin. Tujuh toksin major ini merangkumi 87% daripada jumlah protein bisa dan terutamanya terlibat dalam tindakan toksinologikal utama bisa ular.

Farmakokinetik bisa ular *N. sumatrana* dan *N. sputatrix* juga dikaji dalam arnab. Profil tahap serum toksin – masa apabila kedua-dua bisa ular tersebut disuntik secara intravena membayangkan model farmakokinetik dua-kompartmen dan mempunyai parameter-parameter farmakokinetik yang sama. Kedua-dua bisa ular ini mempunyai keterbiodediaan intraotot yang setanding, iaitu kira-kira 40%. Untuk memahami farmakokinetik komponen toksin-toksin dalam bisa ular dengan selanjutnya, farmakokinetik tiga toksin utama juga telah dikaji. Apabila, toksin disuntik secara intraotot, neurotoksin dan kardiotoxin mencapai C_{max} dalam 30 min, iaitu kadar yang lebih cepat berbanding dengan phospholipase A_2 dan bisa ular, ini menunjukkan kadar penyerapan yang cepat dari tapak suntikan ke dalam peredaran sistemik. Neurotoksin dan kardiotoxin mempunyai kadar eliminasi yang lebih cepat berbanding dengan komponen bisa yang lain. Keterbiodediaan interotot neurotoksin ($F_{i.m.} = 81.5\%$) adalah lebih tinggi berbanding dengan phospholipase A_2 ($F_{i.m.} = 68.6\%$) and kardiotoxin ($F_{i.m.} = 45.6\%$). Neurotoksin mempunyai keterbiodediaan yang tinggi serta T_{max} yang pendek apabila disuntik secara intraotot dan ini menjelaskan neurotoksiti sebagai gejala dominan dalam pembisaan ular tedung. Kesimpulannya, kefahaman yang menyeluruh terhadap toksinologi, proteom dan farmakokinetik bisa ular *N. sumatrana* dan toksin-toksinnya dapat memberikan pandangan yang mendalam mengenai tindakan keseluruhan patofisiologikal bisa ular ini.

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TABLE OF CONTENTS	PAGE
Title Page	i
Original Literary Work Declaration	ii
Abstract	iii
Acknowledgement	vii
Table of Contents	viii
List of Figures	xix
List of Tables	xxii
List of Symbols and Abbreviations	xxvi
List of Appendices	xxix
Chapter 1 Introduction	
1.1 Objectives	5
Chapter 2 Literature Review	
2.1 Introduction to Serpentes	
2.1.1 Evolution and Phylogeny of Serpentes	7
2.1.2 Venomous snakes	8
2.1.3 Venom delivery systems: Evolution and Anatomy	12
2.2 Snake envenomation	
2.2.1 The epidemiology of snake envenomations: Mortality and Morbidity	14
2.2.2 The management of snakebites	16
2.3 Antivenom as effective therapeutics for snake envenomations	17
2.4 Biochemistry and Toxinology of snake venom	19
2.4.1 Snake venom composition and the pathophysiology of	20

snake envenomation with special emphasis on cobra venoms	
2.4.1.1 Phospholipase A ₂	20
2.4.1.2 Three-finger toxins	21
Neurotoxins	21
Cardiotoxins	23
2.4.2 Other protein and enzymes	24
2.4.3 Immunological cross-reactivity of snake venoms	28
2.5 Pharmacokinetics of snake venoms and antivenoms	
2.5.1 Pharmacokinetics of snake venoms and venom components	29
2.5.2 Pharmacokinetics of antivenom: IgG, Fab and F(ab') ₂	31
fragments and their effects on the pharmacokinetics of snake venoms	
2.6 Snake venomics	33
2.6.1 Proteomic tools to unravel the protein compositions of venom	33

Chapter 3 Materials and Methods

3.1 Materials	
3.1.1 Animals	38
3.1.2 Animal ethical clearance	38
3.1.3 Venoms	38
3.1.4 Antivenom	38
3.1.5 Anesthesia	39
3.1.6 Chemicals and general consumables	39
3.1.7 Buffers	42

