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Neurotoxicity Screening of Venom Components from the Philippine Cave Tarantula *Orphnaecus kwebaburdeos*

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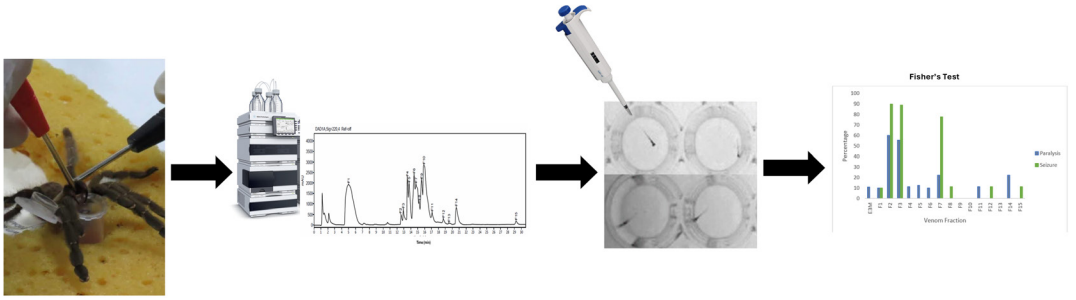
Graphical Abstract



Abstract

We report the neurotoxicity of venom components of a cave-dwelling tarantula from the genus *Orphnaecus*, collected from an island in the eastern part of Luzon, Philippines. The neurotoxicity of the HPLC fractions of the venom extracted from the Philippine tarantula *Orphnaecus kwebaburdeos* was assessed based on their effects on the swimming behavior of zebrafish (*Danio rerio*) larvae and by observing larval swimming patterns. Our results show that several fractions of the spider venom altered the swimming behavior and patterns of the larvae, indicating that they are neurotoxic. Both paralysis and seizure hyperactivity were observed in larvae exposed to Fractions 2 and 3. Only seizure was observed in zebrafish larvae exposed to Fraction 7. These results suggest that several fractions of the *O. kwebaburdeos* venom contain neurotoxic components. The observed neurotoxic phenotypes may be caused by the different neurotoxic components which can further be studied.

Keywords: spider venom; *Orphnaecus*; Philippine tarantula; neurotoxins; zebrafish; paralysis; seizure

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Introduction

Spider venoms are comprised of bioactive molecules that can be tapped as potential sources of therapeutic agents. Spiders efficiently capture, immobilize, and kill other animals by injecting their venom and causing various neurotoxic effects in their target. The venom of spiders is composed of low molecular weight organic compounds, nucleotides, inorganic salts, free amino acids, monoamines, peptides, and proteins [1]. Generally, its neurotoxicity has been attributed to bioactive low molecular weight organic compounds and cysteine-rich peptides [2-4].

Neurotoxic peptides are perhaps the most studied among the bioactive molecules from spiders. These structurally diverse molecules have been attributed to the successful paralysis and killing of tarantula's prey [5]. Venom peptides ranging from 1 to 6 kilodaltons have been reported to bind to ion channels selectively, affecting their activities [6]. Theraphotoxin, a group of sodium ion channel binding molecules that assume an internal cystine knot (ICK) motif, is an example of these neurotoxic peptides that are reported to cause observable neurotoxic behavior in animal models [7-9].

The composition and structural diversity of neurotoxins observed within and across genera, which interestingly produces a variety of distinct toxicological effects, makes this research area a rich subject for biochemical and pharmacological research [10]. In the Philippines, for example, the very limited information on composition and bioactivity of spider venoms, particularly on the genus *Orphnaecus*, the most diverse tarantula in the Philippines, is a clear knowledge gap that needs to be addressed to harness its potential biological applications such as discovery of novel therapeutic molecules [11,12].

Zebrafish (*Danio rerio*) display many characteristics that make them suitable for evaluating the neuroactive effects of spider venom compounds. Zebrafish share many conserved receptors and neuronal architectures with humans [13]. Zebrafish larvae can be bred easily in great numbers, ideal for comprehensively screening spider venom fractions [14,15]. Before the larval zebrafish blood-brain barrier fully matures at 10 days post fertilization (dpf), penetration of pharmaceutical compounds can be observed, making exposure a viable route for screening compounds on the larval zebrafish model [16,17].

This study employed a rapid and high-throughput phenotype-based screening method using zebrafish larvae to evaluate the neurotoxicity of venom fractions of the Philippine tarantula species *Orphnaecus kwebaburdeos*.

