CHAPTER VI

A NOVEL FORMULATION FOR THE IMPROVED TREATMENT OF INDIAN RED SCORPION (*Mesobuthus tamulus*) VENOM-INDUCED TOXICITY TESTED IN *Caenorhabditis elegans* AND RODENT MODELS

6.1 Results

6.1.1 The *in-silico* analysis showed the binding of AAAs to homologous SER6 receptors in *C. elegans*

A comparison of *in-silico* binding efficiency of AAAs to α-adrenergic receptors of humans, mice and homologous receptor (SER6 receptor) in *C. elegans* is shown in Table 6.1. Prazosin-HCL demonstrated the highest binding efficiency to the α1A adrenergic receptor of humans. In contrast, silodosin and terazosin-HCL showed the highest binding efficiency to α1D-adrenergic receptors in mice and SER6 receptor in *C. elegans*, respectively (Table 6.1, Appendix fig. A2).

A molecular docking study showed that prazosin-HCL, binds with AAA via one hydrogen bonding and binds to Gln 345 of the SER6 receptor in *C. elegans*. In contrast, terazosin-HCL showed binding via one hydrogen bonding to Pro 106 and silodosin via three hydrogen bodings to Gln 345, Gln 154, and Thr 363 of the same receptor (binding affinity). The binding relationship of prazosin-HCL, terazosin-HCL, and silodosin HCL with respective receptors was predicted at- 6.6, -7.5, and -6.8 kcal mol^{-1} respectively (Appendix fig. A2, Table 6).

Table 6.1 Docking scores of the α1 adrenoreceptor antagonist (AAA) with the α1 adrenergic receptors from humans, mice, and *C. elegans*. BA: Binding affinity; DR: Docking rank.

Receptor	**Docking score with prazosin hydrochloride [PMCID: 68546]**				**Docking score with terazosin hydrochloride [PMCID: 68546]**				**Docking score with silodosin [PMCID: 68546]**			
	BA	**rmsd/ub**	**rmsd/lb**	**DR**	**BA**	**rmsd/ub**	**rmsd/lb**	**DR**	**BA**	**rmsd/ub**	**rmsd/lb**	**DR**
Alpha1A adrenergic receptor, (AAAR) (human)	-7.7	4.372	2.808	1	-6.8	27.117	24.472	5	-6.9	2.866	1.929	4
Alpha1B adrenergic receptor (ABAR) (human)	-6.4	30.045	26.824	7	-7.1	17.451	13.724	4	-7	33.115	29.702	3
Alpha1D adrenergic receptor (ADAR) (human)	-6.9	44.121	40.803	4	-6.5	41.723	37.361	6	-7.6	2.399	1.596	2
Alpha1A adrenergic receptor (AAAR) (mouse)	-6.7	18.454	16.899	5	-6.5	19.056	16.835	6	-6.7	60.691	57.074	6
Alpha1B adrenergic receptor (ABAR) (mouse)	-7.1	2.835	1.907	3	-7.3	17.909	17.004	3	-6.4	23.825	19.373	7
Alpha1D adrenergic receptor (ADAR) (mouse)	-7.3	27.734	25.635	2	-7.6	18.07	16.309	1	-7.7	37.322	31.764	1
SER6 receptor (*C. elegans*)	-6.6	8.822	4.309	6	-7.5	15.602	10.429	2	-6.8	43.083	40.303	5

6.1.2 Optimum dose of inhibitors in neutralizing the MTV-induced lethality, ROS generation, and depolarization of mitochondrial transmembrane potential in *C. elegans*

The commercial ASAs (PSVPL and HBC) (2 mg), AAAs (100 µM), and ascorbic acid (2 µg) did not show toxicity in *C. elegans* (Table 6.2). The regression analysis determined the LC_{50} value of *M. tamulus* venom towards *C. elegans* after 24 h of incubation as 125 µg/mL (Fig. 6.1).

Table 6.2 Determination of toxicity of ASA, AAA and ascorbic acid in *C. elegans.* Data represent mean ± SD of three determinations.

S. No.	Components	Viability of *C. elegans* (%)	
		0 h	24 h
1	Control	100.00 ± 5	97.20 ± 4.86
2	ASA (PSVPL)	100.00 ± 5	96.10 ± 4.81
3	ASA (HBC)	100.00 ± 5	95.92 ± 4.73
4	AAA (Prazosin)	100.00 ± 5	96.78 ± 4.80
5	AAA (Silodosin)	100.00 ± 5	97.13 ± 4.69
6	AAA (Terazosin)	100.00 ± 5	96.98 ± 4.84

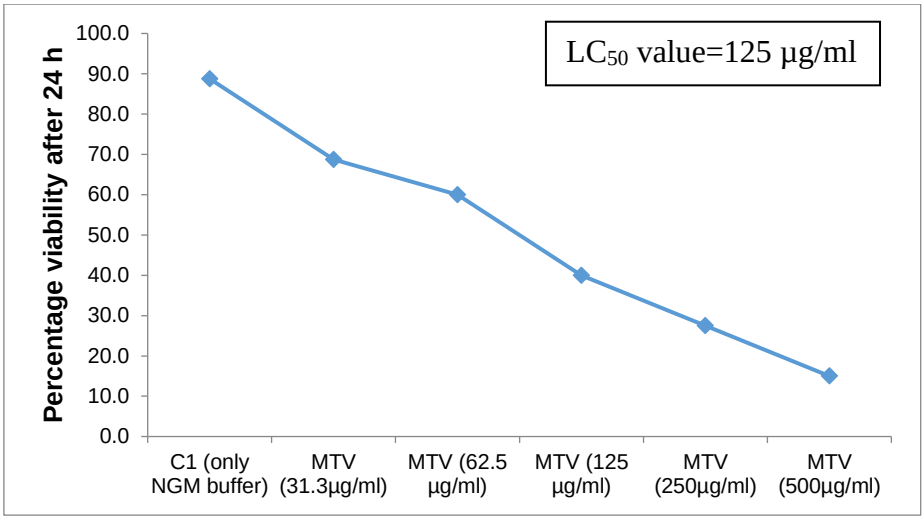


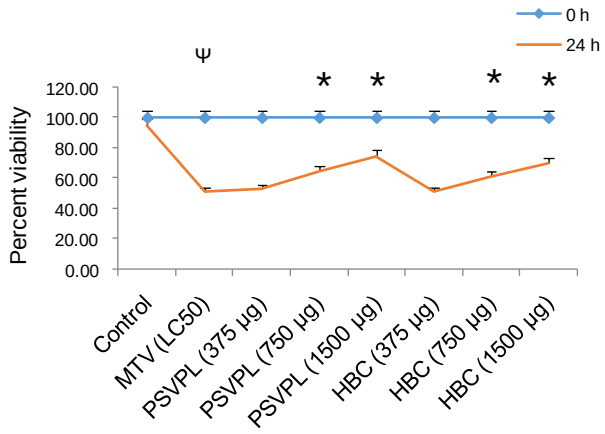
Fig. 6.1. The LC_{50} value of *C. elegans* N_2 calculated after 24 h treatment of scorpion venom (*M. tamulus* venom). The LC_{50} value calculated for scorpion venom towards *C.elegans*, after 24 h incubation, was 125 µg/ml.

The present study provides new information on the *in vivo* MTV neutralisation efficacy of ASAs, AAAs, and ascorbic acid in the *C. elegans* model. All the tested inhibitors, except varespladib, showed dose-dependent neutralisation MTV-induced toxicity (in terms of survivability) in *C. elegans*; however, to a significantly *(*p≤0.05)* different extent. The optimum MTV neutralisation dose of ASA, AAA (prazosin-HCL in this study), and ascorbic acid determined are shown in Table 6.3. The AAAs showed better neutralisation potency than commercial ASAs and ascorbic acid (Fig. 6.2 a-e) against MTV-induced lethality in *C. elegans*. However, silodosin showed better MTV-neutralisation potency than prazosin-HCL and terazosin-HCL (Fig. 6.2 b-d). Further, PSVPL ASA was slightly more effective (**p*≤0.05) than HBC ASA in neutralising the *in vivo* toxicity of MTV *in C. elegans* (Fig. 6.2 a). Interestingly, ascorbic acid (1 µg) and ASAs (1500 µg) showed equipotency in neutralising the MTV-induced toxicity in *C. elegans* (Fig. 6.2 e).

Table 6.3 Determination of *in vivo* optimum dose of commercial ASA, ascorbic acid, and AAAs against LC_{50} of *M. tamulus* venom.

S. No.	Name of samples	Optimum dose
1	**ASA (PSVPL and HBC)**	1500 µg
2	**Ascorbic acid**	1 µg
3	**AAAs:**	
	Prazosin,	50 µM
	Terazosin	50 µM
	Silodosin	25 µM

a



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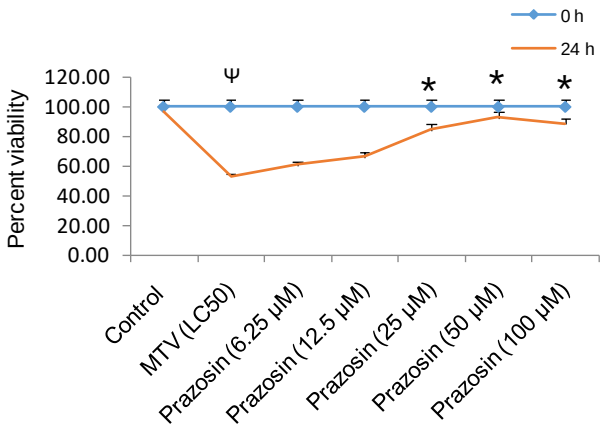