3.2	General Methods	
3.2.1	Determination of protein concentration	
3.2.1.1	Determination of protein concentration by Bradford method	43
3.2.1.2	Determination of protein concentration by 2-D Quant Kit	43
3.2.2	Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	45
3.2.2.1	Preparation of protein samples	46
3.2.2.2	Fixing, staining and destaining	46
3.2.3	Production of antibodies to cobra venom and venom toxins	
3.2.3.1	Immunization Schedule	49
3.2.3.2	Purification of antibody immunoglobulin IgG	49
3.2.3.3	Conjugation of Horseradish Peroxidase (HRP) to the purified IgG	50
3.2.4	Indirect Enzyme-linked Immunosorbent Assay for measurement of antibodies titer	
3.2.4.1	Chequerboard titrations	51
3.2.4.2	Assay Procedure	51
3.2.5	Determination of the median lethal dose (LD ₅₀) of venom and toxins	52

Chapter 4 Biochemical and Toxinological characterization of *Naja sumatrana* (Equatorial spitting cobra) venom

4.1	Introduction	54
4.2	Methods	56

4.2.1 Fractionation of <i>Naja</i> sp. venoms by Resource [®] S Ion Exchange Chromatography	56
4.2.2 Determination of Protein Concentration	56
4.2.3 Determination of enzymatic activities	56
4.2.3.1 Protease assay	56
4.2.3.2 Phosphodiesterase assay	57
4.2.3.3 Alkaline phosphomonoesterase assay	57
4.2.3.4 5'-Nucleotidase assay	58
4.2.3.5 Hyaluronidase assay	58
4.2.3.6 Phospholipase A ₂ assay	59
4.2.3.7 L-amino acid oxidase assay	59
4.2.3.8 Acetylcholinesterase assay	60
4.2.4 Determination of the Median Lethal Dose (LD ₅₀)	60
4.2.5 Statistical analysis	60
4.3 Results	
4.3.1 Fractionation of the <i>N. sumatrana</i> venom by Resource [®] S Ion Exchange Chromatography	61
4.3.2 A comparative study of the toxinological properties of <i>N. sumatrana</i> venom with other Southeast Asian cobra venom	
4.3.2.1 Determination of the enzymatic and lethal activities of venoms from <i>N. sumatrana</i> and three other Southeast Asian cobras	64
4.3.3 Fractionation of the <i>N. sputatrix</i> , <i>N. siamensis</i> and <i>N. kaouthia</i> venom by Resource [®] S Ion Exchange Chromatography	66

**Chapter 5 Proteomic characterization of *Naja sumatrana* (Equatorial
spitting cobra) venom using multidimensional
chromatographic approach**

5.1 Introduction	73
5.2 Methods	
5.2.1 In-solution digestion	75
5.2.2 In-gel tryptic digestion	
5.2.2.1 Destaining for Coomassie blue stained gel plugs	75
5.2.2.2 Destaining for silver stained gel plugs	76
5.2.2.3 Tryptic digestion	76
5.2.2.4 Desalting of digested peptides with ZipTip® Pipette tips	77
5.2.3 Preparation of digested peptides for MALDI-TOF/TOF	77
5.2.4 Fractionation of <i>N. sumatrana</i> venom by Resource® S Ion Exchange Chromatography	78
5.2.5 C ₁₈ reverse-phase HPLC of fractions isolated from Resource® S Ion Exchange Chromatography	78
5.2.6 Assignment of MALDI-TOF/TOF mass spectra of <i>N.</i> <i>sumatrana</i> venom peptides to protein families	79
5.2.7 Shotgun LC-MS/MS of the <i>N. sumatrana</i> venom	79
5.2.8 <i>De novo</i> sequence analysis	80
5.2.9 Isolation of high molecular weight protein (HMW protein) from <i>N. sumatrana</i> venom by Sephadex® G-50 gel filtration column	81