Materials and Methods

Spider Collection and Identification. Tarantula spiders were collected from Burdeos, Polilio Island, Quezon Province, Philippines (Gratuitous Permit No. 318). The collected tarantula specimens were confirmed as *Orphnaecus kwebaburdeos* based on morphological comparison with the type specimens inspected from the University of the Philippines Los Baños Museum of Natural History collection.

Venom Extraction and Fractionation. Venom extraction was performed by electrostimulation and fractionation was done following the methods described by Lopez et al. (2020) with modifications [18]. Individual tarantula spiders were placed in an air-tight container and exposed to a carbon dioxide gas stream for 3-5 minutes. The fangs were retracted and positioned for venom extraction on the brim of a 1.5 mL microcentrifuge tube, and 15 volts of current were applied to the base of the chelicerae until the venom was ejected from the fangs. Collected venoms were stored at -20 °C until use.

Venom fractionation was performed by reversed-phase high-performance liquid chromatography (RP-HPLC) using an InfinityLab Poroshell 120 EC-C18 column (4.6 x 100 mm, 2.7 µm) attached to Agilent 1260 Infinity II Liquid Chromatography System with a diode array detector (Agilent, USA). Separation was performed with a solvent system composed of 0.1% TFA in water (Solvent A) and 0.1% TFA in 95% acetonitrile (Solvent B) using a gradient of 5% to 20% Solvent B in Solvent A from 0 to 6 minutes; 20% to 35% Solvent B in Solvent A from 6 to 11 minutes; 35% to 65% Solvent B in Solvent A from 11 minutes to 35 minutes; and 65% to 95% of Solvent B in Solvent A from 35 minutes to 44 minutes. Fractions collected were lyophilized and stored at -20 °C until use.

To quantify the amount of peptides in each fraction, fractions were reconstituted in distilled, deionized water and aliquots were taken to be tested using the Bradford assay [19]. Aliquots corresponding to the testing amount were lyophilized and reconstituted to E3 medium prior to the assay.

Experimental Animal Care and Breeding. Adult wild-type adult zebrafish were purchased from a local pet shop. Prior to breeding, zebrafish were quarantined and acclimatized in chlorine-free filtered water containing 100 µL/L 1% methylene blue solution for 28 days. Half the tank water was replaced with chlorine-free 3–5-day stock water containing methylene blue during acclimatization. Male and female fish were transferred to separate 10-liter tanks (Gendanio Biotech Inc., Taiwan) in a circulating water filtration maintained at 29 °C. The fish were fed with decapsulated brine shrimp twice daily.

Male and female zebrafish were placed in separate cells of the breeding tank (Gendanio, Taiwan) at a 2:1 male-to-female ratio the night before breeding. The acrylic boundary of the cells separating the male and female fish was removed at dawn to allow mating. The eggs were harvested, rinsed with filtered stock water, and allowed to grow in E3 zebrafish embryo medium (4.96 mM NaCl, 0.18mM KCl, 0.16mM $CaCl_2$, and 0.40mM $MgCl_2$) containing 100 µL/L 1% methylene blue. The protocols used in this study were approved by the University of Santo Tomas Institutional Animal Care and Utilization Committee (UST-IACUC RC2023-100810).

Neurotoxicity Screening. Neurotoxicity of the spider venom fractions was assessed in 7–9 days post fertilization (dpf) larvae (n = 8 to 10 per group). Individual fish larvae were placed in a well of a 24-well microplate containing 900 µL of E3 medium and allowed to acclimate for 30 minutes. Fish swimming was observed and recorded for 5 minutes after acclimatization. After pre-exposure observation, 100 µL of E3 medium containing 5 µg venom fraction was added to each well. Erratic swimming behaviors, which include paralysis, described by absence of movement or loss of gait, and seizure, characterized by whirlpool swimming and convulsive behavior, were recorded and noted as neurotoxicity phenotypes [20,21].

Statistical Analysis. The percentage of fish that exhibited erratic swimming behavior, such as paralysis and seizures, was computed. The proportion of fish expressing the neurotoxicity phenotype among fractions was compared to negative control using Fisher's Exact Probability Test.

Results

Reversed-Phase – High Performance Liquid Chromatography (RP-HPLC) of the crude venom collected from the Philippine cave tarantula *Orphnaecus kwebaburdeos* yielded fifteen distinct peak fractions which were collected separately, lyophilized, and used in the neurotoxicity assay in zebrafish larvae. Most of the fractions eluted between 12 minutes to 22 minutes which can be considered to be in the mid-polar fractions. Figure 1 presents the RP-HPLC chromatogram of the crude venom.

