CHAPTER IV

PROTEOMICS ANALYSIS, BIOCHEMICAL AND PHARMACOLOGICAL CHARACTERIZATION AND IMMUNOLOGICAL PROFILING OF INDIAN RED SCORPION (*Mesobuthus tamulus*) VENOM

4.1 Results

4.1.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of *M. tamulus* venom (MTV)

The SDS-PAGE analysis (non-reduced and reduced) of crude MTV shows the occurrence of toxins (proteins and polypeptides) in the molecular mass range between 3 and 140 kDa (Fig. 4.1). Nevertheless, the densitometry analysis of the SDS-PAGE gels showed that the proteins and polypeptides in the mass range of 3 to 12 kDa are predominant (~80%) in this venom under reducing conditions comprising of 10 gel sections (Fig. 4.1).

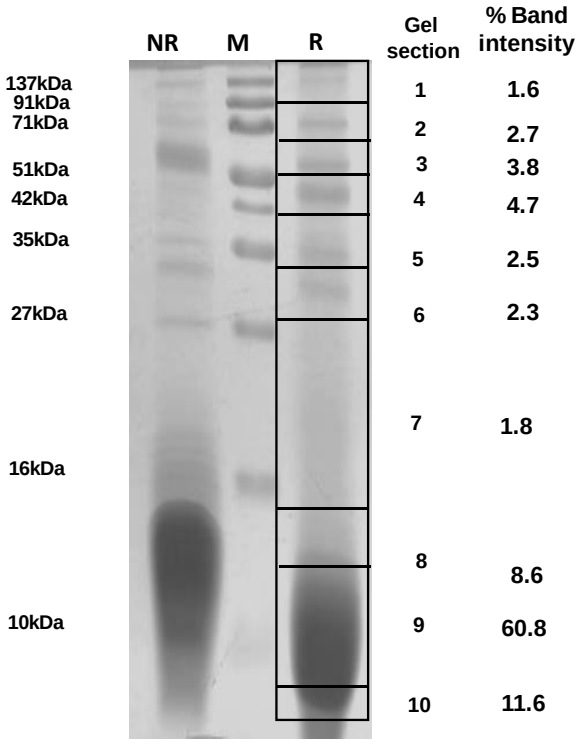

Fig. 4.1. 15% SDS-PAGE analysis of MTV under non-reduced and reduced conditions. Lanes M contain protein molecular markers. Lanes 1 and 2 contain crude MTV (80 µg protein) under non-reduced and reduced conditions, respectively. The per cent band intensity was determined by densitometry analysis of gel.

4.1.2 Tandem mass spectrometry analysis of tryptic peptides

The tandem mass spectrometric analysis of in-gel tryptic peptides from 10 gel sections of MTV (Fig. 4.1) resulted in the identification of approximately 700 proteins in this venom. These proteins were further screened and analyzed to eliminate false identifications, leading to the unambiguous identification of 110 scorpion venom proteins belonging to 13 toxin families (Fig. 4.2, Appendix table A1). The alignment of tryptic peptides derived from LC-MS/MS analysis with the primary structures of the identified toxins is shown in Appendix fig. A1. The LC-MS/MS analysis revealed that MTV is predominately comprised of ion channel toxins making up >80% of the venom proteome. The Na^+ and K^+ ion-channel toxins accounted for 38.4% and 38.2% of the venom proteome, respectively (Fig. 4.2, Appendix table A1).