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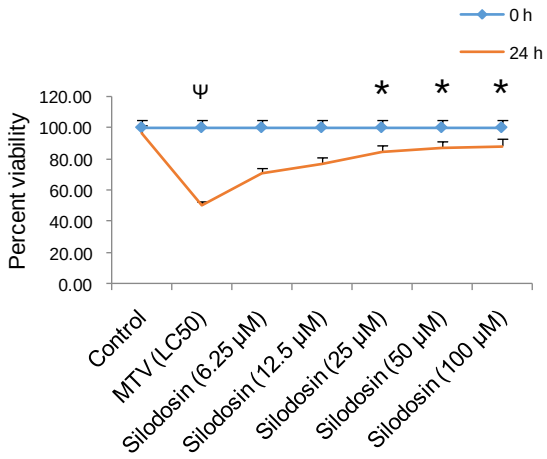
0 h
24 h
Ψ
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120.00
100.00
80.00
60.00
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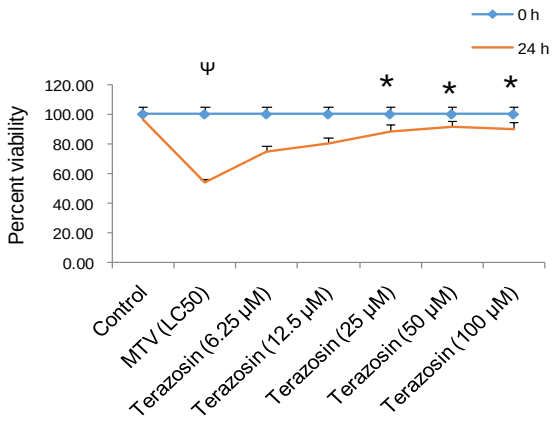
20.00
0.00
Percent viability
Control
MTV (LC50)
Silodosin (6.25 µM)
Silodosin (12.5 µM)
Silodosin (25 µM)
Silodosin (50 µM)
Silodosin (100 µM)

d


0 h
24 h
Ψ
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120.00
100.00
80.00
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0.00
Percent viability
Control
MTV (LC50)
Terazosin (6.25 µM)
Terazosin (12.5 µM)
Terazosin (25 µM)
Terazosin (50 µM)
Terazosin (100 µM)

e

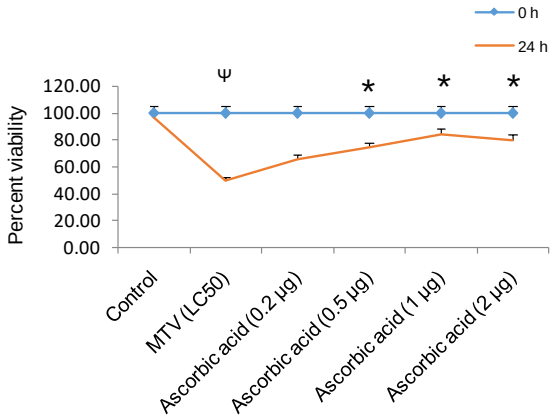
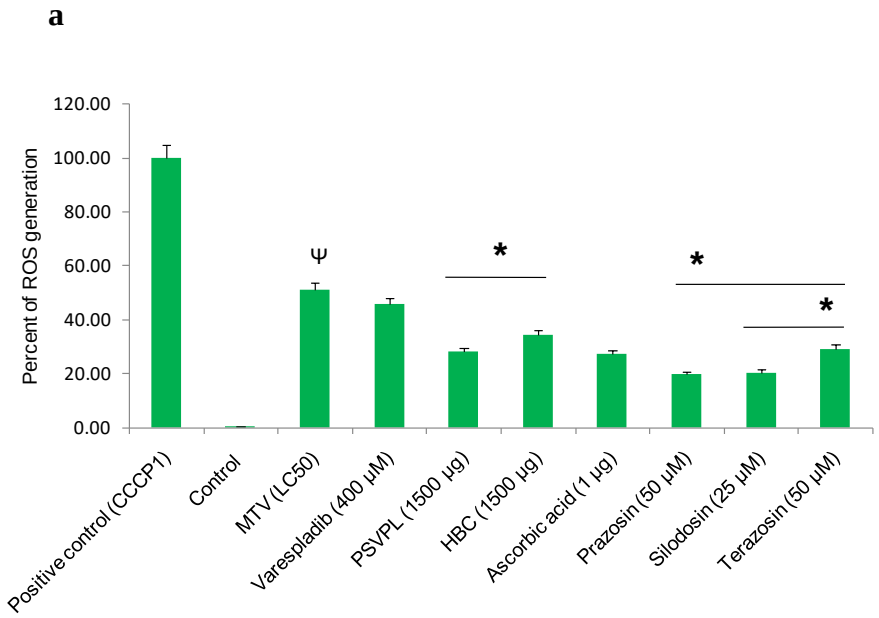

0 h
24 h
Ψ
*
*
*
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Percent viability
Control
MTV (LC50)
Ascorbic acid (0.2 µg)
Ascorbic acid (0.5 µg)
Ascorbic acid (1 µg)
Ascorbic acid (2 µg)

Fig. 6.2. Dose-dependent *in vivo* neutralisation/ inhibition of *M. tamulus* venom (LC_{50})-induced toxicity in *C. elegans* at different time intervals by **(a)** commercial ASAs (375-1500 µg), **(b)** Prazosin-HCL (6.25 µM-100 µM), **(c)** Silodosin (6.25 µM-100 µM), (**d)** Terazosin-HCL (6.25 µM-50 µM) and **(e)** Ascorbic acid (1 µg/mL-10 µg/mL). Data represent ± SD of three determinations. Significance of difference between control and MTV, $^{\Psi}p \leq 0.05$; between MTV and the ASAs, $^{*}p \leq 0.05$. (Abbreviations: LC: lethal concentration; ASAs: anti-scorpion-antivenoms; HCL: hydrochloride; MTV: *M. tamulus* venom)

The ROS production in *C. elegans* after 6 h incubation with LC_{50} concentration of MTV was significantly neutralised in the following order: AAAs> ascorbic acid≥ commercial ASAs (Fig. 6.3a; Appendix fig. A3). The confocal laser microscopic study demonstrated that MTV time-dependently increased the mitochondrial membrane depolarisation in *C. elegans*, significantly neutralised by AAAs followed by ascorbic acid and commercial ASAs (Appendix fig. A3, Appendix fig. A4).



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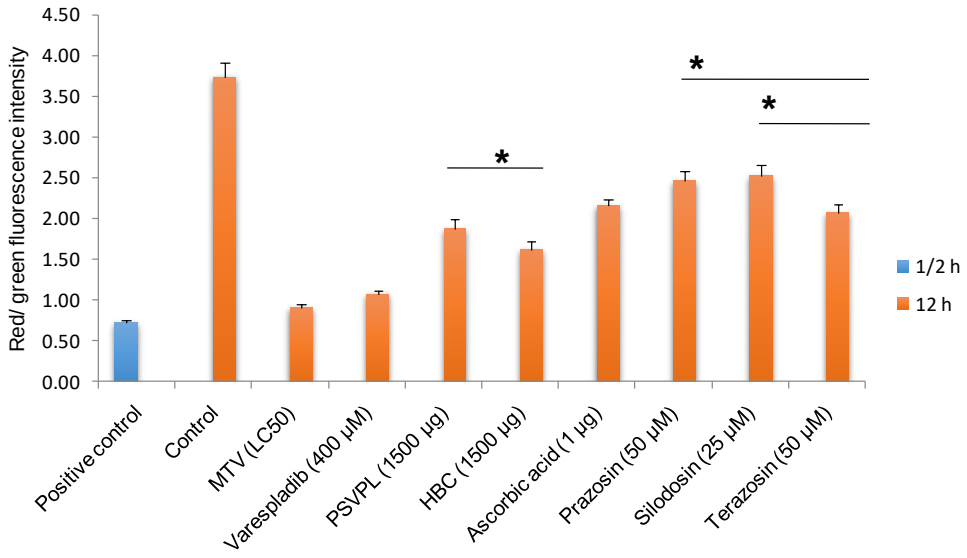
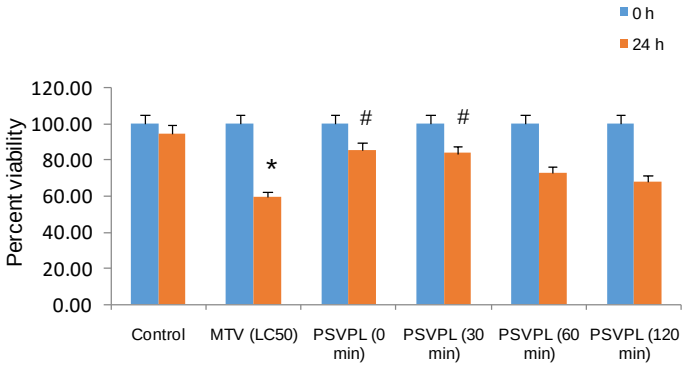


Fig. 6.3. a Illustrates fluorescence intensities of *M. tamulus* venom-induced ROS generation in *C. elegans* after 6 h of *M. tamulus* venom (LC_{50}) treatment and its neutralisation by commercial ASAs, prazosin-HCL, ascorbic acid, silodosin and terazosin-HCL determined by ImageJ software. ROS level in the positive control (CCCP1) *C. elegans* was considered baseline (100%), and other values were compared with that. **b.** Disruption of mitochondrial membrane potential (MMP) in MTV-treated *C. elgans* was observed after 12 h, and its neutralization by commercial ASAs, prazosin-HCL, ascorbic acid, silodosin and terazosin-HCL was determined with measurement of the ratio of red/ green fluorescence intensity by JC-1 staining. Image J software determined the image's intensity, and the bar diagram plotted from the figures shown in Fig. 6. Mitochondrial ROS level in the positive control (CCCP1) *C. elegans* was considered baseline (100%), and other values were compared. Data represent ±SD of three determinations. Significance of difference between control and MTV, $^{\Psi}p \leq 0.05$; between MTV and the ASAs/ AAAs, $^{*}p \leq 0.05$. (Abbreviations: LC: lethal concentration; ROS: reactive oxygen species; ASAs: anti-scorpion-antivenoms; AAA: α1-adrenoreceptor antagonist; HCL: hydrochloride; CCCP1: Carbonyl cyanide 3-chlorophenylhydrazone 1)

6.1.3 Early treatment with AAAs, commercial ASAs, and ascorbic acid showed better neutralisation of MTV-induced toxicity in *C. elegans*

When *C. elegans* were treated with an optimum dose of AAAs (25 µM for silodosin and 50 µM for both prazosin-HCL and terazosin-HCL) and commercial ASAs (1500 µg) at 0, 30, 60, and 120 min post-MTV treatment, per cent viability of worms gradually decreased (Fig. 6.4. a-e). These results suggested early administration (within 30 min post-MTV addition) of AAAs or ASA to scorpion sting patients for better management of the post-envenomation effect. Notably, primary treatment with ascorbic acid (60 and 120 min before the addition of ASAs) compared to treatment with ASAs 60 and 120 min post-addition of MTV was found to be beneficial for efficient therapy of *M. tamulus* venom-induced *C. elegans* (Table 6.4).

