APPENDICES

Appendix A: Isolation and purification of antibody IgG from hyperimmunized serum in rabbits

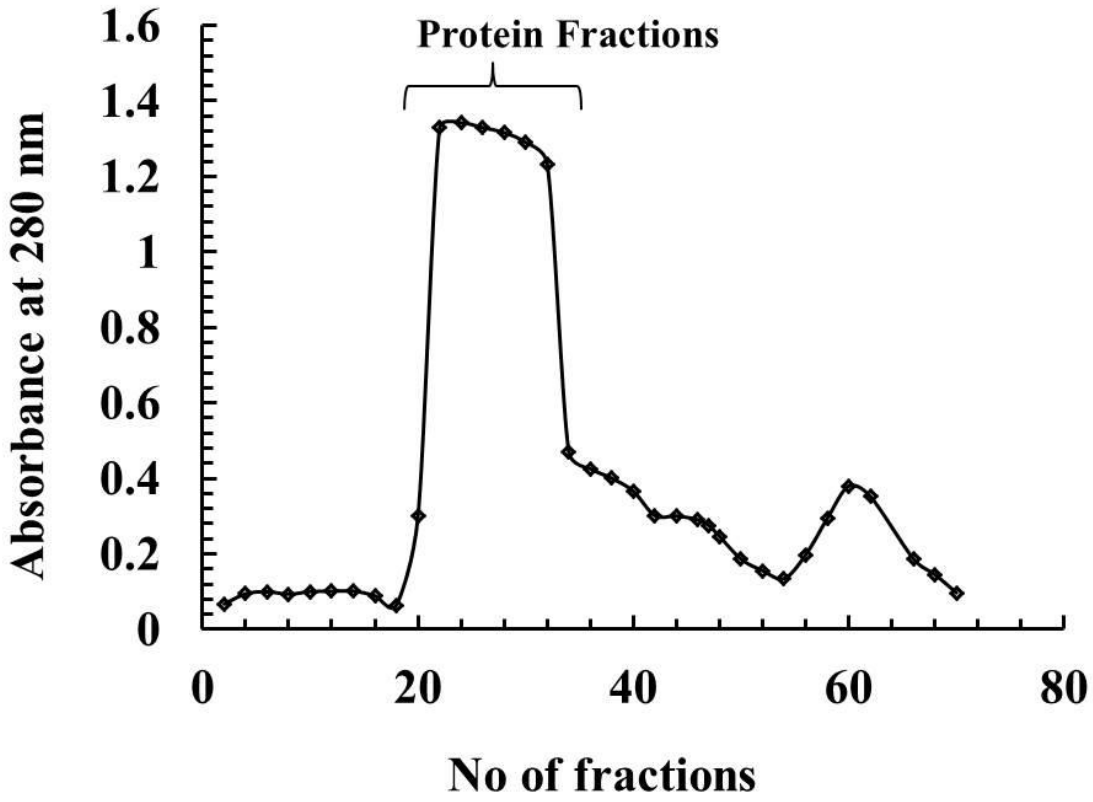

Figure A1: Sephadex® G-25 gel filtration chromatography of protein fractions from hyperimmunized serum in rabbits.

Sephadex® G-25 gel filtration column chromatography (30 mm x 480 mm) was equilibrated with 0.05% (v/v) acetic acid. The flow rate was 30 ml/h and fractions of 5 ml were collected. Protein content was measured by absorbance at 280 nm.

