CIBTech Journal of Zoology ISSN: 2319–3883
Online, International Journal, Available at http://www.cibtech.org/cjz.htm
2026 Vol.15, pp.63-69/Dhananjay et al.
***Research Article** (Open Access)*

VENOM PROFILING OF *HOTTENTOTTA PACHYURUS* AND *HOTTENTOTA TAMULUS* BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

Dhananjay D Tigote[1]*, Mandakini M Thorat[1], Dilip H Dudhmal[2]
[1] Department of Zoology, Sanjeevanee Mahavidyalay, Chapoli, Latur, Maharashtra, India*
[2] Department of Zoology MVP Samaj's ACS College, Dindori, Nashik, Maharashtra, India*

ABSTRACT
The venom of scorpions is a complex cocktail of bioactive proteins and peptides, crucial for their evolutionary success and ecological dominance. In this study, we performed comparative venom profiling of *Hottentotta pachyurus* and *Hottentotta tamulus* using high-resolution mass spectrometry to elucidate their toxin composition. Venom was extracted from specimens collected in the Balaghat Hill Region of Maharashtra and purified using C18 chromatography before analysis. The results revealed that both species possessed serine protease 1 as a dominant component, with high sequence coverage and peptide counts, suggesting its conserved role in proteolysis and prey immobilization. Additionally, numerous neurotoxic peptides, particularly from the alpha-KTx family targeting potassium channels, and species-specific variants such as tamulustoxin, were abundant in both venoms, highlighting their significance in disrupting ion channels and inducing paralysis. Minor components, including delta-buthitoxins, iota-buthitoxins, metalloproteinases, phospholipase A2-like proteins, and defensin-like peptides, were also detected, reflecting the biochemical heterogeneity and multifunctionality of scorpion venoms. Compared to *H. tamulus*, a richer complement of sodium channel toxins and beta-type neurotoxins was found in *H. pachyurus*. This study underscored the taxon-specific yet overlapping nature of venom composition within Hottentotta species and provides foundational data for further evolutionary, pharmacological, and taxonomic investigations. The characterization of these venom components not only advances our understanding of scorpion toxin diversity but also aids in the exploration of their potential therapeutic applications.

Keywords: *Hottentotta pachyurus, Hottentota tamulus, Mass Spectrometry, Orbitrap, Scorpion, Venom*

INTRODUCTION
Scorpions are a group of arthropods whose phenotype has remained largely unchanged over the past 400 million years. Their evolutionary success is closely linked to their potent venom, which serves both to deter predators and immobilize prey (Schaffrath & Predel, 2014). The major components of scorpion venom are peptides (De la Vega *et al.*, 2010), many of which specifically interact with potassium ion channels ("short-chain" peptides) and sodium ion channels ("long-chain" peptides) (Tytgat *et al.*, 1999). It is believed that the diverse array of scorpion venom peptides, each with distinct biological effects, may have originated from a common ancestral gene (Zhijian *et al.*, 2006).
As with venom toxins in other animal groups (Escoubas *et al.*, 1997; Schmidt, 1990; Stöcklin *et al.*, 2000), the composition and efficacy of scorpion peptide toxins are generally taxon-specific, although fundamental peptide types are shared across different clades (Ma *et al.*, 2012). This rapid molecular diversification arises from evolutionary pressures imposed by the ion-channel adaptations of local prey and predators (Brodie & Brodie, 1999). Scorpions become vulnerable when predators develop resistance to their venom (Rowe *et al.*, 2013), necessitating continual refinement of toxin efficacy for survival. In some species, even distinct populations can be differentiated by amino acid substitutions within their peptide sequences (Newton *et al.*, 2007; Pimenta *et al.*, 2003).