a



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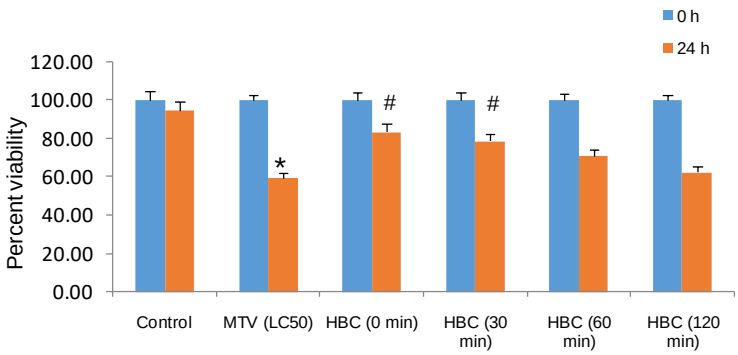

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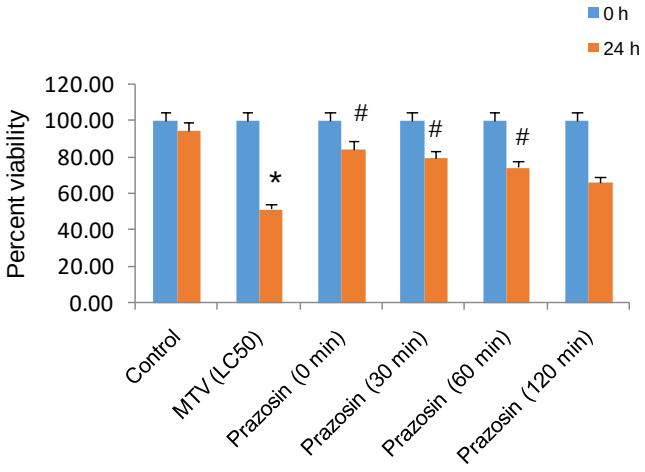
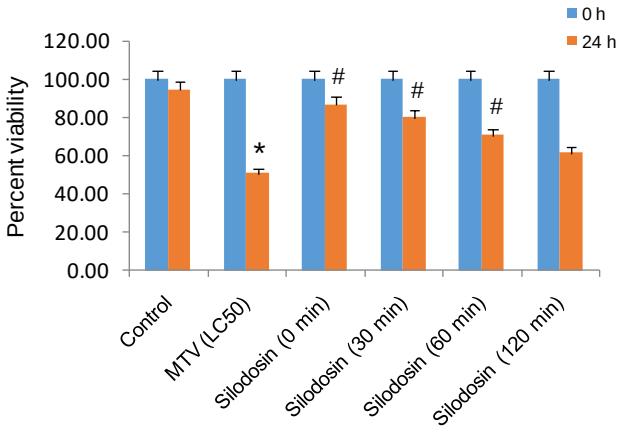

0 h
24 h
Percent viability
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
#
#
#
Control
MTV (LC50)
Prazosin (0 min)
Prazosin (30 min)
Prazosin (60 min)
Prazosin (120 min)

d


0 h
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Percent viability
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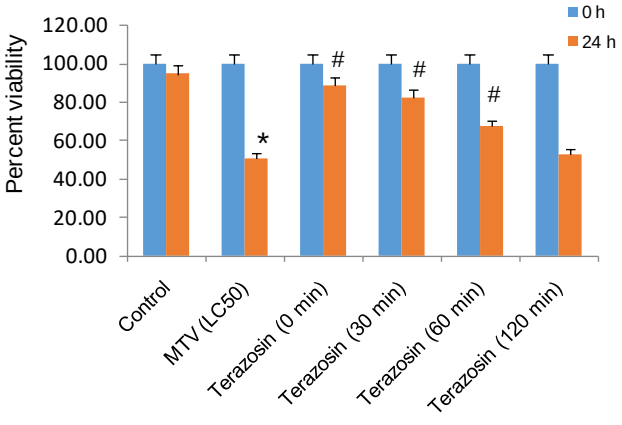
Control
MTV (LC50)
Silodosin (0 min)
Silodosin (30 min)
Silodosin (60 min)
Silodosin (120 min)

e


0 h
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Percent viability
120.00
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60.00
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Control
MTV (LC50)
Terazosin (0 min)
Terazosin (30 min)
Terazosin (60 min)
Terazosin (120 min)

Fig. 6.4. Neutralisation of *M. tamulus* venom (LC_{50})-induced toxicity in *C. elegans* by an optimum dose of ASAs/ AAAs (determined in Fig. 6.2, Table. 6.2), which were added at an interval of 0-120 min post-treatment with venom. **a-b.** PSVPL and HBC ASAs, **c.** Prazosin-HCL, **d.** Silodosin, **e.** Terazosin-HCL against *M. tamulus* venom (LC_{50}) in *C. elegans*. Data represent ±SD of three determinations. Significance of difference between control and MTV, $*p \leq 0.05$; between MTV and the ASAs/ AAAs, $^{\#}p \leq 0.05$. (Abbreviations: LC: lethal concentration; ASAs: anti-scorpion-antivenoms; AAA: α1-adrenoreceptor antagonist; HCL: hydrochloride; PSVPL: Premium Serum and Vaccines Pvt. Ltd.; HBC: Haffkine Bio-pharmaceutical Corporation Ltd.; MTV: *M. tamulus* venom).

Table 6.4. Co-treatment with ascorbic acid and commercial ASA at different time intervals against LC_{50} concentration of *M. tamulus* venom (MTV) in *C. elegans* (n=50). Data represent ± SD of three determinations (n=50). Significance of difference between control and MTV, $^{\Psi}p \leq 0.05$; between MTV and the ASAs, $*p \leq 0.05$.

S. No.	**Components**	**Viability of *C. elegans*(%)**	
		0 h	**24 h**
1	Control	100.00± 5	96.30± 4.8
2	MTV (LC_{50} value, 125 µg/ml)	100.00± 5	51.92± 2.6 (Ψ)
3	Ascorbic acid (0 min) : ASA (60 min)	100.00± 5	77.19± 3.8 (*)
4	Ascorbic acid (0 min) : ASA (120 min)	100.00± 5	69.49± 3.47 (*)

6.1.4 Formulated drug showed significantly higher efficiency compared to individual components of formulation in neutralising the MTV-induced lethality in *C. elegans*

The neutralisation potency of different formulation concentrations, viz formulation 1, formulation 2, and formulation 3 have been evaluated against the lethality of MTV in *C. elegans*. Formulation 1 shows lower potency than formulation 2; however, formulation 3

did not give any additional advantage (p>0.05) as compared to formulation 2 in neutralising the MTV-induced toxicity in *C. elegans* (Table 6.5). Further, formulation 2 demonstrated significantly higher efficiency than individual formulation components and their combinations in neutralising the MTV-induced lethality in *C. elegans* and was considered for further studies (Table 6.5).

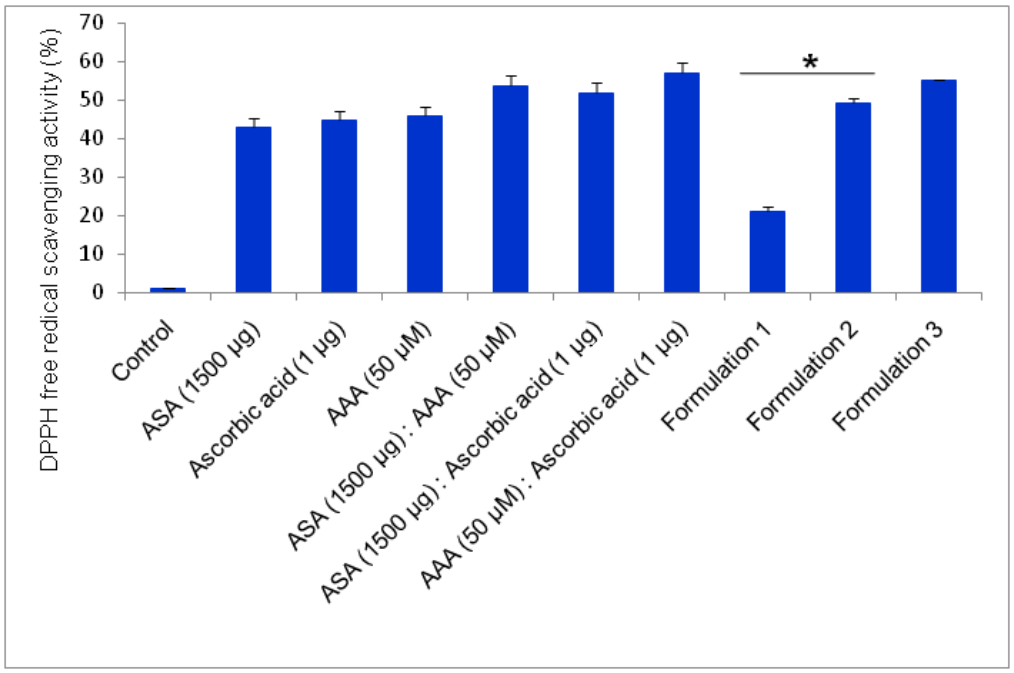
Table 6.5. The *in vivo* neutralization of *M. tamulus* venom (LC_{50} value)-induced toxicity in *C. elegans* with different concentrations of the formulated drugs, individual components of the formulations, and their combinations. Data represent mean ± SD of three determinations (n=50). Significant difference between control and MTV, $^{\Psi}p \leq 0.05$; Significance of difference between formulation 1 and 2, $^{*}p \leq 0.05$.