5.2.10 2-D Clean-Up	81
5.2.11 Two-dimensional electrophoresis (2-DE) of high molecular weight (HMW) protein fractions from <i>N. sumatrana</i> venom.	
5.2.11.1 First dimension electrophoresis (Isoelectric focusing)	82
5.2.11.2 Second dimension electrophoresis (SDS-PAGE)	83
5.3 Results	
5.3.1 Proteomic characterization of the <i>N. sumatrana</i> venom proteins isolated and purified from ion exchange and reverse-phase chromatography	85
5.3.2 Shotgun LC-MS/MS proteomic characterization of <i>N. sumatrana</i> venom	94
5.3.3 Isolation of the high molecular weight proteins of <i>N. sumatrana</i> venom	103
5.3.4 Two dimensional electrophoresis of the high molecular weight protein fraction of <i>N. sumatrana</i> venom and identification of the proteins by MALDI-TOF/TOF	105
5.4 Discussion	113

Chapter 6 Pharmacokinetics of *Naja sputatrix* (Javan spitting cobra) venom and the effect of polyvalent antivenom on its pharmacokinetics

6.1 Introduction	124
6.2 Methods	

6.2.1	Production and purification of antibody IgG against <i>N. sputatrix</i> venom in rabbits	125
6.2.2	Determinations of the antigenic reactivity of anti- <i>N. sputatrix</i> IgG against <i>N. sputatrix</i> venom and venom toxins by Indirect ELISA assay and Western blot	
6.2.2.1	Isolation of <i>N. sputatrix</i> venom toxins	125
6.2.2.2	Indirect Enzyme-linked Immunosorbent Assay (ELISA)	126
6.2.2.3	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (Immunoblotting)	126
6.2.3	Double-sandwich Enzyme-linked Immunosorbent Assay (ELISA)	
6.2.3.1	Determination of serum venom antigen levels in experimental envenomed rabbits	127
6.2.3.2	Determination of serum Neuro Polyvalent Antivenom (NPAV) levels by double-sandwich ELISA	128
6.2.4	Pharmacokinetics of <i>N. sputatrix</i> venom after intravenous (<i>i.v.</i>) and intramuscular (<i>i.m.</i>) administration into rabbits	129
6.2.5	The effect of Neuro Polyvalent Antivenom (NPAV) on the pharmacokinetics of <i>N. sputatrix</i> venom in experimentally envenomed rabbits	
6.2.5.1	Pharmacokinetics of <i>N. sputatrix</i> venom in the presence of a single dose NPAV	130
6.2.5.2	Pharmacokinetics of <i>N. sputatrix</i> venom in the	130

	effect of repeated dosing of NPAV	
6.2.6	Pharmacokinetics of Neuro Polyvalent Antivenom (NPAV) in rabbits	131
6.2.7	Pharmacokinetic analysis	131
6.2.8	Determination of the median lethal dose (LD ₅₀) of <i>N. sputatrix</i> venom	132
6.2.9	<i>In vitro</i> and <i>in vivo</i> neutralization of <i>N. sputatrix</i> venom by Neuro Polyvalent Antivenom (NPAV)	132
6.2.10	Statistical analysis	133
6.3	Results	
6.3.1	Antigenic reactivities of anti- <i>N. sputatrix</i> IgG against <i>N. sputatrix</i> venom and venom toxins	134
6.3.2	Pharmacokinetics of <i>N. sputatrix</i> venom after intravenous administration	137
6.3.3	Pharmacokinetics of <i>N. sputatrix</i> venom after intramuscular administration	138
6.3.4	<i>In vitro</i> and <i>in vivo</i> neutralization of <i>N. sputatrix</i> venom by Neuro Polyvalent Antivenom (NPAV)	141
6.3.5	Serum concentration-time profile of NPAV in rabbits	142
6.3.6	The effect of Neuro Polyvalent Antivenom (NPAV) on the pharmacokinetics of <i>N. sputatrix</i> venom	144
6.4	Discussion	147