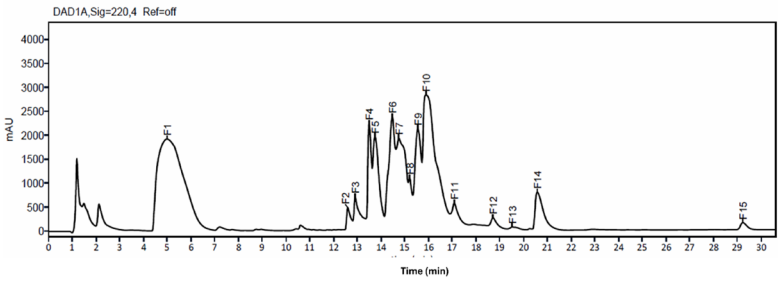

Figure 1. RP-HPLC chromatogram of Venom Extracted from *Orphnaecus kwebaburdeos*.

Paralysis and seizure, characterized by whirlpool swimming patterns, were observed in zebrafish treated with the venom fractions. The percentage of paralysis among the zebrafish treated with Fraction 2 (Rt = 12.589 min) and Fraction 3 are 55.6% and 60%, respectively. These percentages are both significantly higher than the negative control group ($p<0.001$) which only had an 11.1% paralysis. Prior to paralysis, seizure was also observed in 90.0% and 89.9% of the zebrafish treated with Fractions 2 and 3, respectively which is also significantly higher compared to the control group ($p<0.001$) where no hyperactivity was observed. For Fraction 7, only seizure was observed in 77.8% of larvae treated with this fraction, which is also significantly higher ($p<0.001$) than the control group. Figure 2 presents the percentage of zebrafish larvae which expressed neurotoxicity phenotypes from different treatment groups.

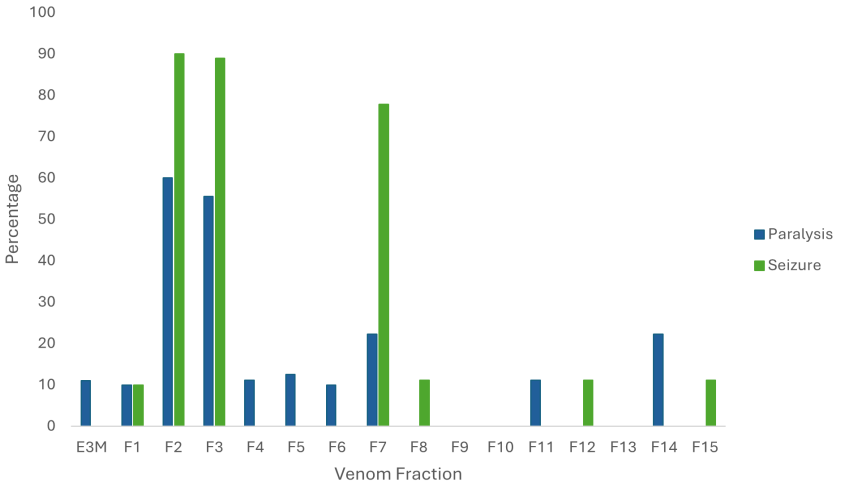

Figure 2. Percentage of zebrafish larvae that exhibited neurotoxic swimming behavior.

Discussion

The majority of the bioactive components of the spider venom are neurotoxins [22]. Neurotoxic components of the venom such as low molecular weight polyamines and cysteine-rich peptides bind to metabotropic or ionotropic receptors and cause an array of effects which include paralysis or seizure [6]. When neurotoxic molecules act on the nervous system of the spider's target, they interfere with neuronal signaling and transport of ions, events that are crucial in the nerve signal transmission process in the brain [23-25].

In this study, we were able to observe the neurotoxic activities of venom fractions of the Philippine cave tarantula *Orphnaecus kwebaburdeos* in 7–9 dpf zebrafish larvae. Zebrafish larvae exposed to Fraction 2 and Fraction 3 exhibited seizure followed by paralysis while zebrafish larvae exposed to Fraction 7 exhibited seizure only after exposure to the venom fraction. These observed motor behavior impairments in zebrafish larvae, which we noted in this study as neurotoxicity behavior, are also used to assess neurotoxicity of compounds from both venom and non-venom toxins [26-28].