S. No.	Components	Viability of *C. elegans* (%)	
		0 h	**24 h**
1	Control	100.00± 5	91.5± 4.51
2	MTV (LC_{50} value, 125 µg/ml)	100.00± 5	51.6± 2.57 (Ψ)
3	MTV (LC_{50} value) pre-treated with ASA (187.5 µg)	100.00± 5	59.5± 2.97
4	MTV (LC_{50} value) treated with AAA (prazosin, 3 µM)	100.00± 5	64.4± 3.22
5	MTV (LC_{50} value) treated with ascorbic acid (0.1 µg)	100.00± 5	63.2± 3.16
6	MTV (LC_{50} value) treated with ASA (187.5 µg) : AAA (3 µM)	100.00± 5	58.9± 2.94
7	MTV treated with ascorbic acid (0.1 µg) : AAA (3 µM)	100.00± 5	63.2± 3.11
8	MTV treated with ASA (187.5 µg) : ascorbic acid (0.1 µg)	100.00± 5	64.5± 3.22
9	MTV treated with ASA (93.75 µg) : ascorbic acid (0.05 µg) : AAA (1.5 µM) [Formulation 1]	100.00± 5	75.0± 3.75
10	MTV treated with ASA (187.5 µg) : ascorbic acid (0.1 µg) : AAA (3 µM) [Formulation 2]	100.00± 5	82.6± 4.13 (*)
11	MTV treated with ASA (375 µg) : ascorbic acid (0.2 µg) : AAA (6 µM) [Formulation 3]	100.00± 5	89.1± 4.45

6.1.5 The Formulated drug (formulation 2) demonstrated optimum efficiency in neutralising the *in vitro* DPPH- free radical scavenging activity and *in vivo* neutralisation of MTV-induced ROS generation and alteration of mitochondrial transmembrane potential in *C. elegans*.

Formulations 2 and 3 compared to formulation 1 and individual components demonstrated higher *in vitro* DPPH-free radical scavenging activity (Fig. 6.5 a,b). There was no significant difference in free-radical scavenging activity between formulations 2 and 3, meaning that formulation 2 has effective free-radical scavenging activity.

a



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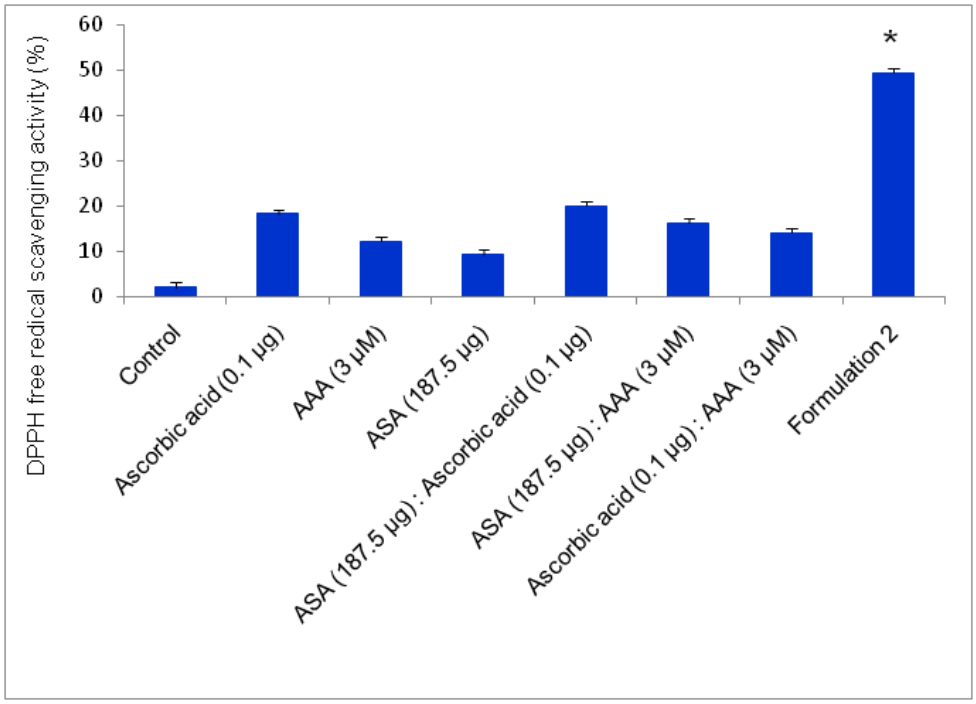
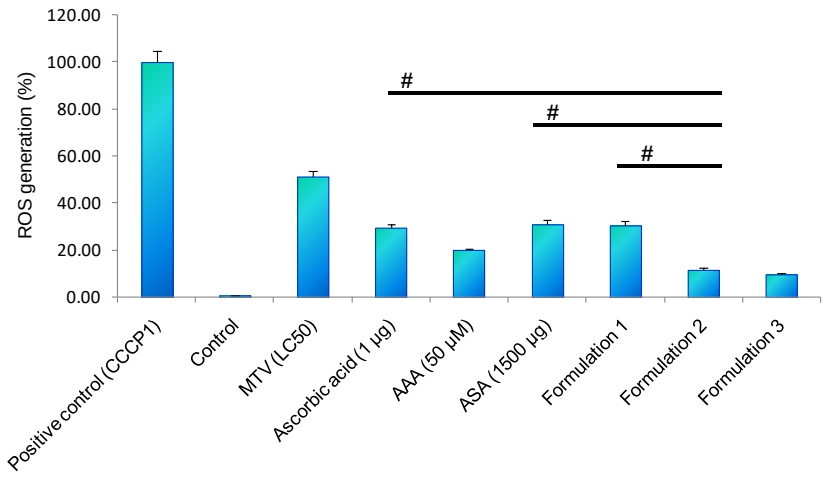


Fig. 6.5. Shows the DPPH free radical-scavenging activity of **(a)** the optimum dose of individual formulation components, their combinations, and different concentrations of formulations, **(b)** Individual components of the formulation and their combinations compared with formulation 2. Data represent mean ± SD of three determinations. Significance of difference, *$p \leq 0.05$ as compared to formulation 2. There was no significant difference ($p > 0.05$) between formulations 2 and 3.

At 6 h of incubation, MTV-induced ROS production was significantly reduced by formulations 2 and 3 (Fig. 6.6 a-b, Appendix fig. A5). However, there was no significant difference ($p > 0.05$) in the inhibition of ROS generation between formulation 2 and formulation 3, but their inhibitory potency was significantly higher compared to formulation 1 (Fig. 6.6 a).

a



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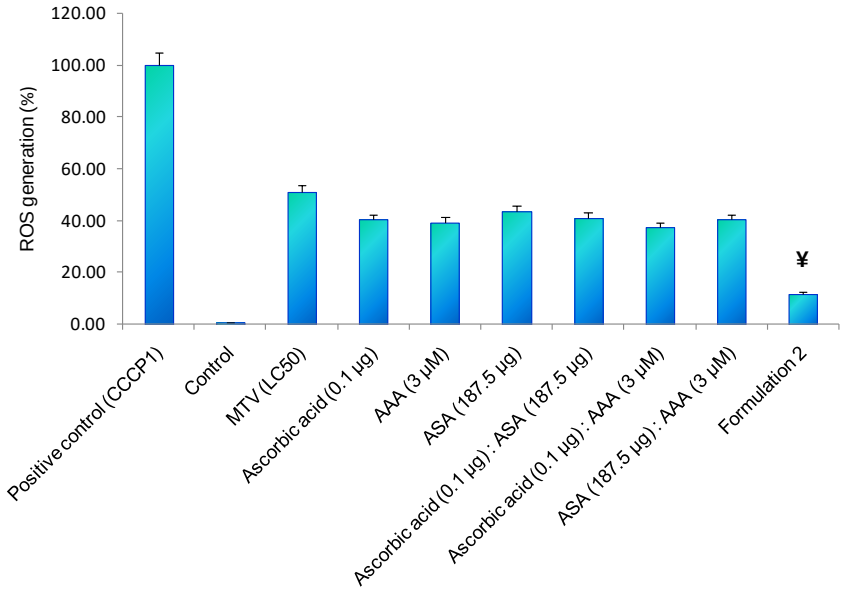
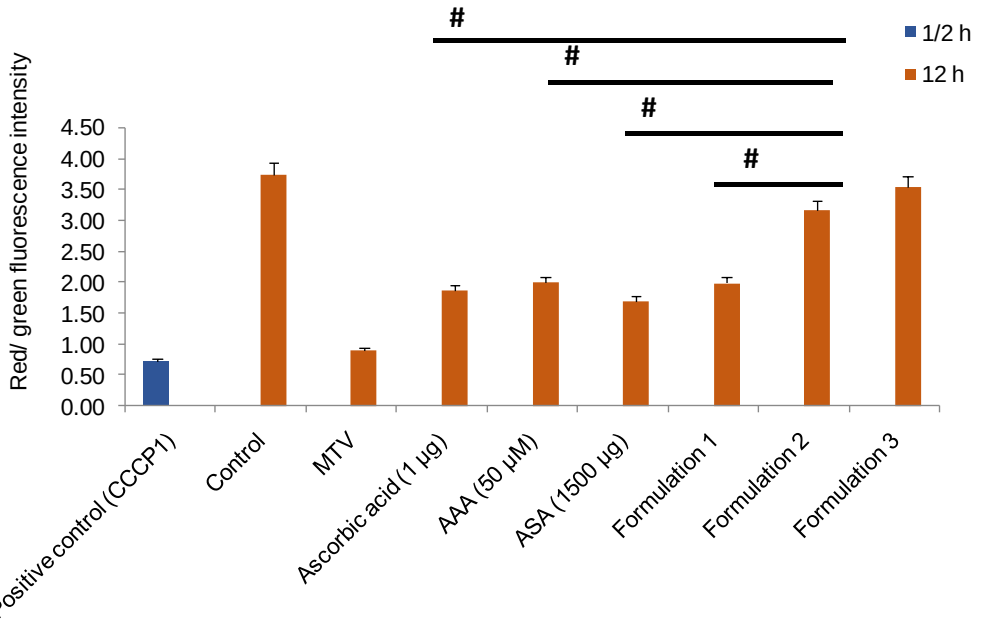


Fig. 6.6. MTV-induced ROS generation in *C. elegans* after 6 h of MTV (LC_{50}) treatment and its neutralisation by **(a)** Optimum dose of individual components of the formulation, their combinations and different concentrations of formulations, **(b)** Individual components of the formulation and their combinations compared with formulation 2. ROS level in the positive control (CCCP1) *C. elegans* was considered baseline (100%), and other values were compared. Fluorescence intensities were determined by ImageJ

software. Data represent ± SD of three Determination. In fig. 9a shows the significance of the difference compared to formulation 2, $^{\#}p \leq 0.05$. In fig. 9b shows the significance of the difference compared to formulation 2, $^{¥}p \leq 0.05$. There was no significant difference ($p > 0.05$) between formulations 2 and 3. (Abbreviations: LC: lethal concentration; ROS: reactive oxygen species; MTV: *M. tamulus* venom; CCCP1: Carbonyl cyanide 3-chlorophenylhydrazone 1)