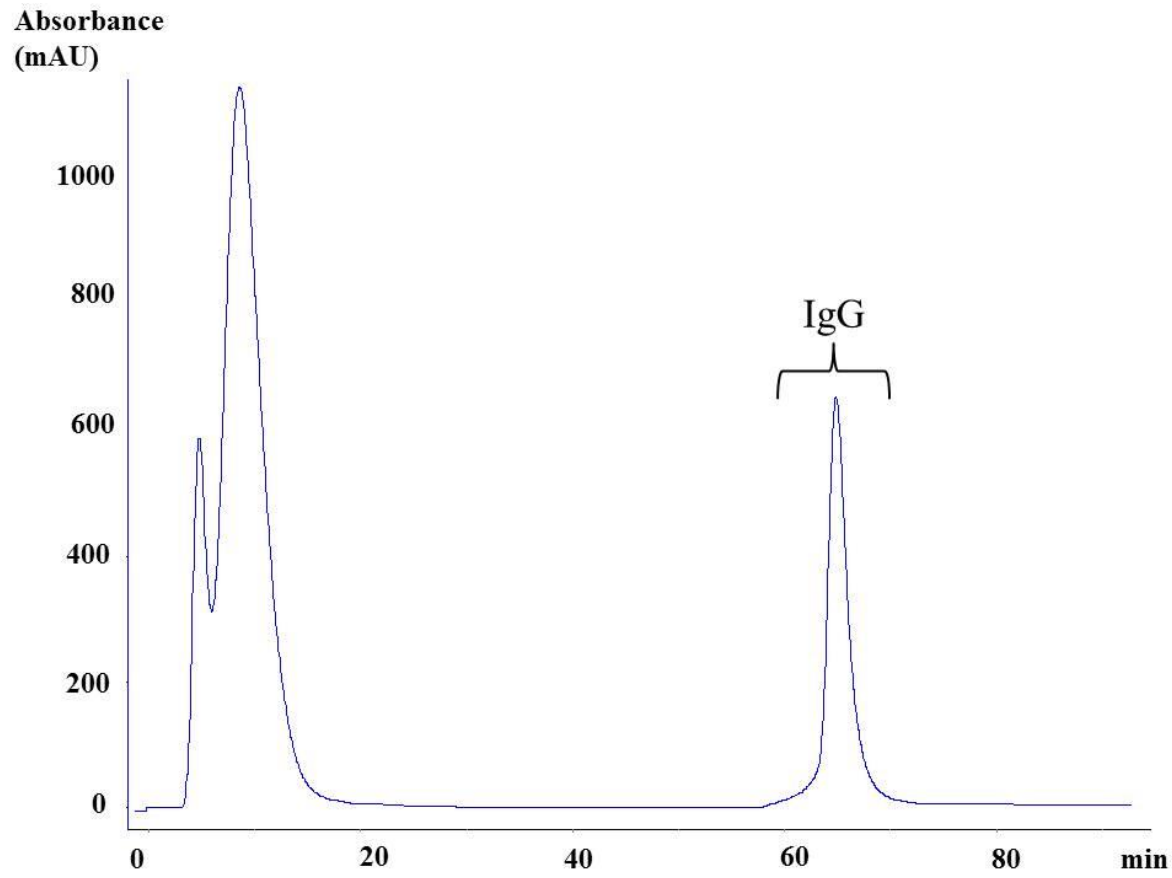

Figure A2 : HiTrap Protein A HP affinity column chromatography (5 ml) of desalted protein fractions.

The antibody IgG was eluted with a linear gradient (0-100%) between binding buffer (20 mM sodium phosphate, pH 7) and elution buffer (0.1 M citric acid, pH 3) at a flow rate of 5 ml/min. The pH of IgG fractions collected manualy was adjusted by adding 100 µl of 1 M Tris-HCl, pH 9.0 per ml of fractions.

Appendix B: Standard curve for protein quantitation

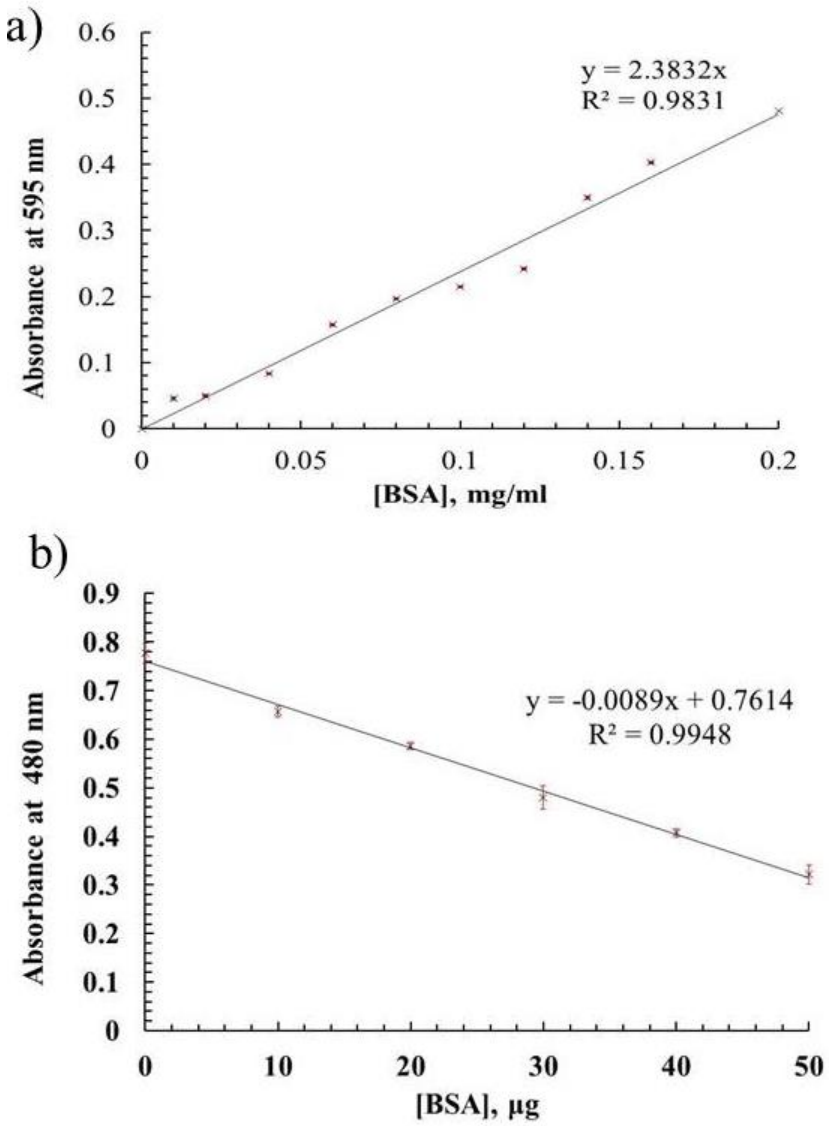

Figure B1: Standard curve of Bovine serum albumin (BSA) for the determination of protein concentration in venom samples.