Chapter 7 Pharmacokinetics of *Naja sumatrana* (Equatorial spitting cobra) venom and its individual toxins following intravenous and intramuscular administration of venom

into rabbits

7.1 Introduction	154
7.2 Methods	
7.2.1 Isolation of <i>N. sumatrana</i> venom toxins	155
7.2.2 Production and purification of antibody IgG against <i>N. sumatrana</i> venom and venom toxins in rabbits	155
7.2.3 Investigation of immunological cross-reactivity of <i>N. sumatrana</i> venom toxins (phospholipase A ₂ , neurotoxin and cardiotoxin)	
7.2.3.1 Indirect ELISA	156
7.2.3.2 Double Sandwich ELISA	156
7.2.3.3 Western Blot (Immunoblotting)	157
7.2.4 Determination of serum venom antigen and toxin antigen levels using double-sandwich ELISA in experimental envenomed rabbits	157
7.2.5 Pharmacokinetics of <i>N. sumatrana</i> venom after intravenous (<i>i.v.</i>) and intramuscular (<i>i.m.</i>) administrations	158
7.2.6 Pharmacokinetics of <i>N. sumatrana</i> venom toxins (phospholipase A ₂ , neurotoxin and cardiotoxin) after intravenous (<i>i.v.</i>) and intramuscular (<i>i.m.</i>) administrations	159
7.2.7 Pharmacokinetic analysis	159
7.2.8 Statistical analysis	160
7.3 Results	
7.3.1 Pharmacokinetics of <i>N. sumatrana</i> venom after intravenous administration	161

7.3.2 Pharmacokinetics of <i>N. sumatrana</i> venom after intramuscular administration	162
7.3.3 Immunological cross-reactions of <i>N. sumatrana</i> venom toxins (phospholipase A ₂ , neurotoxin and cardiotoxin)	165
7.3.4 Pharmacokinetics of phospholipase A ₂ after intravenous administration	169
7.3.5 Pharmacokinetics of phospholipase A ₂ after intramuscular administration	170
7.3.6 Pharmacokinetics of neurotoxin after intravenous administration	171
7.3.7 Pharmacokinetics of neurotoxin after intramuscular administration	172
7.3.8 Pharmacokinetics of cardiotoxin after intravenous administration	173
7.3.9 Pharmacokinetics of cardiotoxin after intramuscular administration	174
7.3.10 Pharmacokinetics of cardiotoxin following intravenous and intramuscular administration of whole <i>N. sumatrana</i> venom	178
7.4 Discussion	182

Chapter 8 Conclusion

8.1 The toxinology and proteome of <i>N. sumatrana</i> venom	191
8.2 The pharmacokinetics of cobra venoms and venom toxins	193
8.3 Future works on the toxinology and proteome of <i>Naja</i> venoms	195
8.4 Future work on pharmacokinetics of <i>Naja</i> venoms and venom	196

toxins

References

197

LIST OF FIGURES

		Page
Figure 1	Equatorial spitting cobra (<i>Naja sumatrana</i>).	4
Figure 2.1	Classifications and divergence of the higher snake taxa.	10
Figure 2.2	A summary of the phylogeny of advanced snakes (Colubroidea).	11
Figure 4.1	Resource [®] S ion exchange chromatography of <i>N. sumatrana</i> venom.	62
Figure 4.2	Fractionation of four Southeast Asian cobra venoms by Resource [®] S ion exchange chromatography.	67
Figure 5.1	Resource [®] S ion exchange chromatography of <i>N. sumatrana</i> venom.	87
Figure 5.2	C ₁₈ reverse-phase chromatography of <i>N. sumatrana</i> venom fractions isolated from Resource [®] S ion exchange chromatography.	88
Figure 5.3	SDS-PAGE of major fractions isolated and purified from <i>N. sumatrana</i> venom.	89
Figure 5.4	Shotgun LC-MS/MS chromatography of trypsin digested <i>N. sumatrana</i> venom peptides.	95
Figure 5.5	Types of proteins determined by shotgun LC-MS approach coupled with <i>de novo</i> peptide sequencing.	102
Figure 5.6	Sephadex [®] G-50 gel filtration chromatography of <i>N. sumatrana</i> venom.	104
Figure 5.7	Two-dimensional gel electrophoresis of the high molecular weight protein fraction isolated from Sephadex [®] G-50 gel filtration chromatography	106

