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Research Article

Purification and Characterization of a Nonenzymatic Neurotoxin from *Hippasa partita* (Lycosidae) Spider Venom Gland Extract

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India is a habitat for nearly one thousand four hundred forty-seven species of spiders under three hundred and sixty-five genera and sixty families. Our initial survey on toxic bite by spider revealed severe edema, itching, acute pain, and hemorrhage following tissue necrosis, which are the general symptoms of envenomation, but there are no reports of mortality. Significantly, *Hippasa partita* spider, commonly called "funnel web spider," which is endemic in hilly regions of the Western Ghats is responsible for envenomation. In this study, a nonenzymatic neurotoxin has been purified from *H. partita* venom gland extract. Gel filtration and ion exchange chromatography were used to purify the toxin into homogeneity as shown by SDS-PAGE, RP-HPLC, and MALDI-TOF. Neurotoxin is devoid of enzymatic activities but causes intense neurotoxic symptoms. Neurotoxin is found to inhibit the twitch response of sciatic nerve gastrocnemius muscle preparation and is found to be postsynaptic in action. Neurotoxin is devoid of coagulant activity, edema, and hemorrhage and is nonlethal to mice (up to 5 mg/kg body weight). In conclusion, a neurotoxin, which is a principle agent in whole venom responsible for induced neurotoxic symptoms, has been purified and characterized.

1. Introduction

Spider venoms are a mixture of pharmacologically active proteins and polypeptides that have led to the development of molecular probes and therapeutic agents. Spider venoms exhibit a wide spectrum of biological activities that results in both local [1, 2] and systemic toxicities [3]. The main purpose of the venom is to immobilize and to digest the prey. *H. partita* spider is endemic in hilly regions of the Western Ghats [4] and is commonly called "funnel web spider" which is mainly responsible for toxic bites. Severe edema and itching, acute pain, and hemorrhage following tissue necrosis are the general symptoms of envenomation, but there are no reports of mortality. Spider bites are often treated with folk medicine. Thus, less attention is paid towards documentation, and no documentation is available in hospitals on clinical pathology.

Spider venoms have been extensively studied for understanding the neurotoxic property [5–7]. Neurotoxins with varied potency have been isolated and studied [6, 7]. Such neurotoxins are acylpolyamines, proteins, and peptides in nature. The venoms from *Agelenopsis aperta* of the American (USA) subcontinent [8–11] and *Phoneutria nigriventer* of the Brazilian subcontinent [12, 13] are the most explored species for neurotoxicity among the spider venoms.

Initial investigations of the *H. partita* whole venom using the mouse model have revealed rapid edema formation at the site of injection and acute neurotoxic signs. However, no study reports towards the isolation and characterization of a neurotoxin from the Indian Subcontinent are taken up so far. In the recent past, we have reported the neurotoxicity of *H. partita* venom on frog's sciatic nerve gastrocnemius muscle preparation [14]. In the present study, we present the result

of purification and characterization of neurotoxin from the venom of *H. partita* spider species.

2. Materials and Methods

H. partita spiders were collected from the Irpu falls region, Madikeri District (Western Ghats of Karnataka, India) (12N11′43.29″–75N48′2.02″) during February to August in the years of 2005, 2006, 2007, and 2008. Irpu region expects heavy rainfall from September to December and in summer from February to May. Spiders live in a funnel-shaped web built in a burrow or crevices of trees. The soil in the region is a mixture of clay and red soil. Spiders (200 in number) were collected during summer season. Spider was identified based on Tikader and Malhotra [15] and confirmed by Dr. Manju Siliwal, Wild Life Information and Liaison Development Society, ZOO Outreach Organization, Coimbatore, Tamil Nadu, India. Sephadex G-100, CM-Sephadex C-25, and Sephadex G-10 were purchased from Sigma Chemicals Co., St. Louis, MO, USA. All other chemicals used were of analytical grade. Swiss Wister albino mice weighing 20–22 g, male frog (*Rana hexadactyla*) weighing 20–22 g were obtained from Animal House Facility, Department of Zoology, University of Mysore, Mysore, India. The animal care and handling were conducted in compliance with the National regulations for animal research. The animal experiments were carried out after reviewing the protocols by the Animal Ethical Committee of the University of Mysore.