a) The absorbance at 595 nm was plotted against concentration of BSA (mg/ml), in Bradford assay (Bradford, 1956).

b) The absorbance at 480 nm was plotted against concentration of BSA (µg), in 2-D Quant Kit.

Appendix C: Standard curves for determination of enzyme activities

C1 Standard curve of phosphate for determination of 5'-nucleotidase activity by ascorbic acid method (Chen *et al*., 1956)

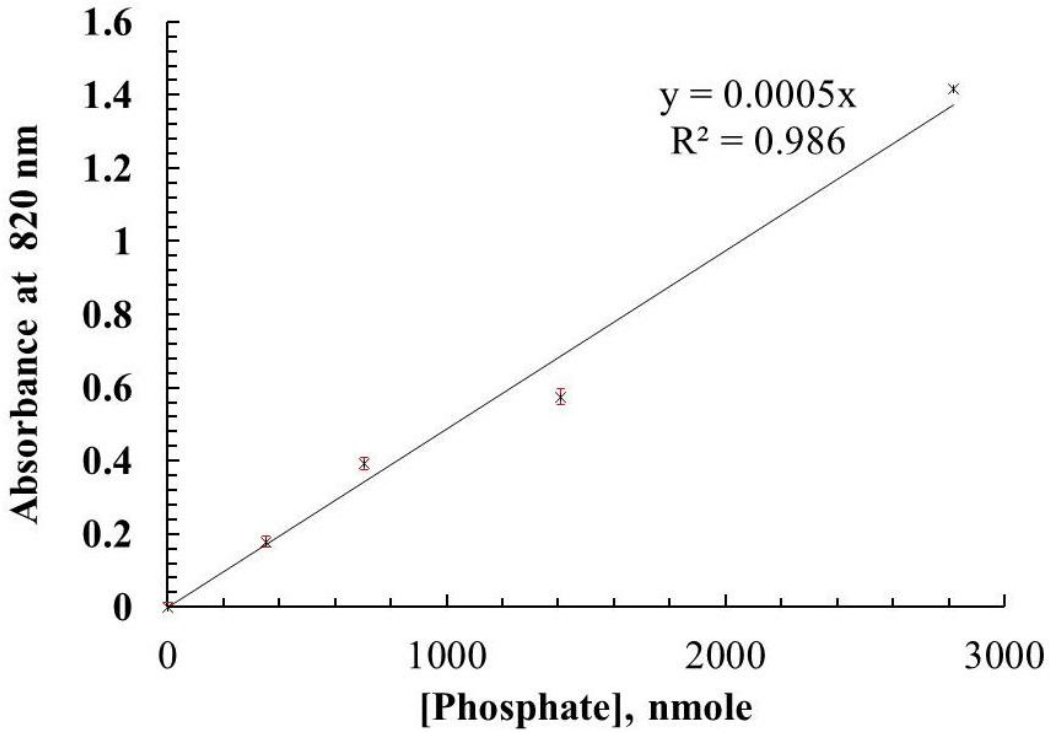

Figure C1: Standard curve of phosphate for the measurement of phosphate concentration liberated in 5'-nucleotidase enzymatic reaction.

The absorbance at 480 nm was plotted against known concentration of phosphate in nmole.

C2 Standard curve of standard Hyaluronidase for determination of hyaluronidase activity by turbidimetric method (Dorfman and Ott, 1948)