Detailed knowledge of specific peptide sequences can aid in exploring their pharmaceutical potential (Lewis & Garcia, 2003) and also serves as a valuable tool for confirming species identity. Since most toxic peptides consist of fewer than 100 amino acids, they are readily detectable by mass spectrometry, which remains the preferred method for rapid venom screening (Favreau *et al.*, 2006; Martin-Eauclaire *et al.*, 2013).

For several scorpion species, especially those of medical importance within the family Buthidae, comprehensive data is already available detailing the peptide composition of their venom. The venom gland peptidome in these species includes both mature peptides and numerous breakdown products derived from larger polypeptides (Rates *et al.*, 2008). However, relatively few studies have focused on comparing venom profiles across different species solely for taxonomic and pharmaceutical purposes. In the current study, a comparative venom characterization will be evaluated from the venom samples of two Hottentotta species *viz*. *Hottentotta pachyurus* and *Hottentotta tamulus* of Buthidae family.

MATERIALS AND METHODS

1.1. Collection of scorpion venom

For venom characterization, *Hottentotta pachyurus* and *Hottentota tamulus* collected from Balaghat Hill Region of Maharashtra were chosen. During the extraction period, the scorpions were maintained under normal light-dark cycles and kept unfed. For the venom collection, a mild electric stimulation using a Testronix 92C DC power supply (0–32V, 0–5A) equipped with modified tweezers was used to deliver the current. To enhance conductivity, the tweezers were coated with conductive gel prior to application. An electric stimulation delivering a controlled shock of 12 Volts, 1 Ampere in pulse of 200 µS (microsecond) was applied on the membrane anterior to the telson. The venom was collected in a 10 µL microtip which each time after collection was immediately to a 0.5 mL Eppendorf containing a 1:1 mixture of acetonitrile and water. The venoms collected separately for each scorpion species were individually preserved at –20°C (Schaffrath & Predel, 2014).

1.2. Purification of venom

The collected venom samples were purified using a packed C18 column (15 cm length, 1.5 µm particle size, 150 µm internal diameter) maintained at 30°C. Separation was carried out on an Ultimate 3000 RS-nano UHPLC system operating at a flow rate of 500 nL/min. The mobile phases consisted of 0.1% formic acid in 98% water (A) and 0.1% formic acid in 80% acetonitrile (B). Peptides were eluted using a gradient of 2–90% mobile phase B over 110 minutes. The gradient conditions were as follows: 0–10 min, 2% B; 10–70 min, ramp to 25% B; 70–96 min, 25–38% B; 96–100 min, ramp to 90% B; 100–105 min, held at 90% B; and re-equilibrated to 2% B at 106 min. The total run time was 110 minutes (Ghezellou *et al.*, 2022). Purified venom fractions were then collected and prepared for mass spectrometric analysis.

1.3. Characterization of venom by Mass Spectrometry

Peptides were analyzed using positive-ion mode on a nano-electrospray ionization source. The capillary voltage was set to 2500 V, and the ion transfer tube temperature was maintained at 275°C. Automated MS/MS data acquisition was performed on an Orbitrap mass analyzer across a mass range of 350–2000 m/z with a minimum threshold of 5.0e3 counts. The twenty most intense precursor ions with charge states from +2 to +8 were selected for MS/MS acquisition using an ion trap mass analyzer. Higher-energy collisional dissociation (HCD) fragmentation was carried out with a normalized collision energy of 30%. For each run, 500 ng of digested protein sample was loaded onto the LC/MS system for analysis (Hempel *et al.*, 2020).

RESULTS

3.1. Venom profiling of *Hottentotta pachyurus*

The mass spectrometry-based venom profiling of *Hottentotta pachyurus* uncovered a complex mixture of proteins and peptides characteristic of buthid scorpion venoms (Table 1). Among the components, serine

protease 1 exhibited the highest sequence coverage (91%) and a substantial number of unique peptides, underscoring its abundance and potential role in venom toxicity and prey digestion. Other prominent components included neurotoxin BmTX3A precursor and several potassium channel toxins such as alpha-KTx 16.1 and alpha-KTx 9.3, reflecting the rich presence of neurotoxic peptides that target ion channels and contribute to the paralytic effects of the venom.