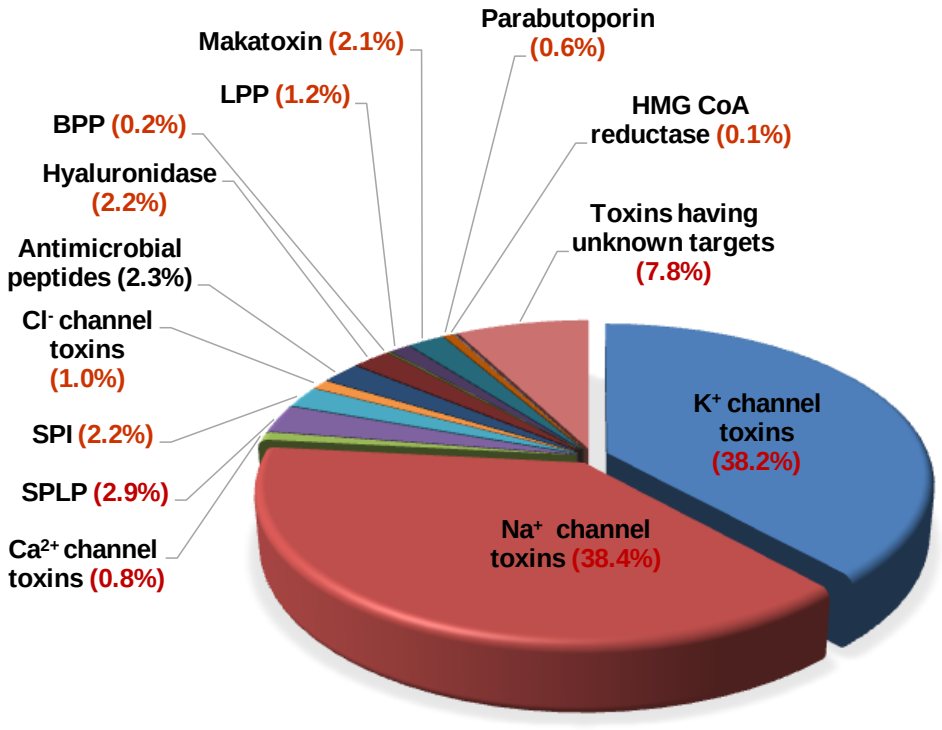

Fig. 4.2. Protein family composition of MTV. The relative abundance of different venom protein families is expressed as an average of relative abundances calculated using MS1 (summed peptide-spectrum Match Precursor Intensity) based on label-free quantitation techniques.

4.1.3 Some enzymatic activities and pharmacological properties of MTV

MTV, under *in vitro* conditions, did not show pharmacological and enzymatic activities. Although the proteomic analysis identified the presence of an serine protease-like protein (SPLP) in this venom (Fig. 4.2, Appendix table A1), it did not show protease activity or influence the clotting time of PPP, presumably because this peptide differs from the conventional snake venom serine proteases that have protease activity and can influence the plasma clotting time [1]. In addition, the low relative abundance of this SPLP (2.9%) in MTV may explain why it did not show enzyme activity. Although hyaluronidase was identified with the LC-MS/MS analysis, the crude venom did not show any hyaluronidase activity by the biochemical analysis. Nevertheless, LC-MS/MS did not identify metalloproteinase, and the biochemical analyses of MTV suggest that this venom lacks metalloproteinase. Thus, the scorpion venom in this study does not influence the haemostatic system of victims and exerts its toxicity mainly via the plentiful ion-channel toxins, which is also well correlated with published clinical data for MT sting [2; 3; 4; 5].

4.1.4 Correlation of MTV toxinome composition with the clinical manifestations of scorpion sting in India

The impact and treatment of scorpion stings have always been neglected, and an in-depth, comprehensive study has not yet been undertaken to correlate the composition of venom toxins with the severity of pathogenesis and clinical manifestations post-MT stings [6]. Among 86 poisonous species of scorpions found in India, MTV is the most lethal, causing fatalities in children and adults [7]. Scorpion stings may cause local reactions such as oedema, intense pain, erythema, and itching [7]. The systemic effects of a sting include cardiovascular disturbances (bradycardia, tachycardia, hypertension, hypotension, and cardiogenic shock), pulmonary edema, systemic circulation (increased cytokines, nitric oxide/ histamine release), hyperglycemia, and priapism in male children [8; 3; 9; 10]. The correlation between clinical symptoms of MT stings and venom components responsible for the pharmacological activity is shown in Table 4.1. The effect of venoms from different scorpion species on the host immune system has been described, [11] though the effect of MTV toxins on the immune system of sting victims is largely unknown. More investigation is needed.

Table 4.1. Correlation between MTV proteome composition and clinical manifestations following a sting.