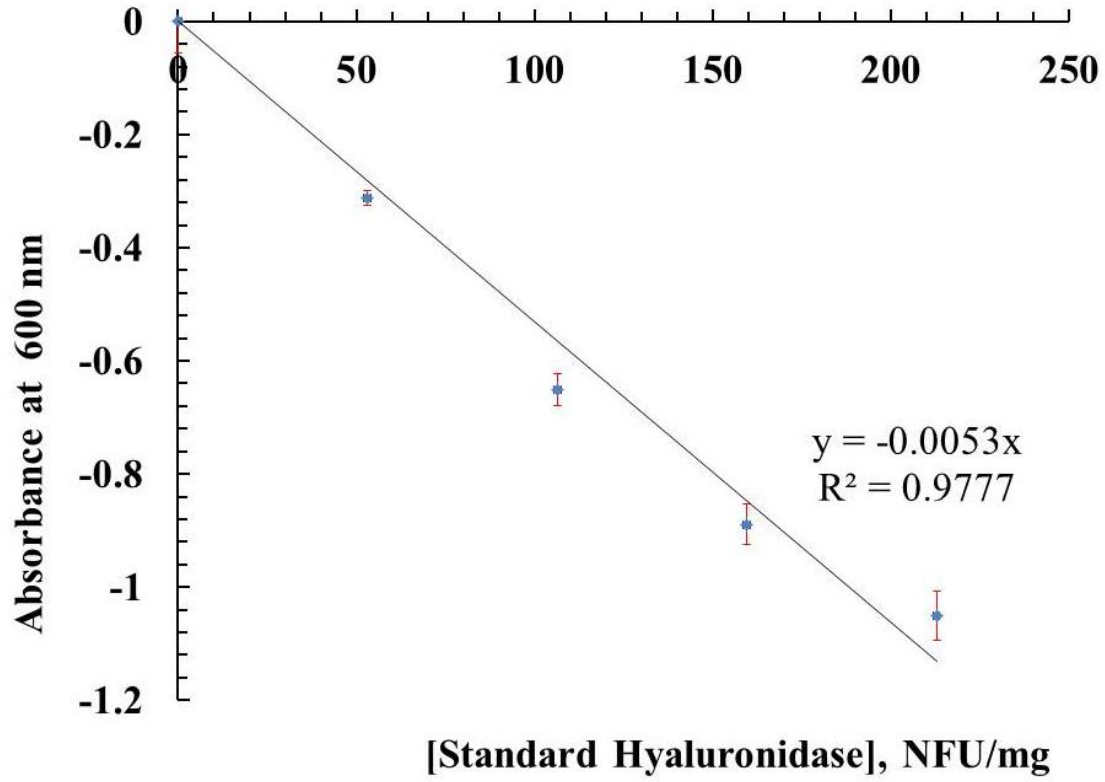

Figure C2: Standard curve of hyaluronidase for the determination of hyaluronidase activity.

The absorbance at 600 nm was plotted against known concentration of standard hyaluronidase in National Formulary Unit (NFU)/mg.