Additionally, the profile revealed minor components such as delta-buthitoxin variants, iota-buthitoxins, and metalloproteinase-related proteins, suggesting potential roles in tissue degradation, prey immobilization, or synergistic enhancement of neurotoxicity. The diversity of molecular weights, peptide counts, and predicted isoelectric points illustrates the biochemical heterogeneity of *H. pachyurus* venom, which is consistent with patterns reported in other buthid species. These findings provided critical insights into the venom composition and form a basis for future functional and evolutionary studies on scorpion venom diversity.

3.2. Venom profiling of *Hottentotta tamulus*

Mass spectrometric analysis of *Hottentotta tamulus* venom revealed abundant serine protease 1, covering 91% of its sequence with high peptide counts, that indicated its dominant role in proteolytic processing and prey immobilization (Table 1). Additionally, several neurotoxic peptides, including alpha-KTx family potassium channel toxins and tamulustoxin variants, were detected with significant sequence coverage, that reflected their crucial function in disrupting ion channels and inducing paralysis.

Other components, such as group XV phospholipase A2-like protein, metalloserrulase 4, neurotoxin BmTX3A precursor, and defensin-like peptides, were also identified, albeit with lower sequence coverage. Together, these results highlight the complex biochemical arsenal of *H. tamulus* venom, combining potent neurotoxins with enzymatic proteins that contribute to prey subjugation and venom diffusion.

Table 1 Venom profiling of *Hottentotta pachyurus* and *Hottentotta tamulus* by Mass Spectrometry

Accession	Description	Coverage [%]	Peptides	Unique Peptides	AA	MW [kDa]
Hottentotta pachyurus						
P00760.3	Serine protease 1 (also known as anionic trypsin I, beta-trypsin, cationic trypsin, pretrypsinogen I, or trypsin I; precursor containing alpha-trypsin chain 1 and 2).	91	32	32	246	25.8
AAN85572.1	Neurotoxin BmTX3A precursor (*Mesobuthus martensii*)	32	3	3	59	6.2
P0C173.1	Potassium channel toxin alpha-KTx 16.1 (also known as alpha-KTx 1.8 or Tamulotoxin; short name TmTX)	42	2	2	36	4.2
P0DQN9.1	Delta-buthitoxin-Hj2a (short name delta-BUTX-Hj2a)	14	1	1	64	7.1
P806691	Potassium channel toxin alpha-KTx 9.3 (also known as Leiuropeptide I or Leiuropeptide-1; short name LpI)	100	1	1	28	3
CCD31420.1	Scorpion toxin Tc48b/Tc49a precursor (*Tityus obscurus*)	37	1	1	86	9.9
ADY39483.1	Iota-buthitoxin-Hj1a, partial (*Hottentotta judaicus*)	14	1	1	77	9.2
AIX87634.1	Sodium channel blocker AbNaTx21 (*Androctonus bicolor*)	14	1	1	87	10
2KY3	Potassium channel toxin alpha-KTx 5.4 (Chain A)	42	1	1	33	3.6