2.1. Spider Venom Gland Extract Preparation. Venom was extracted from wild caught spiders that were kept for five days without food. The venom extract was prepared as previously described [14, 16]. Briefly, adult spiders were dissected out, and a pair of glands was collected into ice cold phosphate buffered saline (PBS). The glands were washed in PBS in order to remove possible contaminants, and venom was harvested in PBS by gentle compressing of the glands. The suspension was clarified by centrifugation at 8000 rpm, and the venom gland extract was stored at −20°C until use.

2.2. Sephadex G-100 Column Chromatography. The lyophilized *H. partita* venom gland extract (100 mg in 1.0 mL of 0.1 M NaCl) was applied onto a Sephadex G-100 column (1.5 × 75 cm) that was equilibrated with 0.1 M NaCl. The column was eluted using 0.1 M NaCl with a flow rate of 22 mL/h, and 2.2 mL fractions were collected. Protein elution was monitored at 280 nm using Shimadzu spectrophotometer (1601A). Peak fractions were analyzed for neurotoxic activity, and peaks having activity were pooled and concentrated by lyophilization.

2.3. CM-Sephadex C-25 Column Chromatography. The neurotoxin fraction recovered from the previous step (30 mg) was desalted on G-10 column and loaded onto the CM-Sephadex C-25 column (1.6 × 25 cm) that was equilibrated with 0.05 M sodium phosphate buffer pH 7.0. Column was eluted stepwise using phosphate buffer of 0.05 M–0.1 M at pH values 7.0–7.5. Fractionation was carried out at a flow rate of 22 mL/h and 2.2 mL; fractions were collected. Finally fractions having neurotoxic activity were pooled and concentrated by lyophilization.

2.4. SDS-Polyacrylamide Gel Electrophoresis. SDS-PAGE (10%) was carried out as previously described method [17]. Electrophoresis was carried out using Tris (25 mM), glycine (192 mM), and SDS (0.1%) for 3 h at 90 V at room temperature. After electrophoresis, gels were stained with 0.1% Coomassie brilliant blue R-250 for detection of the protein bands and destained with 25% ethanol and 8% acetic acid. Molecular weight standards from 14.3 to 200 kDa were used.

2.5. Reversed Phase HPLC (RP-HPLC). Neurotoxin was subjected to RP-HPLC using Vydac C_4 column (5 μm, 0.21 × 25 cm) that had been preequilibrated with 0.1% TFA in water. The column was eluted using linear gradient from solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) for 40 min, and the protein was eluted at a flow rate of 1 mL/min and monitored at 280 nm.

2.6. Mass Spectrometry. The molecular mass of neurotoxin was determined by mass spectrometry using Voyager DE-PRO Matrix-Assisted Laser Desorption Ionization Time Of Flight (MALDI-TOF) machine in positive ionization mode. α-Cyano-4-hydroxycinnamic acid was used as MALDI matrix.

2.7. Circular Dichroism (CD) Measurements. Far UV CD spectra (260 nm–180 nm) were recorded using a Jasco J 725 spectropolarimeter (Jasco, Japan). Data were collected at 0.1 nm with a scan rate of 100 nm/min and a time constant of 0.5 s. The concentration of the neurotoxin was 40 μg/mL. Data were the average of 10 separate recordings and were analyzed by the method of Bohm et al. [18].

2.8. N-Terminal Sequencing. N-terminal sequencing of the neurotoxin was performed by automated Edman degradation using a Procise 494 pulsed liquid-phase protein sequencer (Applied Biosystems) with an on-line 785A phenylthiohydantoin derivative analyzer. The phenylthiohydantoin amino acids were sequentially identified by mapping the respective separation profiles with the standard chromatogram.

2.9. Pharmacological Studies

2.9.1. Neurotoxicity. Neurotoxicity was done by chemographic method as previously described [19]. Briefly, sciatic nerve gastrocnemius muscle was removed from the double-pithed male frog (*Rana hexadactyla*) weighing 20–22 g. The isolated nerve muscle preparation was pinned in a Rhodorsil-lined (Rhone-Poulenc, St. Fons, France) Plexiglas chamber (15 mL capacity) and bathed in Ringers solution. The resting tension was adjusted for each preparation investigated, in order to obtain maximal contractile response upon indirect stimulation. The nerve was stimulated at 5 min intervals by a single impulse (1 ms duration, supramaximal voltage), and the twitches were recorded. Different concentrations

of neurotoxin (10–50 μg/mL) and whole venom (50 μg/mL) were used.