Sl. No.	**Accession no.**	**Protein name**	**Relative abundance in venom**	**Function**	**Symptoms and clinical manifestations of sting**
1	P0DJ47, P0DJ49	Kunitz-type serine protease inhibitor	2.2%	Stops (prevent or reduce) the activity of serine-type endopeptidases	Acute pancreatitis [12; 13; 14]
2	Q8I6X9	Ca^{+} channel toxin	0.8%	Decrease intracellular calcium, thus inhibiting the contraction of pulmonary artery smooth muscle cells.	Pulmonary hypertension [14; 15]
3	Q9NJC4 P59354	Na^{+} channel toxin (α-neurotoxins)	21.4%	Binds to site 3 of Na^{+} channel, respectively and slows the inactivation of Na^{+} channel that stays open during sustained membrane depolarization.	Subsequent depolarization was shown to increase Ca^{2+} influx via voltage-dependent calcium channels, thereby triggering Catecholamine release results in autonomic storm characterized by transient parasympathetic stimulations (vomiting, sweating, salivation, bradycardia) and prolonged sympathetic stimulations (hyperkalaemia, vasoconstriction) that

					leads to tachycardia, pulmonary oedema [16; 4; 10; 17].
4.	Q9UAC8 P0CF76	Na^+ channel toxin (β-neurotoxin)	1.9%	Binds to site 4 of Na^+ channel and shifts the voltage dependence of activation towards more hyperpolarized potential, thus delayingthe recovery from inactivation.	They inhibit the ion flow by blocking the action of Ach or more release of acetylcholine resulting in cholinergic toxicity, cardiovascular abnormalities, and respiratory paralysis [4; 17].
5	P82815, Q86BW9, P59853	Bukatoxin andMakatoxin (α-neurotoxins)	2.3%	Causes a persistent Na^+ channel activation in nitrergic inhibitory fibres resulting in NO release.	Cavernosal smooth muscle relaxation leading to priapism in male children[3; 18; 19].
6	Q17231	Depressant insect beta-toxins.	8.51%	Bind to site-4 of sodium channels (Nav) and shift the voltage of activation toward more negative potentials.	Affecting sodium channel activation and promoting spontaneous and repetitive firing that causes a transient contraction paralysis followed by a slow flaccid paralysis [20].
7	Q8I0K7 M4GX67	Depressant scorpion toxin	6.42%	All ion channels impair toxins (structurally similar to α-toxins but which are inactive in mammals).	Suppress evoked action potentials due to a strong depolarization of the axonal membrane that produces clinical symptoms of progressive flaccid paralysis [21; 5].

8	K7XFK5, E4VP04, Q967F9	K^+ channel toxin.	37.55%	Blocks the activity of potassium channel.	An imbalance of K^+ current in the heart resultsin cardiac arrhythmias [22].
9	Q9TWD3 Q9Y0X4	Bradykinin-potentiating peptide.	0.15%	Potentiate the action of bradykinin.	Inhibition of the angiotensin-converting enzymeshas a hypotensive effect [23; 24].

4.1.5 Assessment of immunological cross-reactivity between MTV and commercial scorpion antivenom by ELISA and immunoblot analyses

The immune-cross-reactivity of commercial ASAs against MTV was evaluated with ELISA and immunoblot analyses. The EC_{50} value of commercial ASAs manufactured by PSVPL and the Haffkine Institute was found to be 101.31± 4.45 µg/mL and 74.6± 3.13 µg/mL, respectively, a difference that is not statistically significant (P>0.05) (Fig. 4.3a). These two antivenoms also did not show a significant difference (p>0.05) in their potency to immune-recognize various toxins of MTV (Fig. 4.3 b) with themimmunoblotting method. In absence of similar data with other scorpion venom, it could not be compared.

The immune-blot analysis demonstrated that toxins of molecular weight >15kDa were better immune-recognized than were the low molecular mass toxins (3-15 kDa) from this venom using commercial antivenom (Fig. 4.4b, c). Most MTV toxins are in the mass range of 3 to 15 kDa. Commercial scorpion antivenom does not contain an adequate titer of antibodies against the most abundant low molecular mass toxins of MTV (Fig.4.3 b, c).