ADY39498.1	Iota-buthitoxin-Hj1b (*Hottentotta judaicus*)	26	1	1	82	9.6
C0HM15.1	Beta-toxin Chui2 (also known as Chui2)	20	1	1	66	7.6
AMO02513.1	A disintegrin and metalloproteinase 1 (*Tityus serrulatus*)	2	1	1	902	99.8
P0DL25.1	Putative beta-neurotoxin	38	1	1	39	4
Hottentotta tamulus						
P00760.3	Serine protease 1 (also known as anionic trypsin I, beta-trypsin, cationic trypsin, pretrypsinogen I, or trypsin I; precursor containing alpha-trypsin chain 1 and 2)	91	29	29	246	25.8
P60209.1	Potassium channel toxin alpha-KTx 9.4 (also known as BTK-2)	78	4	4	32	3.5
P0C173.1	Potassium channel toxin alpha-KTx 16.1 (also known as alpha-KTx 1.8 or Tamulotoxin; short name TmTX)	75	2	2	36	4.2
AAK01860.1	Tamulustoxin 1, partial (*Mesobuthus tamulus*)	43	3	3	35	4
AED93694.1	Defensin-like (DEFL) family protein (*Arabidopsis thaliana*)	35	1	1	80	8.8
AAN85572.1	Neurotoxin BmTX3A precursor (*Mesobuthus martensii*)	32	4	4	59	6.2
C0HJQ5.1	Potassium channel toxin alpha-KTx 8.7 (also known as toxin MeKTx1-2; precursor form)	23	1	1	57	6.4
AMX81474.1	Venom peptide meuPep27 (*Mesobuthus eupeus*)	18	1	1	96	11.1
XP_023217052.1	Group XV phospholipase A2-like isoform X1 (*Centruroides sculpturatus*)	5	1	1	412	47.1
AIJ02112.2	Metalloserrulase 4 (*Tityus serrulatus*)	4	1	1	399	44.1

AA: Amino Acids; MW: Molecular Weight.

DISCUSSION

Peptides constitute the major components of scorpion venoms and are chiefly responsible for their remarkable pharmacological diversity, making them a rich natural source of bioactive molecules (Estrada-Gómez *et al.*, 2021; Estrada *et al.*, 2007). Consequently, numerous recent studies have focused on characterizing the peptide composition of scorpion venoms. In the present study both *H. pachyurus* and *H. tamulus* venoms exhibited a rich array of proteomic components, with several similarities and distinct patterns reflecting evolutionary divergence and potential functional adaptations (Figure 1). In both species, serine protease 1 was the most abundant protein, showing high sequence coverage (91%) and numerous peptides, that suggested a prominent role in proteolytic processing, prey immobilization, and possibly aiding digestion. Serine proteases are recognized for their hydrolytic activity, specifically their ability to cleave peptide amide bonds. Beyond this, they have demonstrated fibrinolytic activity in the venoms of scorpions such as *Tityus bahiensis, T. serrulatus, and T. discrepans*, in contrast to metalloproteases

(Almeida *et al.*, 2002). Interestingly, recent research has identified a novel function of snake venom serine proteases as potassium channel blockers (Boldrini-França *et al.*, 2020).
Potassium channel toxins (alpha-KTx family) were consistently identified across both venoms. *H. tamulus* exhibited particularly high coverage for alpha-KTx 9.4 (78%) and alpha-KTx 16.1 (75%), while *H. pachyurus* also showed substantial representation of alpha-KTx isoforms including alpha-KTx 16.1 (42%) and alpha-KTx 9.3 (100% coverage but fewer peptides). These channel toxins are well-characterized neurotoxins that block voltage-gated potassium channels, contributing to prey paralysis and defensive mechanisms. The present results aligned with earlier proteomic studies of scorpion venoms, which consistently show that toxins targeting Na^+ and K^+ channels constitute the predominant components in Buthidae scorpion venom profiles (Ghezellou *et al.*, 2022).