2.9.2. Coagulant Activity, Edema Inducing Activity, Hemorrhagic Activity, Hemolytic Activity, and LD_{50}. The lethal potency was assessed as previously described method [20]. Briefly, neurotoxin in 0.5 mL saline with dose up to 5 mg/kg bodyweight was injected into a group of mice ($n = 10$) weighing 20–22 g. The mice were constantly observed for the symptom(s) of neurotoxicity, and the survival time was recorded for 24 h. Mice were sacrificed after 24 h, and the postmortem examination was done. Coagulant activity (pro/anti) was determined by prothrombin time according to the described method [21]. Edema inducing activity was determined as previously described method [22]. Hemorrhagic activity was performed according previously described method [23]. Direct and indirect hemolytic activities were assayed using erythrocytes separated from freshly collected blood from the healthy volunteers as previously described method [24].

2.9.3. Protein Estimation. Protein concentration was determined as previously described [25] using bovine serum albumin (BSA) as standard. All data were presented as mean from three independent experiments.

3. Results

A two-step protocol was standardized for neurotoxin purification. The first step involves the Sephadex G-100 gel filtration chromatography, which fractionated *H. partita* venom gland extract into four peaks (Figure 1(a)). Fractions with neurotoxic activity (peak IV) were pooled and concentrated by lyophilization. About 82% of the activity and 30% of the protein loaded onto the column were recovered in the pooled fraction. The second step involves the CM-Sephadex C-25 column chromatography and got resolved into two peaks (Figure 1(b)). Of the two peaks the second peak showed the neurotoxic property. About 74% of activity and 20% of the protein loaded onto the column were recovered. A summary of the purification is given in Table 1. Neurotoxin eluted as a sharp peak with retention time of 13.36 min in reversed phase HPLC on a C_4 column (Figure 1(c)). The purified neurotoxin moved as a sharp band to the same extent in SDS-PAGE under both reduced and nonreduced conditions (Figure 1(d)). It revealed a single symmetrical sharp peak at m/e of 19.725 on MALDI-TOF mass spectrometry (Figure 1(e)), while the apparent calculated mass was found to be 19 kDa in SDS-PAGE. The N-terminal sequence analysis of the neurotoxin gave the sequence of *GVDKASFCIPFKSDENCCKK*.

Neurotoxic property was studied using frog's sciatic nerve gastrocnemius muscle preparation. The isolated sciatic nerve gastrocnemius muscle preparation in Ringer's solution was evoked by indirect stimulation with an interval of 5 min. The twitch height of the control muscle contraction was well maintained and with a gradual decrease in height and attained zero twitch height at about 90 min. Upon addition of the neurotoxin into Ringer's solution, there was observed a gradual decrease in twitching efficiency of the gastrocnemius muscle as there was a decreased twitch height seen. The inhibitory effect was found to be dose dependent. The twitching was inhibited maximally at an optimum concentration of 40 μg/mL, where the twitch was abolished at 25 min following the addition of neurotoxin (Figure 2). While at 40 μg/mL concentration, the whole venom abolished the twitching at 35 min. In either case, the inhibition of twitching was not restored by the addition of submaximal concentration of acetyl choline into Ringer's solution. Therefore, the neurotoxin appears to act postsynaptically.

Neurotoxin was studied for other pharmacological properties such as coagulant activity, edema inducing activity, and hemolytic activity. However, neurotoxin was devoid of any of the activities even when used at a higher concentration of 200 μg/mL. Neurotoxin was found to be non-lethal up to the concentration of 5 mg/kg bodyweight but induced severe neurotoxin symptoms such as hind limb paralysis, respiratory distress, and loss of movement.

The secondary structure of neurotoxin was analyzed by circular dichroism spectrometry in phosphate buffer and in 60% solution of TFE. The aqueous solution of TFE promotes hydrogen bonding, and it is considered to mimic a cell membrane environment. Neurotoxin adopted α-helix structure with negative ellipticity at 208 and 222 nm in 60% TFE (Figure 3).

Partial sequence of neurotoxin from *H. partita* compared for its sequence homology with other neurotoxins showed maximum homology with SNX482 toxin from *Hysterocrates gigas*. Other toxins such as Omega Atracotoxin Hv1a, Hanatoxin, Sgtx 1, Omega agatoxins, and Phospholipase-D from *Loxosceles intermedia* showed partial sequence homology with the neurotoxin (Table 2).