Formulations 2 and 3 significantly decreased MTV-induced increase in the mitochondrial transmembrane potential of *C. elegans* compared to formulation 1 and the individual components of the formulations and their combinations (Fig. 6.7 a-b, Appendix fig. A6). However, there was no significant difference in potency between formulations 2 and 3 (Fig. 6.7 a).

a



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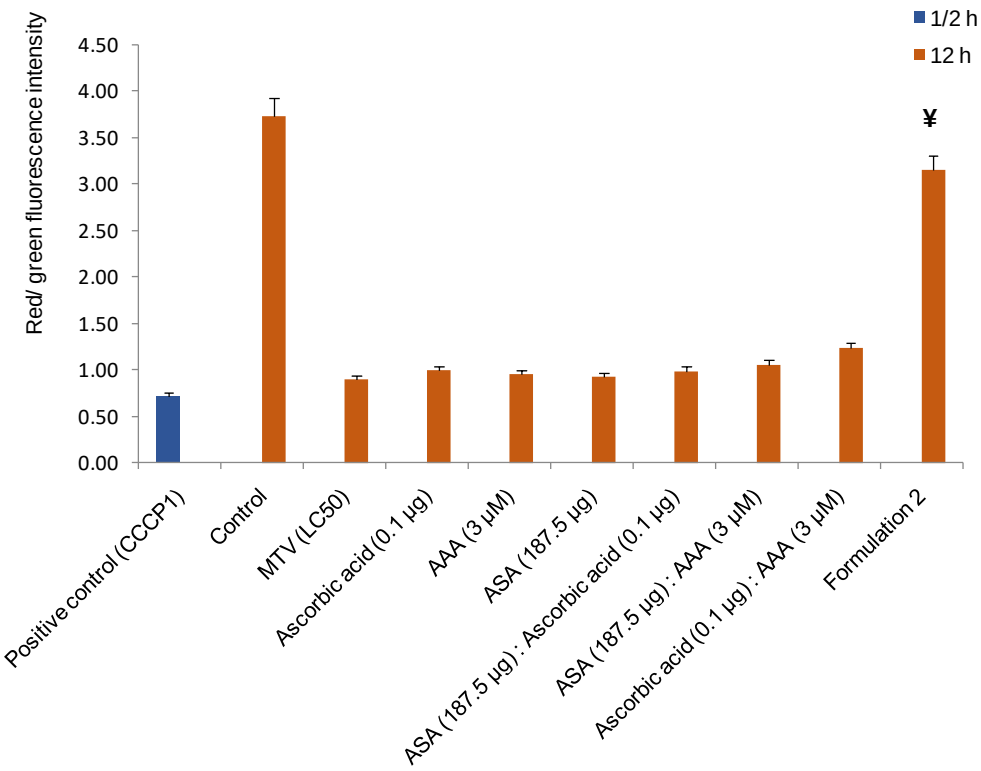


Fig. 6.7. The *in vivo* neutralisation of *M. tamulus* venom-induced (LC_{50} value) alteration of mitochondrial transmembrane potential in *C. elegans* by **(a)** Optimum dose of individual components of the formulation, their combinations and different concentrations of formulation, **(b)** Individual components of the formulation and their combinations compared with formulation 2. Alteration of mitochondrial transmembrane potential induced by positive control (CCCP1) in *C. elegans* was considered baseline (100%), and other values were compared. Fluorescence intensities were quantitated by ImageJ software. Data represent ± SD of three Determination. Significance of difference, $^{\#}p \leq 0.05$ as compared to formulation 2. In Fig. 6.11a, the significance of the difference compared to formulation 2, $^{\#}p \leq 0.05$. In fig. 6.11b shows the significance of the difference compared to formulation 2, $^{¥}p \leq 0.05$. There was no significant difference ($p > 0.05$) between formulations 2 and 3. (Abbreviations: LC: lethal concentration; CCCP1: Carbonyl cyanide 3-chlorophenylhydrazone 1)

6.1.6 Formulated drug restored the MTV-induced upregulation of genes involved in apoptosis, detoxification and stress response to delay MTV-induced programmed cell death in *C. elegans*

Because the composition of formulation 2 was found to be optimum for neutralising the MTV-induced toxic effects in *C. elegans*, further studies were done with these formulations. The expressions of ctl-1 and gst-6 (anti-oxidative genes) were significantly upregulated in MTV-induced *C. elegans* compared to the control (untreated). The expression of hsp60 (heat shock gene) was also upregulated considerably; however, expression of sod-3 and trx-1 was unchanged in MTV-treated *C. elegans* (Fig. 6.8). The qRT-PCR results showed that the MTV-induced expression of upregulated genes was downregulated to normal by formulated drug 2 (Fig. 6.8).

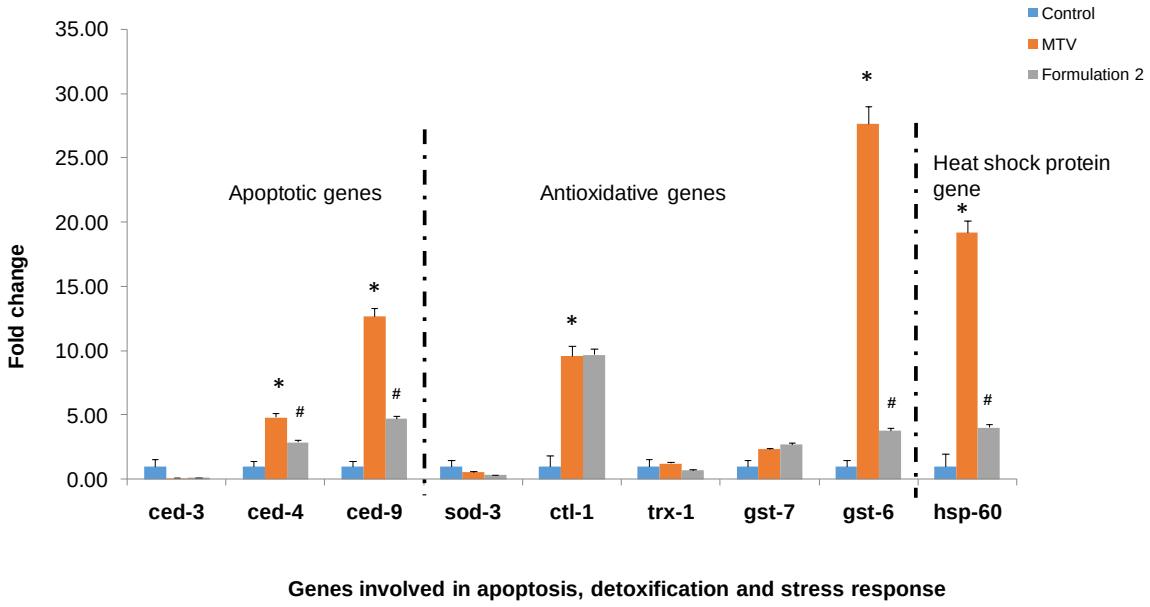


Fig. 6.8. Demonstrated the relative expression of MTV-induced *C. elegans* genes involved in apoptosis, detoxification and stress response compared to control (*$p \leq 0.05$) and improvement by treatment with formulation 2. Significance of difference as compared to MTV ($^{\#}p \leq 0.05$) (Abbreviations: MTV: *M. tamulus* venom).

6.1.7 Neutralisation of MTV-induced hyperglycaemia and pathophysiological symptoms, prolonged tail bleeding time, serum biochemical changes, and morphological alterations in Wistar strain albino rats model by drug formulation 2

As shown in Table 6, MTV-treated Wistar strain albino rats became physically inactive and almost paralysed after 24 h of treatment. They showed high urination, fast breathing, defecation, becoming thirstier, and weak grip strength. However, the formulated drug (formulation 2) restored the regular physiological activity of MTV-treated Wistar strain albino rats.

A significant increase (*$p \leq 0.05$) in the blood glucose level in MTV-treated rats compared to the control group of rats was observed post 30 to 120 min i.v injection of venom (Fig. 6.9 a). The AAA at its optimum dose (determined in *C. elegans*) markedly increased the glucose content at 30 to 120 min post-injection (Fig. 6.9 a). Moreover, the quantity of the combination (two components at a time) of formulation 2 did not effectively lower MTV-induced hyperglycaemia as compared to formulation 2 ($^{¥}p \leq 0.05$) in Wistar strain albino rat suggesting optimum composition of formulation 2 as the most effective formulation to diminish the hyperglycaemic effect (Fig. 6.9 a).

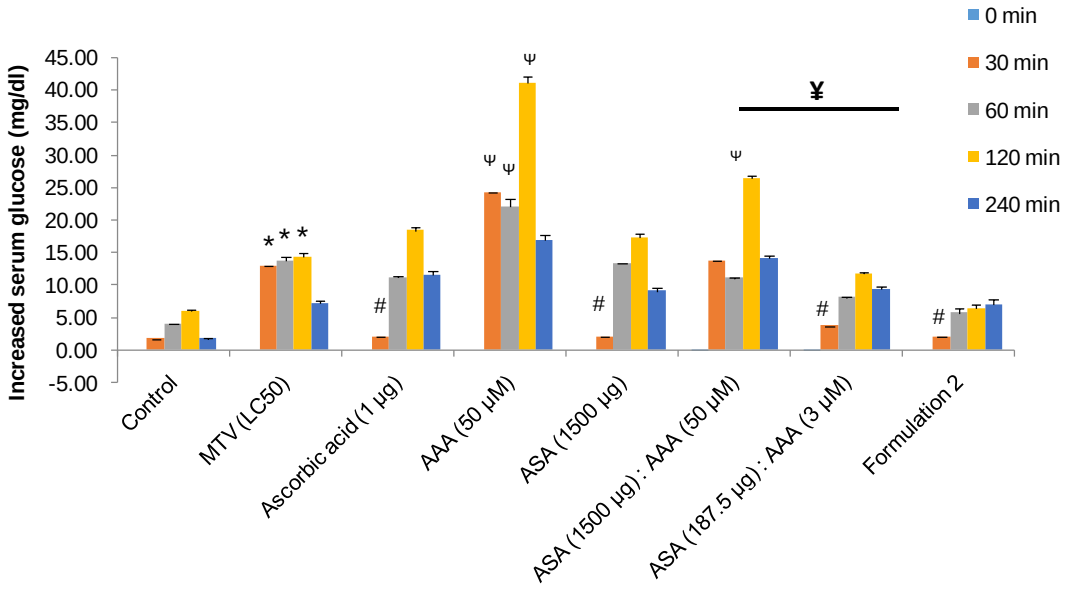
The tail bleeding time was also prolonged in Wistar strain albino rats post-treatment with MTV, which was neutralised by formulation 2 (Fig. 6.9 b). A similar effect was also observed when treated with an optimum dose of individual components and a combination dose of formulation 2 ($^{¥}p \leq 0.05$), the later showed lower potency in neutralising the MTV-induced prolonged tail bleeding time suggesting that formulation 2 and not its components are the most efficient in the treatment of *M. tamulus* sting (Fig. 6.9 b).