Appendix D: Standard curve of venom/venom toxin antigens

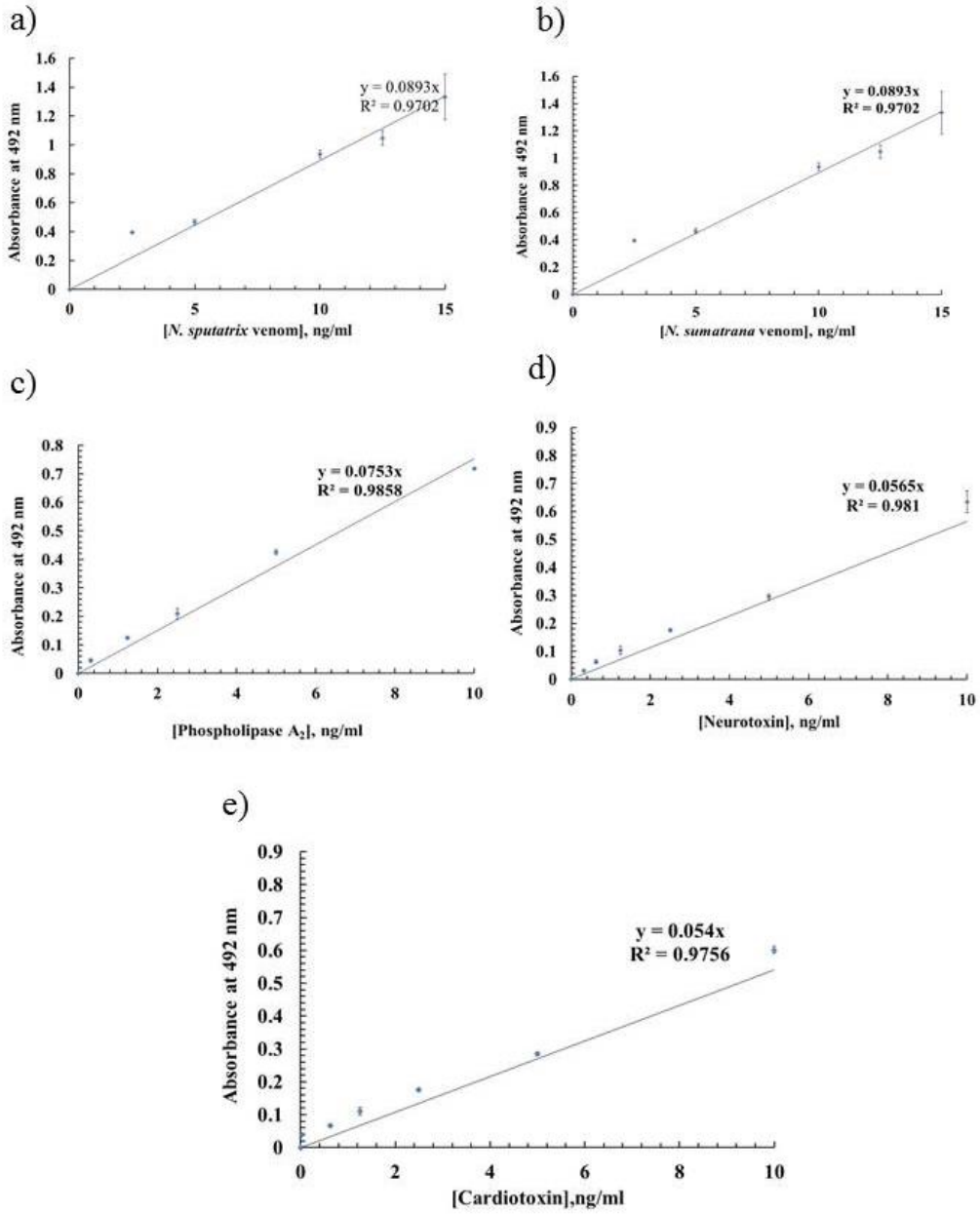

Figure D1: Standard curve of venom/venom toxin antigens in spiked pre-envenomed sera.

a) *N. sputatrix* venom antigens; b) *N. sumatrana* venom antigens; c) Phospholipase A_2 antigens; d) Neurotoxin antigens and e) Cardiotoxin antigens.

Appendix E: Serum concentration of venom/venom toxin antigens

Table E1: Serum concentration of *N. sputatrix* venom antigens following intravenous administration of *N. sputatrix* venom into rabbits (n = 3).

Time (h)	0.01667	0.25	0.5	1	3	6	24
Rabbit 1	223.14	201.99	192.11	167.52	144.69	88.00	45.73
Rabbit 2	190.90	169.38	113.45	98.76	77.45	68.43	37.80
Rabbit 3	276.10	185.43	167.09	112.00	109.88	91.66	36.37
Mean ± S.D. (ng/ml)	230.05 ± 43.02	185.6 ± 16.31	157.55 ± 40.19	126.09 ± 36.48	110.67± 33.63	82.70 ±12.49	39.97 ± 5.04

Table E2: Serum concentration of *N. sputatrix* venom antigens following intramuscular administration of *N. sputatrix* venom into rabbits (n = 3).

Time (h)	0.01667	0.0833	0.1667	0.3333	0.5	0.75	1	2	3	5	8	24
Rabbit 1	213.88	200.99	226.58	220.19	277.32	420.00	320.60	240.30	200.11	168.54	109.54	57.19
Rabbit 2	268.85	206.78	190.00	230.00	391.85	457.99	301.32	236.81	151.76	125.76	108.00	78.67
Rabbit 3	254.98	240.00	217.63	185.79	305.00	409.17	421.35	425.00	397.18	290.00	212.00	95.06
Mean ± S.D. (ng/ml)	245.90 ± 28.59	215.92 ± 21.05	211.40 ± 19.07	211.99 ± 23.22	324.72 ± 59.76	429.05 ± 25.64	347.76 ± 64.46	300.70 ± 107.66	249.68 ± 130.00	194.77 ± 85.20	143.18 ± 59.60	76.97 ± 18.99

Table E3: Serum concentration of *N. sputatrix* venom antigens in the effects of 4 ml of Neuro Polyvalent antivenom (NPAV) in rabbits (n = 3).

Time (h)	0.0833	0.5	1	1.5	2.5	4.5	6.5	12	24	48	72
Rabbit 1	183.76	225.57	304.09	201.13	46.75	83.45	99.43	99.65	50.43	49.66	15.36
Rabbit 2	210.00	243.54	344.32	108.04	134.31	97.67	82.46	69.32	56.169	30.82	21.09
Rabbit 3	178.54	121.73	254.85	84.77	88	96.8	92	74.12	54.38	20.16	7.67
Mean ± S.D. (ng/ml)	190.77 ± 16.86	196.95 ± 65.76	301.09 ± 44.81	131.31 ± 61.57	89.69 ± 43.80	92.64 ± 7.97	91.30 ± 8.51	81.03 ± 16.30	53.66 ± 2.94	33.55 ± 14.94	14.71 ± 6.73

Table E4: Serum concentration of *N. sputatrix* venom antigens in the effects of 4 + 2 ml of Neuro Polyvalent antivenom (NPAV) in rabbits (n = 3).