Figure 6.1	Antigenic reactivity of anti- <i>N. sputatrix</i> IgG against <i>N. sputatrix</i> venom proteins.	136
Figure 6.2	Serum concentration-time profile of <i>N. sputatrix</i> venom following intravenous and intramuscular injections of the venom (in semi-logarithmic plot).	140
Figure 6.3	Serum concentration-time profile of the Neuro Polyvalent Antivenom (NPAV) in rabbits.	143
Figure 6.4	The effects of Neuro Polyvalent Antivenom (NPAV) on the serum concentration-time profile of <i>N. sputatrix</i> venom following intramuscular injection of venom (in semi-logarithmic plot).	145
Figure 7.1	Serum concentration-time profile of <i>N. sumatrana</i> venom following intravenous (<i>i.v.</i>) and intramuscular (<i>i.m.</i>) injection of the venom (in semi-logarithmic plot).	163
Figure 7.2	Western blot analysis (Immunoblotting).	168
Figure 7.3	Serum concentration-time profiles of (A) <i>N. sumatrana</i> venom phospholipase A ₂ , (B) neurotoxin and (C) cardiotoxin; following intravenous and intramuscular injections of the respective toxin.	176
Figure 7.4	Serum concentration-time profile of cardiotoxin following intravenous and intramuscular injection of the whole <i>N. sumatrana</i> venom (in semi-logarithmic scale).	180
Figure A1	Sephadex [®] G-25 gel filtration chromatography of protein fractions from hyperimmunized serum in rabbits.	232
Figure A2	HiTrap Protein A HP affinity column chromatography (5 ml) of desalted protein fractions.	233

- Figure B1** Standard curve of Bovine serum albumin (BSA) for the 234
determination of protein concentration in venom samples.
- Figure C1** Standard curve of phosphate for the measurement of 235
phosphate concentration liberated in 5'-nucleotidase
enzymatic reaction.
- Figure C2** Standard curve of hyaluronidase for the determination of 236
hyaluronidase activity.
- Figure D1** Standard curve of venom/venom toxin antigens in spiked pre- 237
envenomed sera.
- Figure F1** Standard curve of Neuro Polyvalent antivenom (NPAV) in 245
spiked pre-envenomed sera.

LIST OF TABLES

	Page
Table 4.1 Enzymatic and lethal activities of <i>N. sumatrana</i> venom fractions obtained from Resource [®] S ion exchange chromatography.	63
Table 4.2 Lethality and enzymatic activities of four Asiatic cobra venoms.	65
Table 4.3 Cardiotoxin contents in the four Southeast Asian cobra venoms.	68
Table 5.1 Assignment of ion exchange isolated and reverse-phase purified fractions of <i>N. sumatrana</i> venom to protein families by MALDI-TOF-TOF.	90
Table 5.2 Assignment of shotgun LC-MS/MS for trypsin digested <i>N. sumatrana</i> venom proteins to protein families.	96
Table 5.3 Assignment of the trypsin digested peptides of <i>N. sumatrana</i> venom proteins by <i>de novo</i> sequence analysis.	101
Table 5.4 Assignment of the two-dimensional spots of high molecular weight proteins (HMW proteins) isolated from <i>N. sumatrana</i> venom (Figure 5.7) to protein families by MALDI-TOF/TOF.	107
Table 6.1 Indirect ELISA reactions between rabbit anti- <i>N. sputatrix</i> IgG and <i>N. sputatrix</i> venom and venom toxins.	135
Table 6.2 Pharmacokinetic parameters following intravenous and intramuscular administrations of <i>N. sputatrix</i> venom into rabbits.	139
Table 6.3 <i>In vitro</i> and <i>in vivo</i> neutralization of <i>N. sputatrix</i> venom by Neuro Polyvalent Antivenom.	141
Table 6.4 Area under curve (AUC _{0-∞}) value in the absence and presence of antivenom immunotherapy.	146
Table 7.1 Pharmacokinetic parameters of <i>N. sumatrana</i> venom following intravenous and intramuscular administrations of the venom into	164