4. Discussion

This study describes the isolation and characterization of a neurotoxin, which is postsynaptic in nature from *H. partita* spider venom. Neurotoxin has been purified to 74% activity recovery with 20% final protein yield (Table 1). Purity was adjudged by reversed phase HPLC on a C_4 Vydac column, MALDI-TOF mass spectrometry, and N-terminal sequence analysis. Neurotoxin is a single chain protein as it revealed single band in SDS-PAGE under both reduced and nonreduced conditions. The molecular weight was found to be 19.725 kDa in MALDI-TOF mass spectrometry. The N-terminal sequence analysis of the neurotoxin gave the sequence of *GVDKASFCIPFKSDENCCKK*. The CD spectra revealed presence of α-helix structure. Similar kinds of CD spectra were reported earlier [26–28]. The neurotoxin was devoid of enzymatic activities such as caseinolytic, hyaluronidase, and PLA_2 activities which were associated with whole venom [14].

Nonenzymatic neurotoxins from spider venoms interfere in neurotransmission and are highly specific in their action. These have immense potential as lead research tool molecules in drug discovery. Neurotoxins mainly help in acquisition of prey. Neuroparalysis leading to itching, acute pain, and

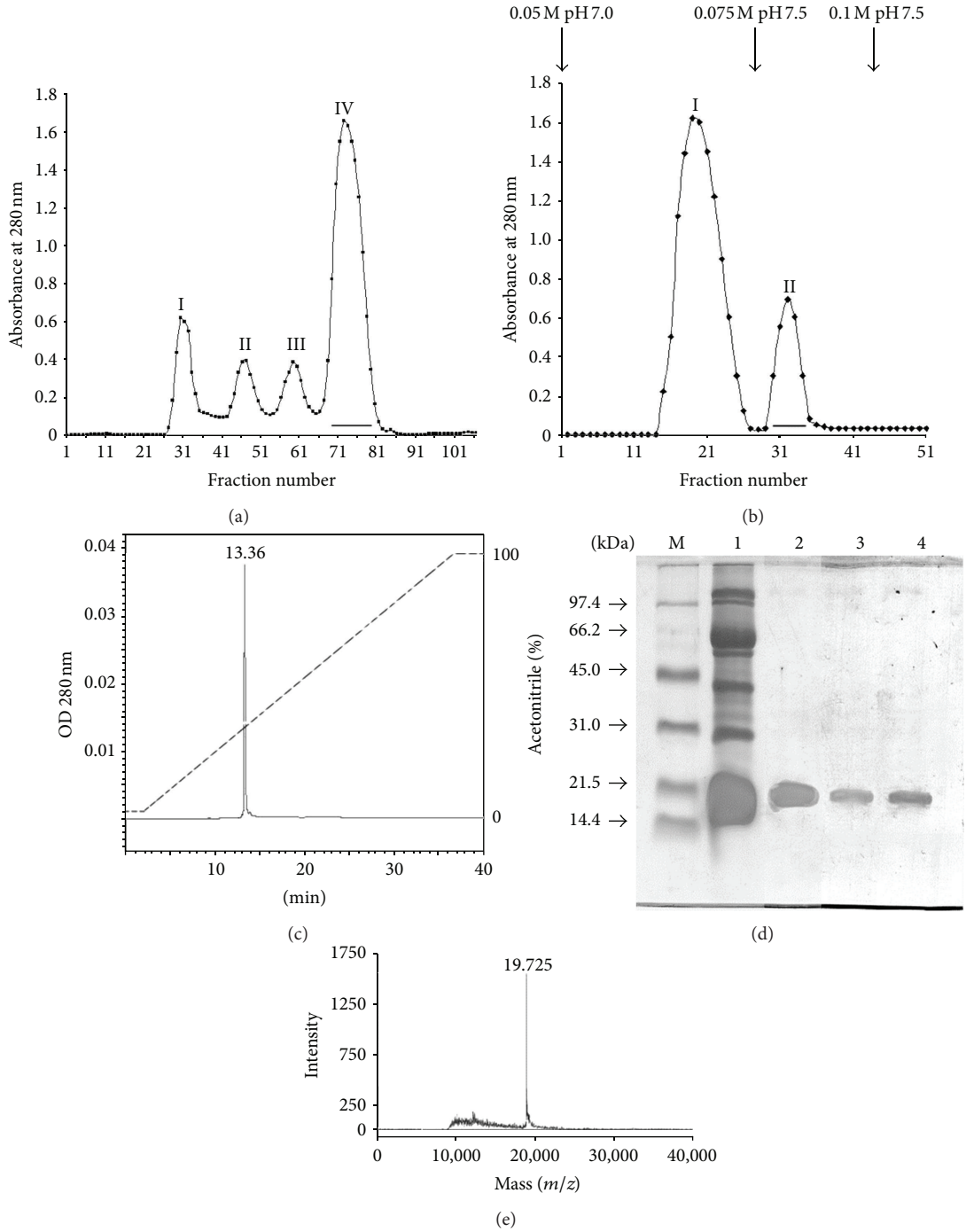

Figure 1: Isolation of neurotoxin from *H. partita* venom. (a) Elution profile from Sephadex G-100 column chromatography. The column (1.5 × 75 cm) was eluted with 0.1 M NaCl at a flow rate of 22 mL/hr, and 2.2 mL fractions were collected. Protein elution was monitored at 280 nm (—). Neurotoxin fractions (bar line) were pooled, concentrated, desalted, and applied onto CM-Sephadex C-25 column for further fractionation. (b) Elution profile from CM-Sephadex C-25 column chromatography. The column (1.6 × 25 cm) was equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). Subfractions were eluted stepwise using sodium phosphate buffer of different