Time (h)	0.0833	0.5	1	1.3	1.83	2.33	2.5	4.5	6.5	12	24	48	72
Rabbit 1	112.46	209.83	302.89	211.67	108.43	85.44	63.21	41.27	33.54	30.15	21.59	15.56	10.31
Rabbit 2	130.23	191.76	296.75	211.67	133.98	93.70	72.11	80.14	36.22	29.11	28.35	12.65	8.82
Rabbit 3	126.43	217.59	318.29	276.13	122.51	86.59	66.70	65.70	38.43	31.12	27.94	10.14	6.59
Mean ± S.D. (ng/ml)	123.04 ± 9.36	206.39± 13.25	305.98 ± 11.10	233.16 ± 37.22	121.64 ± 12.80	88.58 ± 4.47	67.34 ± 4.48	62.37 ± 19.65	36.06 ± 2.45	30.13 ± 1.01	25.96 ± 3.79	12.78 ± 2.71	8.57 ± 1.87

Table E5: Serum concentration of *N. sumatrana* venom antigens following intravenous administration of *N. sumatrana* venom into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	24
Rabbit 1	229.42	182.63	170.04	150.58	130.27	107.59	76.10	32.50
Rabbit 2	191.56	162.36	155.92	133.45	113.72	104.31	70.94	26.40
Rabbit 3	188.34	188.73	172.27	150.88	130.07	106.82	74.54	29.84
Mean ± S.D. (ng/ml)	203.11 ± 22.84	177.91 ± 13.80	166.08 ± 8.87	144.97 ± 9.98	124.69 ± 9.50	106.24 ± 1.72	73.86 ± 2.65	29.58 ± 3.06

Table E6: Serum concentration of *N. sumatrana* venom antigens following intramuscular administration of *N. sumatrana* venom into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	24
Rabbit 1	229.42	182.63	170.04	150.58	130.27	107.59	76.10	32.50
Rabbit 2	191.56	162.36	155.92	133.45	113.72	104.31	70.94	26.40
Rabbit 3	188.34	188.73	172.27	150.88	130.07	106.82	74.54	29.84
Mean ± S.D. (ng/ml)	203.11 ± 22.84	177.91 ± 13.80	166.08 ± 8.87	144.97 ± 9.98	124.69 ± 9.50	106.24 ± 1.72	73.86 ± 2.65	29.58 ± 3.06

Table E7: Serum concentration of *N. sumatrana* phospholipase A_2 antigens following intravenous administration of phospholipase A_2 into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	10	24
Rabbit 1	186.53	177.38	136.38	100.64	72.68	51.33	35.92	27.28	13.21
Rabbit 2	183.37	161.07	130.33	99.84	73.64	46.15	32.04	25.22	13.30
Rabbit 3	181.03	163.67	115.73	95.56	62.42	49.62	36.90	26.33	12.89
Mean ± S.D. (ng/ml)	183.65 ± 2.76	167.37 ± 8.77	127.48 ± 10.62	98.68 ± 2.73	69.58 ± 6.22	49.03 ± 2.64	34.95 ± 2.57	26.28 ± 1.03	13.13 ± 0.22

Table E8: Serum concentration of *N. sumatrana* phospholipase A_2 antigens following intramuscular administration of phospholipase A_2 into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	10	24
Rabbit 1	93.50	103.99	150.42	193.04	232.87	118.76	54.78	36.49	17.36
Rabbit 2	90.70	124.03	149.13	193.41	224.71	116.09	61.34	31.69	13.76
Rabbit 3	89.72	110.65	145.69	187.92	222.35	114.25	56.43	33.29	15.21
Mean ± S.D. (ng/ml)	91.31 ± 1.96	112.89 ± 10.21	148.41 ± 2.44	191.46 ± 3.07	226.64 ± 5.52	116.36 ± 2.27	57.52 ± 3.41	33.82 ± 2.45	15.45 ± 1.81

Table E9: Serum concentration of *N. sumatrana* neurotoxin antigens following intravenous administration of neurotoxin into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	10	24
Rabbit 1	108.71	87.66	67.74	58.78	39.53	34.26	26.47	17.61	5.79
Rabbit 2	125.21	100.95	78.04	67.68	45.55	39.44	30.48	20.28	6.67
Rabbit 3	108.78	87.67	67.81	58.83	39.57	34.28	24.45	17.66	7.29
Mean ± S.D. (ng/ml)	114.23 ± 7.76	92.10 ± 6.26	71.20 ± 4.84	61.76 ± 4.18	41.55 ± 2.83	35.99 ± 2.44	27.13 ± 2.50	18.52 ± 1.25	6.58 ± 0.62

Table E10: Serum concentration of *N. sumatrana* neurotoxin antigens following intramuscular administration of neurotoxin into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	10	24
Rabbit 1	61.97	69.53	123.10	95.67	59.90	38.39	28.52	18.34	7.50
Rabbit 2	58.94	65.84	118.35	79.47	57.23	47.32	31.27	18.47	7.26
Rabbit 3	60.49	67.69	120.68	87.53	58.57	42.93	29.92	18.41	7.40
Mean ± S.D. (ng/ml)	60.47 ± 1.24	67.69 ± 1.51	120.71 ± 1.94	87.56 ± 6.62	58.57 ± 1.09	42.88 ± 3.64	29.90 ± 1.12	18.40 ± 0.050	7.39 ± 0.098

Table E11: Serum concentration of *N. sumatrana* cardiotoxin antigens following intravenous administration of cardiotoxin into rabbits (n = 3).