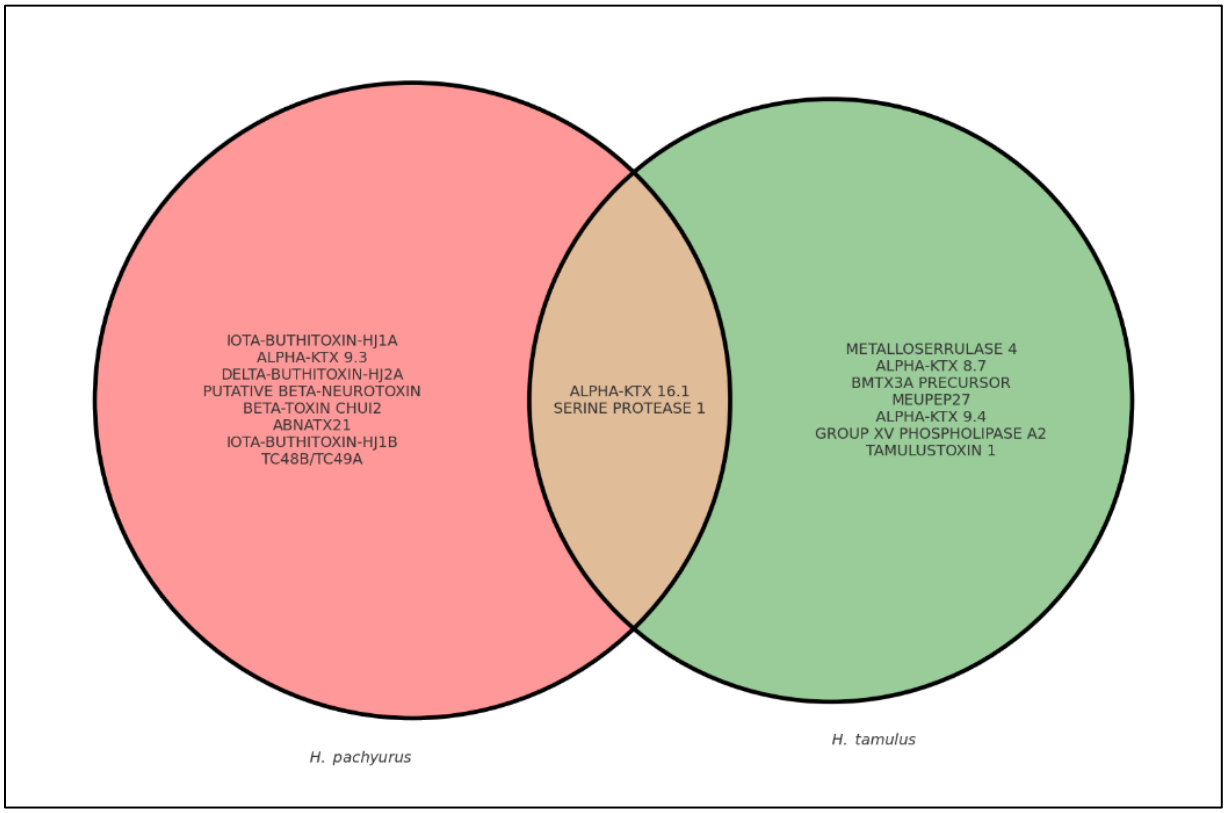

Figure 1 Comparative venom profiling of *H. pachyurus* and *H. tamulus*

Distinctively, in the current study *H. pachyurus* venom revealed a broader diversity of sodium channel toxins and beta-type neurotoxins, including delta-buthitoxin-Hj2a, beta-toxin Chui2, putative beta-neurotoxin, and Tc48b/Tc49a precursors, which were either absent or less represented in *H. tamulus*. These molecules target sodium channels, producing potent neurotoxic effects and enhancing venom efficacy against prey. The primary distinction between the venoms of Buthidae and non-Buthidae scorpion families lies in the abundance of long chain toxins in Buthidae venoms. These toxins largely account for the neurotoxic properties of scorpion venom and are central to the clinical manifestations of scorpion envenomation (Almaaytah & Albalas, 2014). Notably, transcriptomic analyses of venom glands in two Hottentotta species have revealed that they possess relatively fewer long chain toxin-encoding sequences compared to other Buthidae members (Kovařík, 2007).
Conversely, *H. tamulus* venom uniquely contained tamulustoxin 1 (43% coverage), potassium channel toxin alpha-KTx 8.7, and several peptides derived from other scorpion genera (e.g., meuPep27 from *Mesobuthus*

eupeus), signifying possible convergent evolution or conserved venom strategies within Buthidae. The presence of group XV phospholipase A2-like proteins and metalloserrulase components in *H. tamulus* further points to differential expansion of enzymatic toxins that may facilitate tissue penetration and prey subjugation.