In zebrafish larvae, increased swimming activity, rapid darting, whirlpool-like movements, clonus-like tail beats, and convulsions followed by brief loss of posture are the common seizure swimming phenotypes observed in larvae exposed to the chemoconvulsant agent pentylenetetrazole (PTZ) [29,30]. These seizure swimming behaviors, particularly whirlpool-like movements and rapid darting, were observed in zebrafish treated with Fraction 2, Fraction 3, and Fraction 7.

Seizure is one of the effects of insect and arthropod bites [31]. Arthropod venom-associated seizures are linked to the venom component's activity towards sodium and potassium voltage-gated ion channels (Na_Vs and K_Vs) and regulation of synthesis and reuptake of the neurotransmitters γ-amino butyric acid (GABA) and glutamate [32,33]. Venom toxins induce seizure either by activation of ion channels or inhibition of ion channel inactivation [34]. BmK NT, a Na_V-activating peptide toxin from the Chinese golden scorpion *Olivierus martensii*, and tityustoxin-K(alpha) and pandinustoxin-K(alpha), K_V-blocking peptides from the Brazilian and Venezuelan scorpion from the Titiinae subfamily, are examples of arthropod venom which induces seizure by ion-channel regulation while the α-dendrotoxin from the from the African green mamba snake *Dendroaspis angusticeps* affects release of neurotransmitters causing epileptiform movement in rats [33,35,36].

There are several molecules present in the spider venom that can cause paralysis. Phospholipase A2, a secreted component of spider and snake venom, for example, causes paralysis by hydrolyzing membrane phosphatidylcholine and phosphatidylethanolamine causing the destabilization of membrane integrity allowing calcium influx thereby increasing intracellular calcium concentration. This elevation of intracellular calcium affects the peripheral neuromuscular system dysfunction; hence, its paralytic effect [37,38]. Disulfide-rich peptides from animal venoms, an example of which is the ICK peptide VdTx-1 from the Brazilian tarantula *Vitalius dubius*, induce paralysis by blocking neurotransmitter release in the neuromuscular junction causing muscular dysfunction and paralysis in zebrafish as well as in other mammals [39-41]. Spider venom peptides, such as Aps III from the American trapdoor spider *Apomastus schlingeri*, Hm-3 from the Macedonian crab spider *Heriaeus melloteei*, and Ae1a form the African tarantula *Augacephalus ezendami* induce paralysis by inhibiting the activity of voltage-gated ion channels (VGICs) [42-44].

The two neurotoxic behavior phenotypes, paralysis and seizure, were observed successively in zebrafish larvae exposed to Fractions 2 and Fraction 3. These two neurotoxic effects, which have also been observed in animals exposed to the *Tityus serrulatus* scorpion toxin TsTx-I and the black widows *Latrodectus* species spider toxin α-latrotoxin, are not only phenotypically opposite but also contrasting in terms of molecular mechanisms [45,46]. Two amino acid insertions into spider peptides from *Poecilotheria metallica* have been observed to reverse activity in NaV1.7 from activation to inhibition [47]. Both peptides eluted less in a single 1-min fraction, indicating similarity in hydrophobicity despite the 2-amino-acid difference. This effect of the spider venom on animals is evidence of the complexity of the venom's neurotoxicity. At one point, venom toxins may promote neuronal firing and glutamate release which induce seizure and hyperactivity and, at another point, it will be succeeded by paralysis which happens due to progressive failure of neuromuscular transmission caused by depletion of presynaptic vesicles [48].

Conclusion

This study on the neurotoxicity of *Orphnaecus kwebaburdeos* spider venom from the Philippines provides preliminary insights into the neurotoxic activity of its components. Additional research, particularly focused on the purification and characterization of these components, is necessary to gain a comprehensive understanding of their molecular mechanisms and pharmacotoxicity. Such work is essential for the development of spider venom components as therapeutics.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, LAG, LMMD, MRSB, and CMVR; methodology, LAG and CMVR; data collection, LAG and CMVR; analysis and interpretation of data, LAG and CMVR; original draft preparation, LAG; review and editing of the draft, LAG, LMMD, MRSB, and CMVR. All authors have read and agreed to the final version of the manuscript.

Institutional Review Board Statement

Protocols used in this experiment were approved by the University of Santo Tomas Institutional Animal Care and Utilization Committee.

Informed Consent Statement

Not applicable.

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