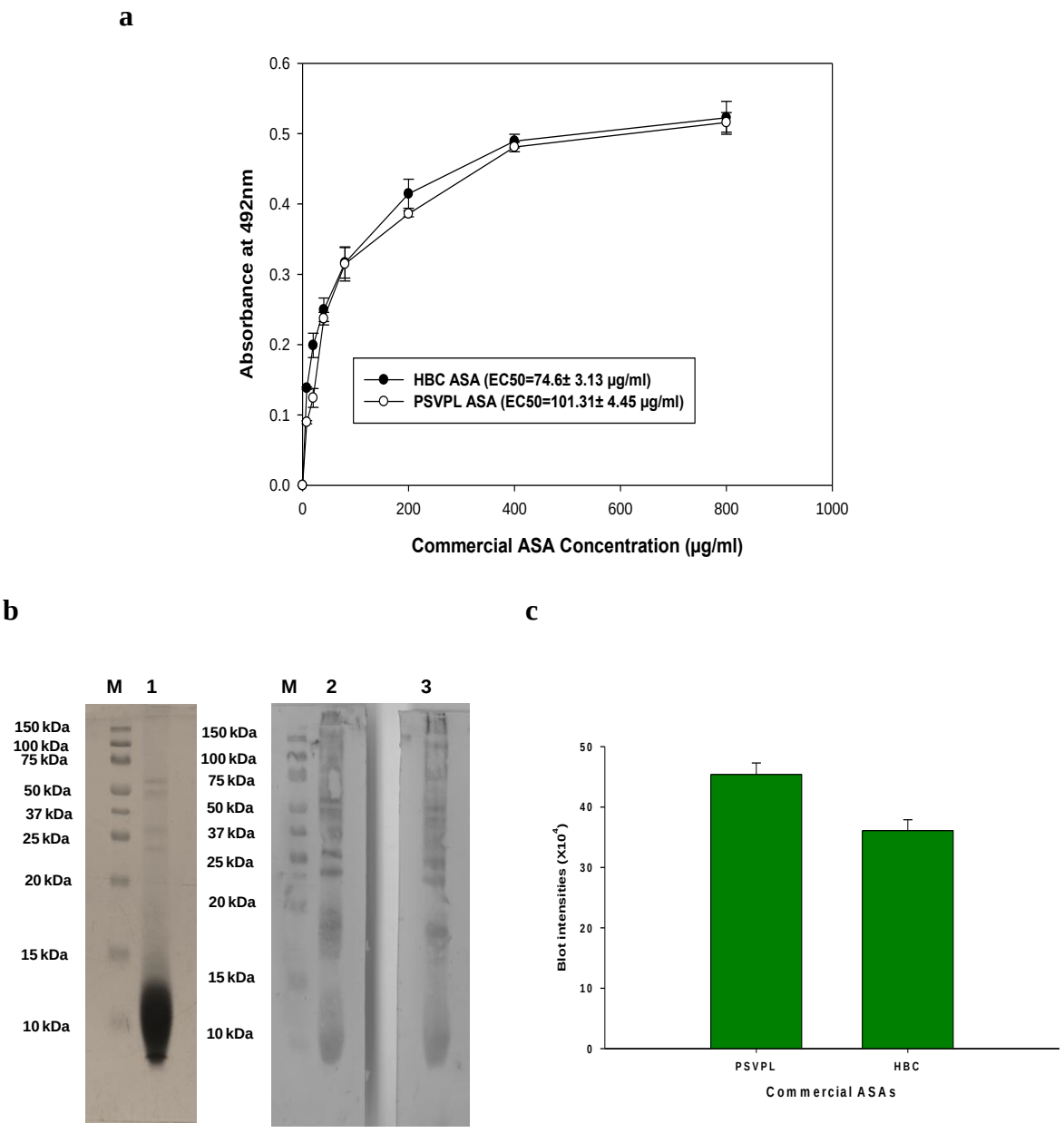

Fig 4.3. Assessment of immunological cross-reactivity of MTV against commercial scorpion antivenom by ELISA and immunoblot analysis. **a.** Immunological cross-reactivity of MTV against commercial ASAs (PSVPL and HBC) by ELISA; **b.** Immunoblot analysis. Lanes M and 1 contain protein molecular markers and MTV (80 µg protein, reduced), respectively. Lanes 2 and 3 represent blots of MTV immune-detected by commercial ASAs produced by Haffkine Institute and PSVPL, respectively;

c. Densitometry analyses of whole blot intensities of MTV detected by commercial ASAs.Values are shown as mean ± SD of triplicate determination.