T(h)	0.0833	0.1667	0.5	1	2	3	6	10	24
Rabbit 1	101.185	92.97	71.80	56.27	41.41	34.34	23.70	18.31	6.10
Rabbit 2	101.20	92.43	72.06	55.80	41.08	34.10	26.32	18.23	6.08
Rabbit 3	101.31	91.90	70.59	55.34	40.74	33.86	23.31	18.04	6.01
Mean ± S.D. (ng/ml)	101.23 ± 0.057	92.43 ± 0.44	71.48 ± 0.64	55.80 ± 0.38	41.08 ± 0.27	34.10± 0.20	24.44 ± 1.33	18.19 ± 0.11	6.06 ± 0.038

Table E12: Serum concentration of *N. sumatrana* cardiotoxin antigens following intramuscular administration of cardiotoxin into rabbits (n = 3).

T(h)	0.0833	0.1667	0.5	1	2	3	6	10	24
Rabbit 1	69.01	77.16	138.70	93.09	67.10	55.43	36.67	21.67	8.52
Rabbit 2	66.08	73.90	132.80	89.18	64.97	53.67	35.45	20.99	8.41
Rabbit 3	63.36	70.88	127.33	85.47	62.85	51.91	34.34	20.31	8.19
Mean ±S.D. (ng/ml)	66.15 ± 2.31	73.98 ± 2.57	132.95 ± 4.64	89.25 ± 3.11	64.97 ± 1.73	53.67 ± 1.44	35.491 ± 0.95	20.99 ± 0.55	8.37 ± 0.14

Table E13: Serum concentration of *N. sumatrana* cardiotoxin antigens following intravenous administration of whole venom into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	24
Rabbit 1	101.22	83.67	64.65	50.63	37.29	30.93	23.02	8.63
Rabbit 2	102.27	85.86	65.40	51.77	38.51	32.70	24.50	8.84
Rabbit 3	102.27	85.80	65.44	51.77	38.51	32.70	24.29	8.84
Mean ± S.D. (ng/ml)	101.92 ± 0.61	85.13 ± 1.26	65.15 ± 0.44	51.39 ± 0.66	38.10 ± 0.70	32.11 ± 1.02	23.94 ± 0.80	8.77 ± 0.12

Table E14: Serum concentration of *N. sumatrana* cardiotoxin antigens following intramuscular administration of whole venom into rabbits (n = 3).

Time (h)	0.0833	0.1667	0.5	1	2	3	6	24
Rabbit 1	88.98	99.54	178.89	120.19	73.89	61.30	45.56	17.13
Rabbit 2	83.33	105.83	166.39	116.11	71.39	64.72	46.39	16.94
Rabbit 3	86.67	111.94	162.78	119.17	68.89	61.39	43.61	19.17
Mean ± S.D. (ng/ml)	86.33± 2.84	105.77 ± 6.20	169.35± 8.45	118.49 ± 2.12	71.39 ± 2.50	62.47 ± 1.95	45.19 ± 1.43	17.75 ± 1.23

Appendix F: Standard curve of Neuro Polyvalent antivenom (NPAV)

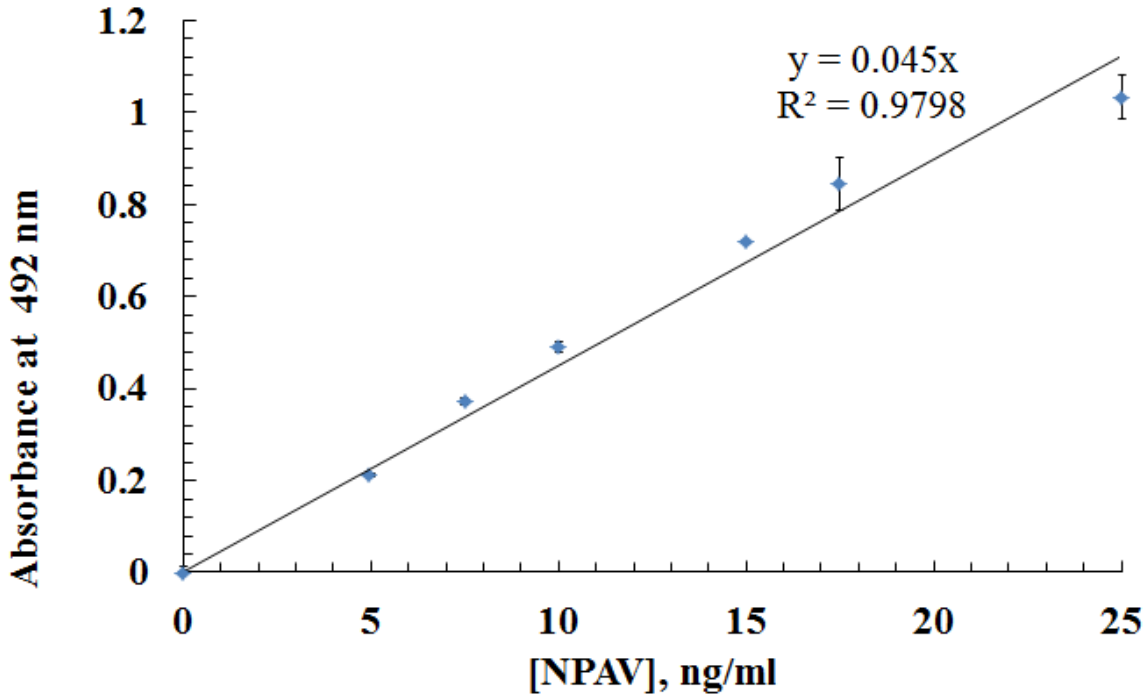

Figure F1: Standard curve of Neuro Polyvalent antivenom (NPAV) in spiked pre-envenomed sera.