Table 6.6. Physical or behavioural changes in MTV-treated albino Wistar strain rat and their recovery by formulation 2 [ASA (187.5 µg), ascorbic acid (0.1 µg), AAA (3 µM)]. The symptoms were noted 24 h post-injection of MTV.

S. No.	Pathophysiological symptoms post MTV-treatment	Treatment with the formulation 2
1	High urination	Recovered

2	Fast breathing	Recovered
3	Defecation	Recovered
4	Become more thirsty	Recovered
5	Weak grip strength	Recovered

a



b

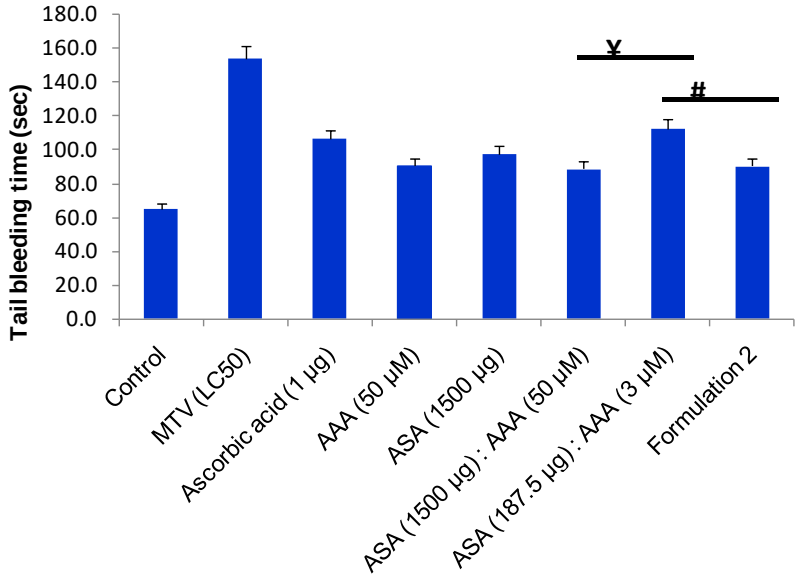


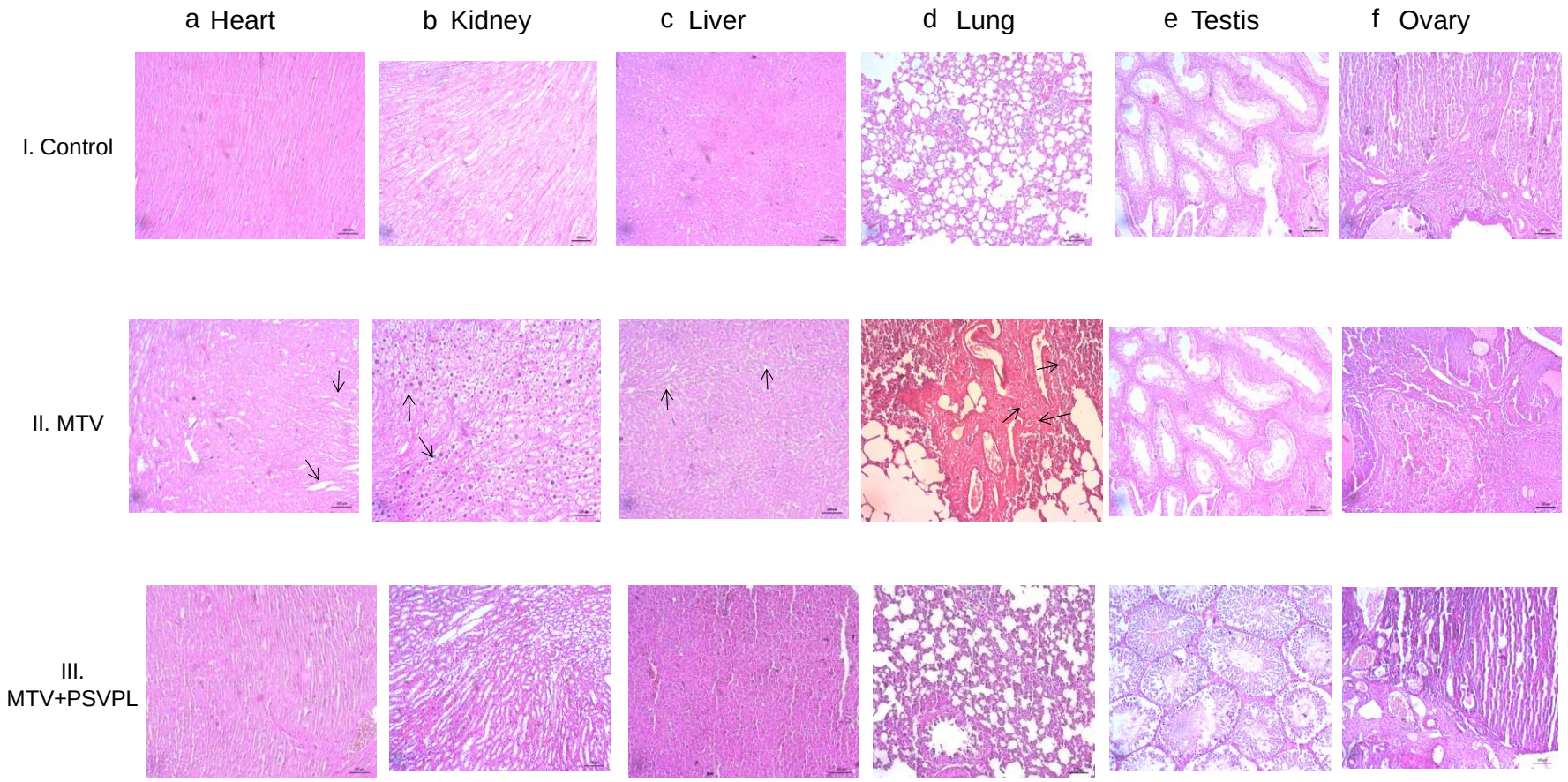
Fig. 6.9. a Illustrates the time-dependent increase in blood glucose content in *M. tamulus* venom (MTV)-treated (25 µg/ 200 g, i.v.) Wistar strain albino rats and its neutralisation by formulation 2, individual component of the formulation at their optimum dose (the dose of individual component where they showed best MTV neutralisation (LC_{50} value) potency in *C. elegans*, (Fig.3 b, c, d)) and combinations of commercial ASA and AAA against MTV (25 µg/ 200 g, i.v.) in Wistar rats. Significance of difference $*p \leq 0.05$, as compared to control; $^{\#}p \leq 0.05$, as compared to MTV; AAA (50 µM) and [AAA (50 µM): ASA (1500 µg] instead increase the blood glucose content in MTV treated rat, $^{\Psi}p \leq 0.05$. Significance of difference, $^{¥}p \leq 0.05$, between the combination doses [ASA (1500 µg): AAA (50 µM)] and [ASA (187.5 µg): AAA (3 µM)]**. b.** Increase in tail bleeding time (sec) in MTV-treated (25 µg/ 200 g, i.v) Wistar strain albino rats and its neutralisation by formulation 2, individual components of the formulation at their optimum dose and combinations of commercial ASA and AAA against MTV (25 µg/ 200 g, i.v.) in Wistar rats. Data represent ± SD of three determinations. Significance of difference $^{\#}p \leq 0.05$ compared to formulation 2. The significance of difference, $^{¥}p \leq 0.05$, between the combination doses [ASA (1500 µg): AAA (50 µM)] and [ASA (187.5 µg): AAA (3 µM)]. (Abbreviations: LC: lethal concentration; ASAs: anti-scorpion-antivenoms; AAA: α1-adrenoreceptor antagonist; MTV: *M. tamulus* venom).

The MTV-treated rats showed increased serum SGPT, ALKP, and creatinine levels compared to the control group of rats. The MTV-induced increased liver enzymes (ALKP and SGPT) and metabolic product (creatinine) were better reduced by formulation 2 (Table 7). Further, the study also suggested that formulation 2 is effective compared to individual components of the formulation for restoration of MTV-induced serum biological changes (Table 7).

Table 6.7. Neutralisation of *M. tamulus* venom (25 µg/ 200 g, i.v)-induced elevation in ALKP, SGPT, and creatinine level in the blood of rats (24 h post-injection) by formulation 2, individual components of the formulation at their optimum dose (the dose of individual component where they showed best MTV neutralisation (LC_{50} value) potency in *C. elegans,* (Fig.3 b, c, d)) and combinations of commercial ASA and AAA against MTV in Wistar rats (n=6). Data represent mean ± SD of three determinations. Significant difference between control and MTV, $^{\Psi}p \leq 0.05$; Significance of difference between MTV and the other components of formulation 2, $^{*}p \leq 0.05$.