4.2 Discussion

4.2.1 The venom proteome of *M. tamulus* is predominately comprised of low molecular mass ion-channel peptides

The pharmacological properties and toxicity of venom are influenced by the qualitative and quantitative occurrence of different toxins responsible for exerting a wide variety of biological activities, either alone or in unison. The median lethal dose (LD_{50}, s.c. injection) of MTV in young and adult rats was determined to be 1.3 ± 0.14 mg/kg and 2.2 ± 0.21 mg/kg, respectively [25]. The occurrence of 3-140 kDa ranged toxins (proteins and polypeptides) found in MTV was also observed in Russell's viper venom proteins, [26] suggesting the occurrence of multimeric proteins, and/or interactions between MTV proteins.

De-complexion of venom proteins before LC/MS-MS analysis is a prerequisite for better separation and proteomic identification of many proteins. Different strategies for protein de-complexation; for example, liquid chromatographic separation techniques (gel-filtration chromatography, ion-exchange chromatography, RP-HPLC) and SDS-PAGE separation are commonly used for the separation of venom proteins [27; 28; 29; 30]. Moreover, the separation of venom proteins by SDS-PAGE analysis has provided reliable proteomic results and is often used in our laboratory and by other toxinologists worldwide to decipher the venom proteome composition [31; 32; 29]. The method is economical, easy to perform, and requires less venom.

The result of LC-MS/MS analysis correlates well with previous data on the proteomic analyses of scorpion venoms like those from *Mesobuthus martensii* and *Hetrometrus longimanus* where the Na^+ and K^+ ion-channel toxins comprise the most copious non-enzymatic protein families of scorpion venom [33; 34].

The Na^+ channel toxins, responsible for the neurotoxic effects of scorpion stings, are classified as α-type and ß-type toxins, according to their sensitivity to the voltage changing post binding to the target. The Na^+ channel scorpion toxins may also show a

target specificity towards mammals and/or insects and may also show activity against other organisms [35]. The proteomic analyses identified both α- and ß-type toxins in MTV. The K^+ channel toxins (KTxs) are short peptides comprised of 30-40 amino acids. Some of these KTxs demonstrate a very high affinity towards one or more subtypes of K^+ channels [36]. Based on their sequence homologies, 3D folding pattern, and activity, the KTxs are classified into six families [37]. The proteomic analysis showed that MTV is predominately comprised of α-KTxs (Table 4.1), which are considered as the most important toxins. They contain about 200 peptides [36; 37]. In addition, β-KTxs and γ-KTxs were identified in MTV.

The other minor toxin classes of the MTV proteome with less relative abundances (as determined by proteomic analysis) are SPLP (2.9%), serine protease inhibitor (SPI) (2.2%), antimicrobial peptide (2.3%), hyaluronidase (2.2%), makatoxin (2.1%), lipolysis potentiating peptides (LPP) (1.2%), Cl^- channel toxin (1%), parabutoporin (0.6%), Ca^{2+} channel toxin (0.8%), bradykinin potentiating peptide (BPP) (0.2%), HMG CoA reductase inhibitor (0.1%), and some other toxins with unknown targetsor pharmacological activity (7.7%). The relative abundances of these toxins in a particular venom would be expected to vary depending on the genus and scorpion species. The geographical origin of the scorpion species may also influence them. Individually or in unison, these toxins exert various pharmacological effectsin a scorpion sting, and some also show an anticancer potential. Venoms of some scorpions, such as *Hemiscorpius lepturus*, *Scorpio maurus*, and *Tityus serrulatus* are reported to contain phospholipase A_2 and hyaluronidase, [38; 39; 37]. A proteomic analysis of Indian MTV has demonstrated the lack of phospholipase A_2, supporting the genus- and species-specific variation in scorpion venom composition.