Together, these findings indicated that while both species share core venom components essential for neurotoxicity (notably potassium channel toxins), *H. pachyurus* has a richer complement of sodium channel toxins and beta-type neurotoxins, potentially reflecting divergent ecological niches, prey preferences, or defensive strategies. These differences underscore the importance of comparative venom profiling in understanding scorpion venom evolution and functional adaptation.

Conclusion

The present study successfully characterized and compared the venom proteomes of *H. pachyurus* and *H. tamulus* using mass spectrometry. Both species exhibited abundant serine protease 1, highlighting its conserved role in prey digestion and envenomation. Diverse neurotoxic peptides targeting ion channels, including alpha-KTx family toxins and species-specific variants such as tamulustoxin, were prominent components in both venoms. Minor constituents like metalloproteinases, phospholipases, and defensin-like peptides further underscored the biochemical complexity and multifunctionality of these venoms. Overall, this profiling provided valuable insights into the toxin repertoire of medically important scorpions and offers a basis for future evolutionary, pharmacological, and taxonomic research.

REFERENCES

Almaaytah, A., & Albalas, Q. (2014). Scorpion venom peptides with no disulfide bridges: a review. *Peptides*, **51**, 35-45.

Almeida, F., Pimenta, A., De Figueiredo, S., Santoro, M., Martin-Eauclaire, M., Diniz, C., & De Lima, M. (2002). Enzymes with gelatinolytic activity can be found in Tityus bahiensis and Tityus serrulatus venoms. *Toxicon*, **40**(7), 1041-1045.

Boldrini-França, J., Pinheiro-Junior, E. L., Peigneur, S., Pucca, M. B., Cerni, F. A., Borges, R. J., Costa, T. R., Carone, S. E. I., Fontes, M. R. d. M., & Sampaio, S. V. (2020). Beyond hemostasis: a snake venom serine protease with potassium channel blocking and potential antitumor activities. *Scientific Reports*, **10**(1), 4476.

Brodie, E. D., & Brodie, E. D. (1999). Predator-prey arms races: asymmetrical selection on predators and prey may be reduced when prey are dangerous. *Bioscience*, **49**(7), 557-568.

De la Vega, R. C. R., Schwartz, E. F., & Possani, L. D. (2010). Mining on scorpion venom biodiversity. *Toxicon*, **56**(7), 1155-1161.

Escoubas, P., Célérier, M. L., & Nakajima, T. (1997). High-performance liquid chromatography matrix-assisted laser desorption/ionization time-of-flight mass spectrometry peptide fingerprinting of tarantula venoms in the genus Brachypelma: chemotaxonomic and biochemical applications. *Rapid Communications in Mass Spectrometry*, **11**(17), 1891-1899.

Estrada-Gómez, S., Vargas-Muñoz, L. J., Saldarriaga-Córdoba, M. M., & Meijden, A. v. d. (2021). MS/MS analysis of four scorpion venoms from Colombia: a descriptive approach. *Journal of Venomous Animals and Toxins Including Tropical Diseases*, **27**, e20200173.

Estrada, G., Villegas, E., & Corzo, G. (2007). Spider venoms: a rich source of acylpolyamines and peptides as new leads for CNS drugs. *Natural Product Reports*, **24**(1), 145-161.

Favreau, P., Menin, L., Michalet, S., Perret, F., Cheneval, O., Stöcklin, M., Bulet, P., & Stöcklin, R. (2006). Mass spectrometry strategies for venom mapping and peptide sequencing from crude venoms: case applications with single arthropod specimen. *Toxicon*, **47**(6), 676-687.