	rabbits.	
Table 7.2	MALDI-TOF/TOF identification of phospholipase A ₂ , neurotoxin and cardiotoxin.	166
Table 7.3	Immunological cross-reactivity of <i>N. sumatrana</i> venom toxins by Indirect ELISA and Double Sandwich ELISA.	167
Table 7.4	Pharmacokinetic parameters of <i>N. sumatrana</i> venom toxins (phospholipase A ₂ , neurotoxin and cardiotoxin) following intravenous and intramuscular administration of the venom toxins into rabbits.	177
Table 7.5	Pharmacokinetics parameters of cardiotoxin following intravenous and intramuscular administration of whole <i>N. sumatrana</i> venom into rabbits.	181
Table E1	Serum concentration of <i>N. sputatrix</i> venom antigens following intravenous administration of <i>N. sputatrix</i> venom into rabbits (n = 3).	238
Table E2	Serum concentration of <i>N. sputatrix</i> venom antigens following intramuscular administration of <i>N. sputatrix</i> venom into rabbits (n = 3).	238
Table E3	Serum concentration of <i>N. sputatrix</i> venom antigens in the effects of 4 ml of Neuro Polyvalent antivenom (NPAV) in rabbits (n = 3).	239
Table E4	Serum concentration of <i>N. sputatrix</i> venom antigens in the effects of 4 + 2 ml of Neuro Polyvalent antivenom (NPAV) in rabbits (n = 3).	239
Table E5	Serum concentration of <i>N. sumatrana</i> venom antigens following intravenous administration of <i>N. sumatrana</i> venom into rabbits	240

	(n = 3).	
Table E6	Serum concentration of <i>N. sumatrana</i> venom antigens following intramuscular administration of <i>N. sumatrana</i> venom into rabbits (n = 3).	240
Table E7	Serum concentration of <i>N. sumatrana</i> phospholipase A ₂ antigens following intravenous administration of phospholipase A ₂ into rabbits (n = 3).	241
Table E8	Serum concentration of <i>N. sumatrana</i> phospholipase A ₂ antigens following intramuscular administration of phospholipase A ₂ into rabbits (n = 3).	241
Table E9	Serum concentration of <i>N. sumatrana</i> neurotoxin antigens following intravenous administration of neurotoxin into rabbits (n = 3).	242
Table E10	Serum concentration of <i>N. sumatrana</i> neurotoxin antigens following intramuscular administration of neurotoxin into rabbits (n = 3).	242
Table E11	Serum concentration of <i>N. sumatrana</i> cardiotoxin antigens following intravenous administration of cardiotoxin into rabbits (n = 3).	243
Table E12	Serum concentration of <i>N. sumatrana</i> cardiotoxin antigens following intramuscular administration of cardiotoxin into rabbits (n = 3).	243
Table E13	Serum concentration of <i>N. sumatrana</i> cardiotoxin antigens following intravenous administration of whole venom into rabbits (n = 3).	244
Table E14	Serum concentration of <i>N. sumatrana</i> cardiotoxin antigens	244

following intramuscular administration of whole venom into rabbits (n = 3).

Table G1 Serum concentration of Neuro Polyvalent antivenom (NPAV) in 246 rabbits (n = 3).