S. No.	Components	ALKP activity (U/L)	SGPT activity (U/L)	Creatinine activity (U/L)
1	Control	108.00± 5.13	48.11± 2.40	0.27± 0.01
2	MTV (LC_{50})	180.50± 5.02 (Ψ)	75.77± 3.78 (Ψ)	0.44± 0.02 (Ψ)
3	ASA (1500 µg)	122.20± 3.45 (*)	39.90± 1.20 (*)	0.31± 0.01 (*)
4	Ascorbic acid (1 µg)	113.00± 5.31 (*)	45.66± 2.28 (*)	0.29± 0.01 (*)
5	AAA (50 µM)	121.50± 4.56 (*)	40.75± 2.03 (*)	0.33± 0.02 (*)
6	ASA (1500 µg): AAA (50 µM)	106.90± 4.96 (*)	42.00± 2.13(*)	0.30± 0.01 (*)
7	ASA (187.5 µg): AAA (3 µM)	130.00± 3.12 (*)	51.00± 2.55 (*)	0.39± 0.01 (*)
8	Formulation 2	102.71± 4.20 (*)	37.00± 1.85 (*)	0.28± 0.01 (*)

Histological analysis of the MTV-treated rats' hearts, kidneys, livers, and lungs revealed some gross morphological alterations (Fig. 6.10 a-f (i-iii)). MTV-induced heart muscle showed massive deleterious degeneration, and almost no intact muscle tissue remained (Fig. 6.10 a (i-ii). The kidney showed some black spots on the tissues along with necrosis (Fig. 6.10 b (i-ii), and the liver also suffers from tissue necrosis (Fig. 6.10 c). Inflammation with pulmonary oedema was also observed in MTV-induced lung tissues filled with proteinaceous fluid (Fig. 6.10 d (i-ii)). Ovary and testis of the MTV-induced rat did not show any morphological change compared to the control (Fig. 6.10 e-f, i-ii). The toxic effect of *M. tamulus* venom in a rat model was neutralised when treated with formulation drug 2 (Fig. 6.10 a-f, iii).


a Heart
b Kidney
c Liver
d Lung
e Testis
f Ovary
I. Control
II. MTV
III. MTV+PSVPL

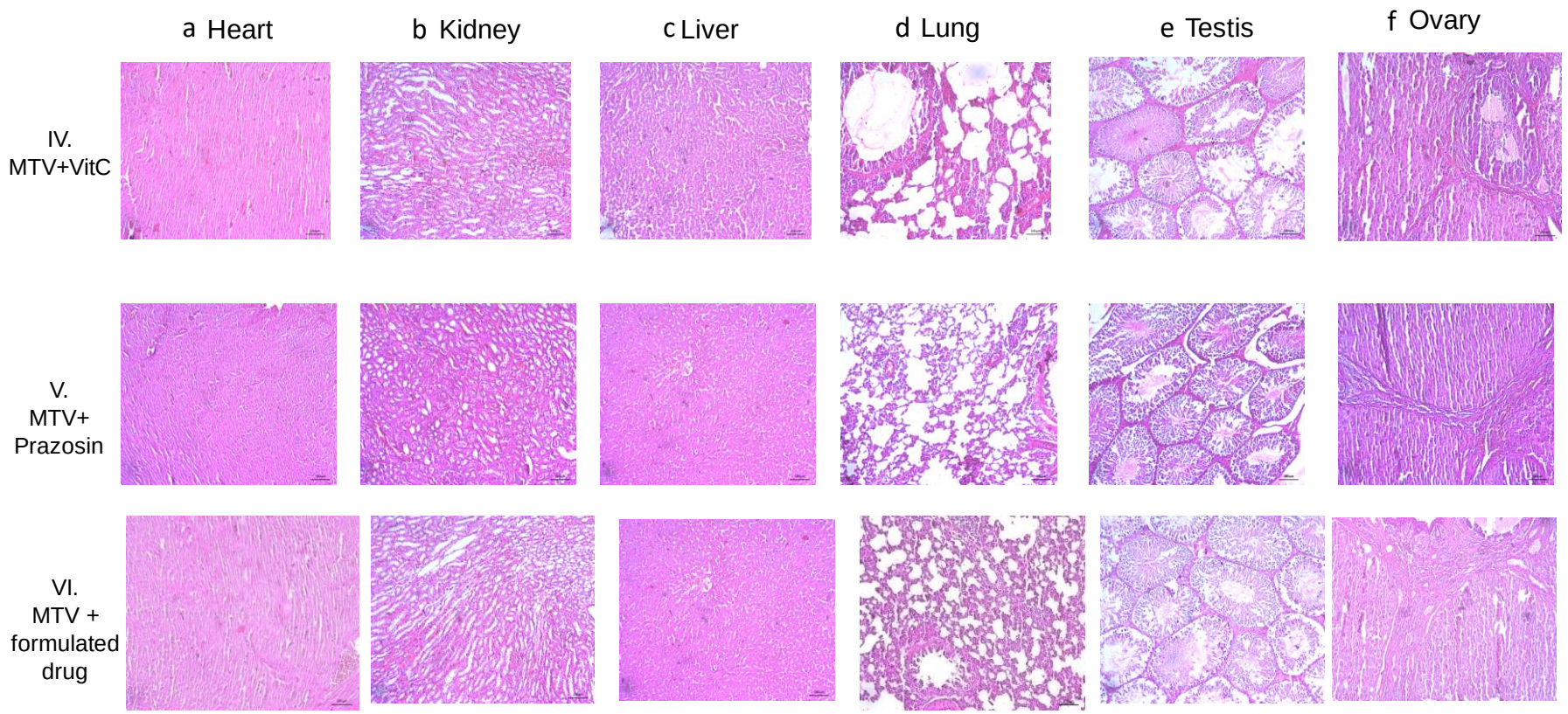


Fig. 6.10. Histopathological analysis of the *M. tamulus* venom-induced Wistar rat tissues and their neutralisation by formulation 2. Light microscopic observation of a) Heart, b) Kidney, c) Liver, d) Lung, e) Testis and f) Ovary from control and treated groups, Bar-100µM. The black arrow indicates the morphological changes observed in MTV-induced rat tissue compared to the control.

6.1.8 Decrease of pro-inflammatory cytokines in MTV-treated Swiss albino mice

The level of pro-inflammatory cytokines in the MTV-treated Swiss albino mice was significantly decreased compared to the control mice (Fig. 6.11).

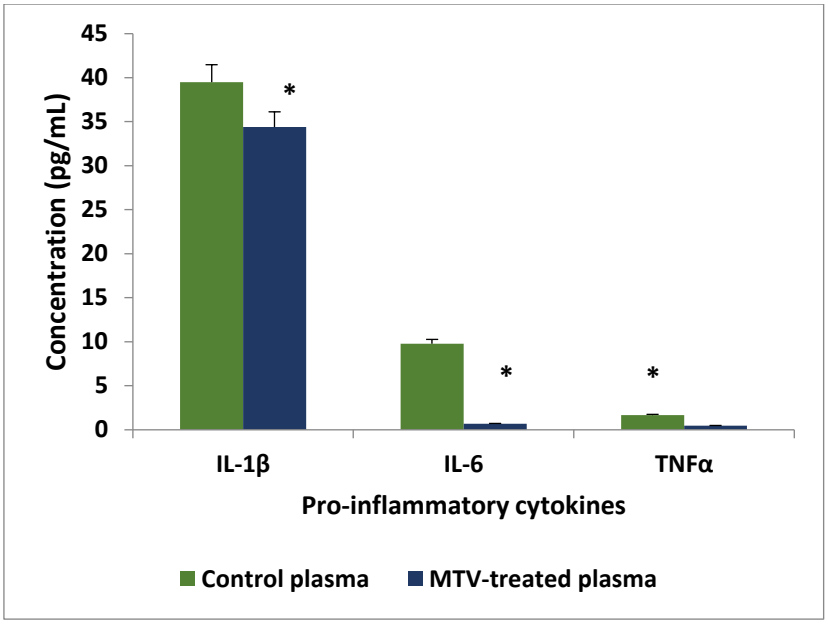


Fig. 6.11. Circulating levels of pro-inflammatory cytokines in the Swiss albino mice with and without *M. tamulus* envenomation, MTV-treated plasma and control plasma, respectively. Significance of difference, *p≤0.05 as compared to control

6.2 Discussion

In vivo, toxicity studies with mammalian models are expensive [1; 2] and have ethical concerns. Nevertheless, the prediction of toxicity study can be augmented by using more than one mammalian species, but it will also increase the cost and moral significance and decrease throughput [3]. To overcome these limitations, for studying the toxic effects of scorpion venom and screening the venom neutralisation potency of drugs, *C. elegans,* a tiny non-parasitic nematode, is one of the best-established *in vivo* models that have contributed significantly to understanding numerous human diseases such as neurodegenerative disease, diabetes, etc [4; 5]. It also serves as a model organism for genetic studies on the aging process, age-related illnesses, mechanisms of longevity, and

screening for compounds that increase lifespan [4; 6]. Notably, *Caenorhabditis elegans* is a prominent in vivo model for neuronal research because of its wide acceptance in understanding the evolution of neural lineages and neuronal differentiation. The benefits of using *C. elegans* as a model organism include its transparent body, small size, short life cycle (about three days at 20 °C), ability to self-fertilize, high reproductive rate (> 300 offspring per hermaphrodite), and prominent and well-developed nervous system [7; 8; 9; 10]. As a result, they are a commonly used model organism for neuronal research. They have a higher ethical value for utilising laboratory experimental animals than do experimental rats, and they don't need any space to grow. They also require easy and affordable maintenance. Different studies throughout the globe have already shown that *C. elegans* is a good choice for studying the neurotoxic effects of toxic chemicals (such as pesticides) [11; 12].

Considering the above, in our study, *C. elegans* has been used as a model organism to study the neurotoxic effects of MTV and the screening of the neutralization potency of a novel therapeutic formulation against MTV-induced toxicity. The formulated drug has successfully inhibited the mammalian pathophysiology associated with neurotoxic symptoms, such as free radicals scavenging, ROS inhibition, and inhibition of depolarization of MMP, resulting from MTV envenomation. However, we agree that *C. elegans* is not the final model for establishing the therapeutic efficacy of drug molecules, instead of that our aim of the study is to find an alternative *in vivo* screening model to solve the ethical issue associated with the animal experiment and also to follow 3R model (Replacement, Reduction, and Refinement of tested animals) suggested by WHO to reduce the animal experiments. Further, the best-screened formulation was validated in rodent models where the formulated drug has shown superior efficacy compared to the individual components of the same drug to neutralize the toxicity of Indian red scorpion venom, thus establishing the suitability of C. *elegans* model to screen drugs against MTV. This *in vivo* model may also be used to screen drugs against other scorpions and neurotoxic venoms.