Appendix G: Serum concentration of Neuro Polyvalent antivenom (NPAV)

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

Time (h)	0.0833	0.5	1.5	2.5	6.5	12	24	48	72
Rabbit 1	1410.97	1237.98	1098.73	748.52	618.57	702.11	326.58	257.38	188.19
Rabbit 2	1403.38	1246.4	979.75	683.54	628.69	603.38	254.01	263.29	183.12
Rabbit 3	1419.41	1227.00	921.52	750.21	616.88	600.00	330.80	262.45	239.66
Mean ± S.D. (ng/ml)	1411.25 ± 6.55	1237.13 ± 7.95	1000 ± 73.75	727.43 ± 31.04	621.38 ± 5.22	635.16 ± 47.36	303.80 ± 35.25	261.04± 2.61	203.66 ± 25.54

Appendix H: List of publications in ISI-indexed journals

a) Yap Michelle KK, Tan NH and Fung SY (2011). Biochemical and toxinological characterization of *Naja sumatrana* (Equatorial spitting cobra) venom. *J Venom Anim Toxins incl Trop Dis*. 17:4, 451-459

b) Yap Michelle KK, Tan NH, Sim SM, and Fung SY (2013). Toxicokinetics of *Naja sputatrix* (Javan spitting cobra) venom following intramuscular and intravenous administration of venom into rabbits. *Toxicon.* 68:18-23

Appendix I: List of conference proceedings

a) Pharmacokinetics of *Naja sputatrix* (Javan spitting cobra) venom and the effect of a neuro polyvalent antivenom, 11-15 Sep 2011, 17th Congress of EU section of International Society of Toxinology, International Society of Toxinology (International).

b) Biochemical and toxinological studies of *Naja sumatrana* (Equatorial spitting cobra) venom, 27-28 July 2011, 36th Annual Conference of MSBMB, Malaysian Society of Biochemistry and Molecular Biology (National).

c) Pharmacokinetics of *Naja sputatrix* (spitting cobra) venom in rabbits, 22 -24 Mar 2011, 11th Southeast Asian Western Pacific Regional Meeting of Pharmacologist, The Japanese Pharmacological Society (International).

d) Serum kinetics of *Naja sputatrix* (spitting cobra) venom in rabbit and immunological responses in rabbits immunized with *Naja sputatrix* venom and venom components, 27 -28 July 2010, 35th Annual Conference of The Malaysian Society of Biochemistry and Molecular Biology (National).

Appendix J: List of manuscripts submitted

1. Michelle Khai Khun Yap, Shin Yee Fung, Kae Yi Tan, Nget Hong Tan. Proteomic characterization of venom of the medically important Southeast Asian *Naja sumatrana* (Equatorial spitting cobra), submitted to Acta Tropica (ISI-Indexed Publication).
2. Michelle Khai Khun Yap, Nget Hong Tan, Si Mui Sim, Shin Yee Fung. The effect of a polyvalent antivenom on the pharmacokinetics of *Naja sputatrix* (Javan spitting cobra) venom in experimentally envenomed rabbits, submitted to International Immunopharmacology(ISI-Indexed Publication).
3. Michelle Khai Khun Yap, Nget Hong Tan, Si Mui Sim, Shin Yee Fung, Choo Hock Tan. Pharmacokinetics of *Naja sumatrana* (Equatorial spitting cobra) venom and its major toxins in experimentally envenomed rabbits, submitted to PLoS NTD (ISI-Indexed Publication).

Appendix K Ethical Clearance for laboratory animal use

UNIVERSITY OF MALAYA
KUALA LUMPUR

3rd July 2013

Dr. Fung Shin Yee
Department of Molecular Medicine
Faculty of Medicine

Dear Researcher,

Biomedical and Toxinological Studies on Snake Venom Proteins

This is to inform you that the FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC) has approved your Animal Use Protocol with the above mentioned title for duration of one (1) year until **June 2014**.

Please be advised that should you require changes to be made to the approved protocol, you are responsible to submit an application for amendments. Failure to do so may result in the Approval of your Animal Research Protocol be withdrawn by FOM IACUC.

Your Ethics Reference no. : **2013-06-07/MOL/R/FSY**

Thank you.

Yours sincerely,

Dr. Kiew Lik Voon
Acting Chairperson
Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC)

Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC)
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