LIST OF SYMBOLS AND ABBREVIATIONS

sp.	Species	™	trademark
<i>i.v.</i>	intravenous	pI	isoelectric point
<i>i.m.</i>	intramuscular	kDa	kilodalton
FCA	Freund's complete adjuvant	ml	milliliter
FIA	Freund's incomplete adjuvant	mg	milligram
F(ab') ₂	fragment antigen-binding (bivalent)	kg	kilogram
IgG	immunoglobulin G	μl	microliter
Fc	fragment crystallizable region	xg	g force
Fab	fragment antigen-binding	μg	microgram
CRISPs	cysteine-rich secretory proteins	g	gram
SVMP	snake venom metalloproteinase	μm	micrometer
3FTXs	three-finger toxins	mm	millimeter
LAAO	L-amino acid oxidase	nm	nanometer
PLA ₂	phospholipase A ₂	h	hour
NTX	neurotoxin	min	minute
CTX	cardiotoxin	Å	Angstrom
CDF	complement depleting factor	mA	milliampere
CVF	cobra venom factor	ng	nanogram
VNGF	venom nerve growth factor	mM	millimolar
NGF	nerve growth factor	M	molar
A	alpha	%	percent
K	kappa	w/v	weight per volume
β	beta	v/v	volume per volume
γ	gamma	°C	degree Celsius

μ	micro	Sec	seconds
®	registered	V	Volt
L	liter	Cm	centimeter
ADP	adenosine monophosphate	NPAV	Neuro Polyvalent Antivenom
mRNA	messenger ribonucleic acid	$T_{1/2\alpha}$	initial phase half-life
Tyr	tyrosine	$T_{1/2\beta}$	terminal phase half-life
Phe	phenylalanine	$V_{d,area}$	volume of distribution by area
Cys	cysteine	V_c	volume of central compartment
NaCl	sodium chloride	V_p	volume of peripheral compartment
H_2SO_4	sulphuric acid	F	bioavailability
HCl	hydrochloric acid	CL	systemic clearance
APS	ammonium persulfate	k_{12}	transfer rate constant from central to peripheral compartment
DTNB	dithionitrobenzoate	k_{21}	transfer rate constant from peripheral to central compartment
MES	2-(N-Morpholino) ethanesulfonic acid	C_{max}	maximum concentration
PBS	phosphate buffered saline	AUC	area under the curve
PBS-Tween	phosphate buffered saline-Tween 20	ANOVA	analysis of variance
BSA	bovine serum albumin	C.I.	confidence interval
TEMED	NN,N,N'-N'-tetramethylethylenediamine	NFU	National Formulary Unit
PVDF	polyvinylidene fluoride	ED ₅₀	median effective dose
TMB	3,3',5,5'-Tetramethylbenzidine	LD ₅₀	median lethal dose

HRP	horseradish Peroxidase	ADME	absorption, distribution, metabolism, elimination
EDTA	ethylenediaminetetraacetic acid	ELISA	enzyme-linked immunosorbent assay
DTT	dithiothreitol	UV	ultraviolet
IAA	iodoacetamide	Vis	visual
TFA	trifluoroacetic acid	HMW	high molecular weight
WHO	World Health Organization	MW	molecular weight
SEARO	WHO-Southeast Asia Regional Office	<i>m/z</i>	mass-to-charge ratio
NCBI	National Center for Biotechnology Information	MALDI	matrix-assisted laser desorption/ionization
CIOMS	Council for International Organizations of Medical Sciences	ESI	electrospray ionization
CID	collision-induced	TOF	time-of-flight
HPLC	high performance liquid chromatography	2DE	two-dimensional gel electrophoresis
MS	mass spectrometry	LC- MS/MS	liquid chromatography-tandem mass spectrometry
1DE	one-dimensional gel electrophoresis	S.D.	standard deviation

LIST OF APPENDICES

Appendix A	Isolation and purification of antibody IgG from hyperimmunized serum in rabbits	232
Appendix B	Standard curve for protein quantitation	234
Appendix C	Standard curves for determination of enzyme activities	235
Appendix D	Standard curve of venom/venom toxin antigens	237
Appendix E	Serum concentration of venom/venom toxin antigens	238
Appendix F	Standard curve of Neuro Polyvalent antivenom (NPAV)	245
Appendix G	Serum concentration of Neuro Polyvalent antivenom (NPAV)	246
Appendix H	List of publications in ISI-indexed journals	247
Appendix I	List of conference proceedings	248
Appendix J	List of manuscripts submitted	249
Appendix K	Ethical clearance for laboratory animal use	250