Several workers have used quantifying the relative abundance of different venom proteins by label-free quantification [32; 26; 29; 40]. Nevertheless, the label-free quantification method has disadvantages, such as lacking a comprehensive database [41; 29]. In any case, a reasonable correlation of the relative abundances of protein families with the SDS-PAGE band intensities of a class of proteins with a particular mass range can support the accuracy of this identification method [32; 26; 29]. In this study, the relative distributions of MTV toxins are well correlated with the SDS-PAGE protein band intensitiesin a given mass range of proteins. For example, the percent band

intensity of the proteins in gel sections 8, 9, and 10 (proteins of 3 to 13 kDa mass range) containing Na^+ and K^+ channel toxins, SPLP, SPI, antimicrobial peptide, hyaluronidase, makatoxin, LPP, Cl^- channel toxin, parabutoporin, Ca^{2+} channel toxin, and other toxins with unknown targets was found to be 81% of the aggregate band intensity. This data correlates well with the cumulative relative abundance of these proteins (80.4%), determined by label-free quantitative proteomics. A small quantity of low molecular mass proteins, identified in gel section 9 with a 10 to 15 kDa mass range, were also associated with high molecular mass protein bands (>45 kDa) of MTV. At the same time, no high molecular mass proteins were identified in MTV by LC-MS/MS, possibly because of the scarcity of databases for the Buthidae family of venom proteins (toxins). Similarly, database-dependent proteomic analyses fail to identify some high molecular mass toxins from the venom of the Viperidae family of snakes [32; 26; 40]. Possibly, like the snake venom protein complexes, [42; 26] scorpion venom toxins may also form cognate protein complexes that act synergistically to augment the toxicity of individual components of venom [42; 43]. Further studies are warranted to identify and characterize the protein complexes in scorpion venom.

4.2.2 Characterization of some pharmacological properties of MTV

MTV, under *in vitro* conditions, did not show pharmacological and enzymatic activities. Interestingly, the venom of *H. laoticu*, a scorpion of Vietnam origin, contains two low molecular mass di peptides Leu-Trp and Ile-Trp, with anticoagulant activity [44]. At the same time, such di-peptides have not been detected in Indian MTV. This finding justifies the lack of anticoagulant activity in Indian MTV. Moreover, the results of the enzyme assays did not show the presence of enzymes in MTV. The biochemical analysis did not observe the enzyme activity of hyaluronidase in MTV. However, it was identified with the LC-MS/MS analysis, possibly because this enzyme constitutes only 2.2% of MTV, which is insufficient to show *in vitro* hyaluronidase activity. Hyaluronidase is reported to act as a spreading factor to promote hyperemia and cause intense pain in the sting victim.

The Brazilian scorpion *Tytius serrulatus* venom showed the presence of metalloproteinase enzyme [45] whereas Valdez-Vela´zquez et al. [46] demonstrated the presence of three protein sequences that correspond to three different putative metalloproteinases in the venom of the scorpion *Centruroides tecomanus*. Nevertheless,

LC-MS/MS and the biochemical analyses of MTV did not recognise metalloproteinase, which suggests that its venom proteome was devoid of the enzyme metalloproteinase. Thus, the scorpion venom in this study does not influence the haemostatic system of victims and exerts its toxicity mainly via the plentiful ion-channel toxins, which is also well correlated with published clinical data for MT sting [2].

4.2.3 Immuno-cross reactivity studies show that commercial anti-scorpion antivenom is deficient in specific antibodies against the low molecular mass toxins of MTV

Administration (i.v.) of equine scorpion antivenom is the most specific treatment against scorpion stings. At the same time, the safety and efficacy of commercial antivenom are the two most important factors in the hospital management of scorpion stings. Cardiac manifestations due to scorpion stings have been reported to lead to possible fatality even after antivenom therapy [11]. Reis et al. [11] hypothesized that the "host response to the venom is perpetuated by molecular mechanisms functioning after the venom toxins have been neutralized by antiserum, which could promote further pathology". In our study, however, commercial ASA lacked antibodies against the low molecular mass toxins of MTV.

Notably, the abundance of low molecular mass neurotoxins is responsible for the cardiovascular dysfunction and other deleterious pharmacological effects of MT sting [11]. The failure of commercial ASAs to recognize the most abundant toxins in this venom is a severe problem for efficient antivenom therapy and hospital management of scorpion sting victims. Innovative immunization protocols that can enhance the antibody response (in the horse) against partially immunogenic, toxicologically relevant MTV components need to be developed [32].

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