molarities (0.05 M–0.1 M) and pH (7.0–7.5). Neurotoxin fractions (bar line) were pooled, concentrated, and desalted. (c) RP-HPLC profile of neurotoxin on a Vydac C_4 column (5 μm, 0.21 × 25 cm), that had been equilibrated with 0.1% TFA in water. Protein was eluted using linear gradient from solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) over 40 min. Protein was eluted at a flow rate of 1 mL/min and monitored at 280 nm. (d) SDS-PAGE pattern of purified neurotoxin. Purification of neurotoxin as shown in SDS-PAGE (10%). Samples containing 80 μg *H. partita* venom (1), 75 μg Sephadex G-100 fraction (2), and 20 μg of neurotoxin under nonreduced (3) and reduced (4) conditions. M represents the molecular weight markers in kDa (from top to bottom: phosphorylase b (97.4), bovine serum albumin (66.2), ovalbumin (45.0), carbonic anhydrase (31.0), soya bean trypsin inhibitor (21.5), and lysozyme (14.4)). (e) Mass spectrometry of neurotoxin using Voyager DE-PRO (PerSeptive Biosystems) MALDI-TOF machine in positive ionization mode. 3,5-Dimethoxy-4-cinnamic acid is used as matrix.

Table 1: Summary of purification of neurotoxin from *H. partita* venom. Values are average of three independent experiments.

Procedure	Total protein (mg)	Protein recovery (%)	Activity recovery (%)
Venom gland extract	100	100	100
Sephadex G-100 column (peak IV)	30	30	82
CM-Sephadex C-25 column (peak II)	6	20	74
Purified neurotoxin (considering the protein loaded on first step, after desalting and lyophilizing)	**5**	**5**	**74**

Values are average of three independent experiments.

Table 2: Primary amino acid sequence homology of *H. partita* neurotoxin, SNX482, Omega Atracotoxin Hv1a, Hanatoxin, Sgtx1, Omega agatoxins, and Phospholipase-D from *Loxosceles intermedia*. Underlined residues in the alignment are amino acids found in *H. partita* neurotoxin either as identical amino acids or as conserved amino acids.