Ghezellou, P., Jakob, K., Atashi, J., Ghassempour, A., & Spengler, B. (2022). Mass-spectrometry-based lipidome and proteome profiling of Hottentotta saulcyi (Scorpiones: Buthidae) Venom. *Toxins*, **14**(6), 370.

Hempel, B.-F., Damm, M., Mrinalini, Göçmen, B., Karış, M., Nalbantsoy, A., Kini, R. M., & Süssmuth, R. D. (2020). Extended snake venomics by top-down in-source decay: investigating the newly discovered Anatolian meadow viper subspecies, Vipera anatolica senliki. *Journal of proteome research*, **19**(4), 1731-1749.
Kovařík, F. (2007). A revision of the genus Hottentotta Birula, 1908, with descriptions of four new species (Scorpiones, Buthidae). *Euscorpius*, **2007**(58), 1-107.
Lewis, R. J., & Garcia, M. L. (2003). Therapeutic potential of venom peptides. *Nature reviews drug discovery*, **2**(10), 790-802.
Ma, Y., He, Y., Zhao, R., Wu, Y., Li, W., & Cao, Z. (2012). Extreme diversity of scorpion venom peptides and proteins revealed by transcriptomic analysis: implication for proteome evolution of scorpion venom arsenal. *Journal of proteomics*, **75**(5), 1563-1576.
Martin-Eauclaire, M.-F., Granjeaud, S., Belghazi, M., & Bougis, P. E. (2013). Achieving automated scorpion venom mass fingerprinting (VMF) in the nanogram range. *Toxicon*, **69**, 211-218.
Newton, K. A., Clench, M. R., Deshmukh, R., Jeyaseelan, K., & Strong, P. N. (2007). Mass fingerprinting of toxic fractions from the venom of the Indian red scorpion, Mesobuthus tamulus: biotope-specific variation in the expression of venom peptides. *Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry*, **21**(21), 3467-3476.
Pimenta, A. M., Almeida, F. D. M., de Lima, M. E., Martin-Eauclaire, M. F., & Bougis, P. E. (2003). Individual variability in Tityus serrulatus (Scorpiones, Buthidae) venom elicited by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, **17**(5), 413-418.
Rates, B., Ferraz, K. K., Borges, M. H., Richardson, M., De Lima, M. E., & Pimenta, A. M. (2008). Tityus serrulatus venom peptidomics: assessing venom peptide diversity. *Toxicon*, **52**(5), 611-618.
Rowe, A. H., Xiao, Y., Rowe, M. P., Cummins, T. R., & Zakon, H. H. (2013). Voltage-gated sodium channel in grasshopper mice defends against bark scorpion toxin. *Science*, **342**(6157), 441-446.
Schaffrath, S., & Predel, R. (2014). A simple protocol for venom peptide barcoding in scorpions. *Eupa open proteomics*, **3**, 239-245.
Schmidt, J. O. (1990). Hymenoptera venoms: striving toward the ultimate defense against vertebrates.
Stöcklin, R., Mebs, D., Boulain, J.-C., Panchaud, P.-A., Virelizier, H., & Gillard-Factor, C. (2000). Identification of snake species by toxin mass fingerprinting of their venoms. *Mass Spectrometry of Proteins and Peptides: Mass Spectrometry of Proteins and Peptides*, 317-335.
Tytgat, J., Chandy, K. G., Garcia, M. L., Gutman, G. A., Martin-Eauclaire, M.-F., Van Der Walt, J. J., & Possani, L. D. (1999). A unified nomenclature for short-chain peptides isolated from scorpion venoms: α-KTx molecular subfamilies. *Trends in pharmacological sciences*, **20**(11), 444-447.
Zhijian, C., Feng, L., Yingliang, W., Xin, M., & Wenxin, L. (2006). Genetic mechanisms of scorpion venom peptide diversification. *Toxicon*, **47**(3), 348-355.