The hyperactivity of α-adrenergic receptors by scorpion venom toxins leads to cardiovascular effects with a first phase of transient cholinergic hyperfunction, followed by dose-dependent adrenergic hyperactivity characterized by hypertension, tachycardia, and alteration of myocardial function. Along with the cardiovascular effect, hyperactivity of the α-adrenergic receptor activates the neurotransmitter nor-epinephrine and the

neurohormone epinephrine, also known as catecholamines. The massive release of sympathetic (catecholamine) post-scorpion sting results in a mixed neuroexcitatory syndrome [13; 14]. Therefore, assessing the neurotoxicity related pathophysiology such as ROS generation and disruption of mitochondrial membrane potential of scorpion venom in *C. elgans* has a rationale. However, there are some limitations to using *C. elegans* as a model organism, such as studying scorpion sting-induced cardiovascular alterations. Nevertheless, *C. elegans* also exhibit limitations as they lack many mammalian organs such as eyes, lungs, heart, kidney, and liver and are devoid of an adaptive immune system. Despite these, they are considered good toxicity testing models that help bridge between *in vitro* assays and mammalian toxicity studies [15]. Therefore, the scorpion venom-neutralizing potency of screened drug molecules must be validated in the rodent model.

Notably, *C. elegans* also poses a homologous receptor of the mammalian α1-adrenergic receptor, SER6 [12] which is demonstrated by *in silico* analysis in this study. SER6 is an octopamine receptor, and its stimulation causes an octopaminergic signal that involves an array of neuropeptides that activate receptors and induce a cAMP response in the CNS thus show pathophysiology associate with neurotoxicity such as ROS generation, and disruption of mitochondrial membrane potential [11; 16].

Therefore, at the outset, we tested the MTV neutralisation potency of formulated drug and individual components of the formulation in *C. elegans* to save the experimental animals. This study was followed by determining the MTV toxicity neutralising potency of the formulated drug in a rodent model (Wistar strain albino rats).

Stimulation of α1-adrenergic receptors (a classic post-synaptic α-receptor found on vascular smooth muscle) by scorpion venom α-neurotoxins induce hyperkalemia and hyperglycemia, accumulation of free radicals in the myocardium, and coronary spasm and thus initiating lethal ventricular arrhythmias and sudden death [17]. Most clinical manifestations post scorpion sting envenomation result from the massive release of neurotransmitters such as catecholamines leading to autonomic storm [18; 19]. Sepsis, a multiorgan dysfunction condition in extreme case, is caused by a combination of immune cell malfunction (macrophages, neutrophils, and lymphocytes), endothelial cell dysfunction, and epithelial cell failure. ROS and reactive nitrogen species (RNS) both contribute considerably to these cells' dysfunction during sepsis [20]. The balance of pro- and anti-inflammatory activity determines the intensity and degree of inflammation, which

results in various clinical outcomes. Cytokine imbalances induced by scorpion venom toxins play a role in developing organ damage and fatality during severe sepsis. Although the sepsis induced by scorpion sting cannot be assessed in *C. elegans*; however, ROS generation and alteration of mitochondrial transmembrane potential, the primary causes of sepsis, can be determined in *C. elegans*. We have demonstrated that the formulated drug effectively inhibits ROS production and restore the disruption of MMP. For management of sepsis, along with the antivenom treatment, antibiotic treatment is suggested [21; 22; 23]. Further, few studies have also reported that use of prazosin to mitigate the sepsis in scorpion stinged patients. Furthermore, a histopathological study in rodent models has shown a deleterious effect of MTV in different organs of rats and its effective neutralization by formulated drug, much better than the individual components of the drug. The studies of marker enzyme in the serum of MTV-treated rat showed increase of the SGPT, ALKP, and creatinine indicating damage in liver and kidney. Our formulated drug restore the level of marker enzyme significantly higher than the individual components. Nevertheless, an in-depth study into the mechanism of MTV-induced sepsis and its neutralization is utmost needed.

Hyperglycemia, one of the scorpion venom-induced symptoms, is caused by an increase in counter-regulatory hormones that inhibit gluconeogenesis and glycogenolysis, leading to respiratory failure, multisystem organ failure, pulmonary oedema, and increased mortality rate [24; 25; 26; 27]. Prazosin, a post-adrenergic receptor blocker with 1000-fold more affinity to the α1-adrenergic receptor, has been clinically used with commercial ASAs to reduce a post-scorpion sting's pharmacological and toxic effects [17].

Before initiating the wet lab experiment, the *in silico* analysis was done to predict whether or not the AAA could bind to the SER6 receptor of *C. elegans*. The computational analysis predicted the efficient binding of AAA with the homologous SER6 receptor indicating that *C. elegans* is a good model for assaying the effect of AAAs against scorpion venom. Our present study revealed that all tested AAAs showed more efficient neutralisation than commercial ASA against MTV, which is in close agreement with the previous clinical data showing prazosin as an effective drug compared to scorpion antivenom [28].

Scorpion venom induces apoptosis and cell death by elevating reactive oxygen species (ROS) [29]. Thus, this study has presented evidence that *MTV* also generates free radicals, which induce the production of ROS, the oxidative stress response, and alter the

mitochondrial trans-membrane potential in *C. elegans*. Our present study revealed that MTV-induced toxicity (increased ROS production and depolarisation of mitochondrial transmembrane potential) was better neutralised by AAA than commercial ASA.

Commercial ASA generally neutralizes scorpion venom toxicity [30; 31]. However, our previous study has revealed that a high concentration of ASA (1:60; venom: antivenom) was required to neutralise (*in vitro*) the toxicity of MTV; the reason may be due to the presence of a low abundance of venom-specific antibodies (5.36%-6.29%) in commercial ASAs [32]. Due to their insufficient venom-specific antibodies and some adverse side effects (e.g. hypersensitivity, anaphylactic reactions, serum sickness, etc.) [33], the commercial ASA may prevent scorpion sting patients from recovering completely, suggesting improving scorpion sting management protocol. Besides, prazosin at higher concentrations used to treat Indian red scorpion stings also induces hyperglycemia [34]. Our present study also evidenced that MTV-induced hyperglycemia was not reduced when treated with prazosin-HCL (50 µM); instead, it raised glucose levels in rat serum. The sudden increase in blood glucose can produce a hyperglycemic shock in the patients. It is a severe life-threatening problem for diabetic patients.

The therapeutic advantages of early administration of AAAs, or ASA, for effective treatment of scorpion stings and consequently saving a patient's life are reinforced in this study. ASA is only sometimes available in most rural health centres in developing countries, and patients must travel a long distance to get the treatment [35]. Therefore, based on the results of these studies, we proposed that immediate administration of ascorbic acid can delay the onset of pharmacological effects post-scorpion envenomation, and the patient can get sufficient time to reach a hospital where an envenomation treatment facility is available. In this study, both the models (*C. elegans* and Wistar strain rats) demonstrated improved neutralisation efficiency of the formulated drug 2 (composed of ASA, AAA and ascorbic acid) against MTV-induced toxicity by increasing the percent worm survivability rate, lowering free radical production in *C. elegans.* We showed that MTV causes upregulation of genes involved in apoptosis, detoxification, and stress response in *C. elegans* after 24 h of MTV treatment. The formulated drug stored this upregulation of *C. elegans* genes mentioned above.

Scorpion venom causes hyperglycemia via different mechanisms such as massive catecholamine release, glucagon release, and glucocorticoid release, affecting renin–

angiotensin–aldosterone system, causing hyperinsulinemia, and cytokines release, etc. [36; 37]. However, the precise mechanism of MTV-induced hyperglycemia is unknown. In our present study, drug formulation 2 also envisaged reducing MTV-induced hyperglycemia in Wistar strain albino rats, much better than commercial ASA or ascorbic acid. It also improved the pathophysiological changes and altered tissue morphological deformation caused by MTV toxicity in Wistar strain albino rats. With formulation 2, insulin treatment may not be required for hospital management of scorpion sting patients. Moreover, the bleeding time evaluates the function of platelet.

Clinical reports of MTV-induced rat serum indicated a significant increase of liver enzymes such as ALKP and SGPT; creatinine (an indicator of kidney function) after 24 h post-treatment. However, the SGOT (ALT) level did not change compared to the control group. Studies have shown the rise in serum levels of SGOT, SGPT (AST), and/or glucose, cholesterol, SGOT, SGPT, uric acid, bilirubin, and urea in experimental animals post-treatment with scorpions (*Hemiscorpius lepturus, Odonthobuthus doriae, Androctonus crassicauda,* and *Palamneus gravimanus)* venom samples [38; 39; 40; 41]. In this study, formulated drug 2, compared to its components, has also shown an impressive result in lowering MTV-induced increased biochemical parameters such as ALKP, SGPT, and creatinine in Wistar strain albino rats suggesting its therapeutic application in treating MT sting.

MTV-treated rats showed histological symptoms, including tissue necrosis, oedema, and muscular injury observed in tissue such as the heart, kidney, liver, and lung. In our study, myocardial damage was observed in rat heart muscle. Scorpion venom-induced myocardial damage has also been reported as one of the major clinical manifestations that may lead to death [42]. The present study also revealed the disorganization of liver tissue with a necrotic lesion; many experimental studies have also reported histopathological changes in the liver caused by scorpion sting envenomation which may lead to toxic hepatitis and coagulopathy [43]. Pulmonary oedema is a common and significant systemic clinical manifestation, which was also observed in MTV-treated rat lung tissue [44]. These morphological alterations were diminished after treatment with the most effective formulated drug 2, which helped to reverse the imbalance caused by MTV without causing adverse reactions.

Many cardiogenic and non-cardiogenic factors are involved in the pathogenesis of acute pulmonary oedema after scorpion stings [45; 46]. But no such effect was observed on MTV-induced rat testis and ovary tissue. The study of *in vivo* neutralization of MTV-induced toxicity by the formulated drug in *C. elegans* was well correlated with results with the Wistar strain albino rats model, supporting the proposed hypothesis that *C. elegans* can also serve as a model to screen the antidotes against scorpion venom.

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