H. Partita neurotoxin	GVDKASFCIPFKSDENCCKK
SNX 482 from *Hysterocrates gigas*	GVDKAGCRYMFGGCSCNDDCCPRLGCHSLFSYCAWDLTF
Omega Atracotoxin Hv1a	CIPSGQPCPYNENCCSQSCT GGRCD
Hanatoxin	SDCCK
Sgtx 1	DCCK
Omega agatoxins	NCECK
PL-D from *L. intermedia*	NCKK

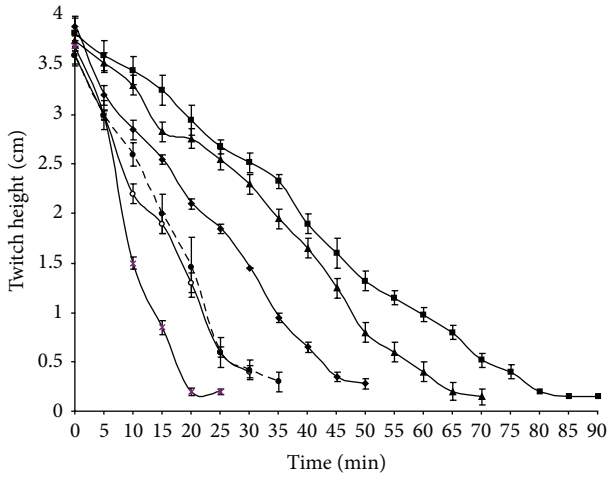

Figure 2: Effect of neurotoxin and *H. partita* venom on frog's sciatic nerve gastrocnemius muscle preparation. Sciatic nerve gastrocnemius muscle was indirectly stimulated at 5 min intervals by single impulse (1 ms duration). Different concentrations of neurotoxin (0 [-■-], 10 [-▲-], 20 [-◆-], 30 [-○-], and 40 [-x-] μg/mL) (solid lines) and *H. partita* whole venom 40 [-●-] μg/mL (broken line) added to Ringer's solution in organ bath, the twitching was followed independently, and the twitch height was recorded. Each point represents mean ± SD of three independent experiments. *indicates significant differences compared to control group as determined by one-way ANOVA, DMRT test at $P < 0.001$.

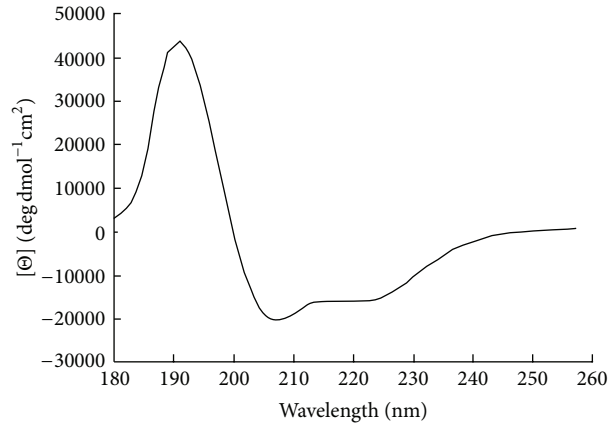

Figure 3: Far UV-CD spectrum of neurotoxin. The protein was dissolved in MilliQ water (0.5 mg/mL), and the CD spectra were recorded using a 0.1 cm path length cuvette.

muscle cramp were the preliminary symptoms of *H. partita* spider envenomation [14]. We observed the similar kind of behavioral reactions after intraperitoneal injection of neurotoxin into mice during the assay of LD_{50}.

The neurotoxin purified from *H. partita* was found to be not lethal up to the tested dose of 5 mg/kg weight in mouse model but caused intense neurotoxic symptoms such as hind limb paralysis; respiratory distress, loss of movement, and the postmortem examination did not reveal any visible damage to any of the vital organs. Similarly, a postsynaptic neurotoxin affecting the frog's neuromuscular junction was isolated from *Agelenopsis aperta* spider venom [29]. The toxin HwTx-I from *Selenocosmia huwena* venom inhibited indirectly evoked twitches in mouse phrenic nerve diaphragm preparation without affecting directly evoked twitches [26, 30]. The neurotoxicity of spider venoms and their purified neurotoxins were also tested on the neuromuscular junction of insects. The neurotoxin ω-Aga IA isolated from *Agelenopsis aperta* spider venom blocks voltage gated

calcium ion channels in *Drosophila* neuromuscular junction [27], and Huwentoxin-II from the venom of the spider *S. huwena* causes reversible paralytic effect in cockroaches [28]. The sequence homology showed maximum homology with SNX482 toxin from *Hysterocrates gigas* which is a specific Ca^{+} and Na^{+} channel blocker. This further supports the probable mode of action of neurotoxin.

In conclusion, this study presents the first neurotoxin from *H. partita* spider venom. Further studies revealing the mechanism of action including its effect on specific ion channels appear to be interesting.

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