Research Paper:
Cardiotoxic Effects of *Hemiscorpius Lepturus* Scorpion Venom Fractions in Rats

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**Background:** Scorpion stings are responsible for many deaths in humans; however, the toxicity mechanisms of the venoms from many species are not well studied. We investigated the cardiotoxicity of the crude venom from *H. lepturus* scorpion and its isolated fractions, F-I to F-VI.

**Methods:** The scorpion’s venom was extracted into six fractions by chromatography. Healthy male Wistar rats (N=72) were equally divided into eight groups of nine: G1: Controls (0.5ml. normal saline), G2: Crude venom (1000µg/kg), G3: F-I (120µg/kg), G4: F-II (430µg/kg), G5: F-III (80 µg/kg), G6: F-IV (180µg/kg), G7: F-V (60µg/kg), and G8: F-VI (130µg/kg). Blood samples were obtained by cardiac puncture at 1, 3 and 24 hours after the venom injection. The serum levels of AST, LDH, CPK, CK-MB and troponin-I were determined. Upon euthanasia, the hearts were removed from the rats and examined microscopically for histopathology.

**Results:** In groups receiving crude venom and F-VI, we observed multifocal fragmentation of myocardial fibers, hemorrhage, degeneration and disappearance of striations in cardiac muscles as compared to the controls. The findings showed that AST and LDH activity in groups 2, 4 and 8, CPK activity in groups 2, 4, 6 and 8 and CK-MB activity and troponin-I levels in groups 2 and 8 increased significantly compared to those in the control group.

**Conclusion:** There was evidence of significant cardiotoxicity in the group receiving crude venom and F-VI. Although alterations in the enzymatic and troponin-I levels were observed in other groups, the greatest cardiotoxicity of *H. lepturus* venom was caused by fraction VI.

**Keywords:** *Hemiscorpius lepturus*, Scorpion, Cardiotoxicity, Venom, Fractions, Rats

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**ABSTRACT**

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**Introduction**

Scorpion stings are responsible for many deaths in infants, children and adults in developing countries worldwide [1]. There are six types of venomous scorpions in Iran, which are grouped into Hemiscorpiidae and Buthidae species. *Hemiscorpius lepturus* (*H. lepturus*) scorpions belong to the Hemiscorpiidae family and are endemic to the southern region of Iran. Exclusive and morphologic characteristics of this species include small bead-shaped and jointed tail, resembling cow’s tail [2].

The *H. lepturus* sting is rather painless and leaves a cutaneous mark ranging from a small erythema to a large ulceration and necrotic spot at the sting site. Additional symptoms following the initial sting may include lethargy, pyrexia, hypotension, perspiration, tachycardia, gastrointestinal symptoms, convulsions, internal hem-
orrhage, and severe hematuria. Constituting only 10 to 25% of all scorpion stings in Khuzestan, a southwestern province in Iran, H. lepturus sting is the cause of 92% of the hospitalized cases and 89% of the deaths due to scorpion stings [2, 3]. As such, H. lepturus is recognized as the most dangerous scorpion in Iran [1], which is considered an emergency requiring immediate medical attention and clinical care [2].

The H. lepturus venom contains various pathological enzymes including Hemitoxin, Hemicalcin and Heminecrolysin, which contribute to its hemolytic, neurotoxic and cytotoxic effects in various human and animal organs and tissues [4, 5]. Likewise, experimental envenomation with H. lepturus venom has caused myocardial damage, bradycardia and low cardiac output associated with the inhibition of acetylcholine and epinephrine release [6]. Cardiotoxicity has been reported in experimental and clinical studies due to a variety of scorpion venoms. For instance, Bouimeja et al found that the venom from Buthus occitanus scorpion induced acute histopathological alterations in kidneys, lungs, liver and heart, causing hemorrhage and myofibrilar degeneration of the myocytes [7].

In addition, Nouira et al reported that the high levels of epinephrine and norepinephrine in the blood were significantly correlated with the intravenous injection of Androctonus australis venom [8]. The venom induced hemodynamic adverse effects, including low cardiac output, increased mean systemic and pulmonary arterial blood pressure [8]. Likewise, in three clinical cases of scorpion stings, in addition to myocardial dysfunction, pulmonary edema and shock were reported as the most serious complications following Androctonus mauritanicus sting [9]. In another study, Hadruroides lunatus venom was shown to have cardiotoxic effects, possibly through the action of neurotoxins on the voltage gated ion channels in the myocardium [10].

Materials and Methods

Despite the high population of scorpions in Iran and the clinical consequences of their stings in humans and animals, details of the mechanism of toxicity for many species have not been sufficiently investigated. Studying the venom toxicity of the Iranian scorpions by the isolated fractions enables researchers to identify the target organs associated with each fraction and the route of toxin spread. This will assist the clinicians to develop the necessary therapeutic measures and effective antidotes against the specific, toxic fractions. In this context, this study was conducted to identify the various fractions of H. lepturus scorpion venom and investigate the mechanism of toxicity. We also compared the cardiotoxic effects of the crude venom versus those of the fractions in rats, using specific biochemical markers.

Collection and assessment of the crude venom: H. lepturus scorpions from Khuzestan were provided by Razi Vaccine and Serum Research Institute of Iran (Karaj, Iran). The venom was collected by mild electrical stimulation of each scorpion’s telson, lyophilized by Freeze drying at -75°C and stored at 20°C. Then, 50 mg of the crude venom was dissolved in 10 ml deionized water, vortexed on ice for three hours and stored at 4°C overnight. The solution was then centrifuged at 13000g for 5 min at 4°C to remove the undissolved materials. Subsequently, the venom was dissolved in 5 ml 20 mM ammonium acetate buffer (pH: 8.6), and purified by gel chromatography on a Sephadex G-50 column (2.5×125 cm). The prepared venom was then injected into the column, and the components were collected at a flow rate of 3 mL/min by a fraction collector over 18 hours. The optical absorbance of the eluent was later measured at 280 nm and the absorbance peaks were mixed and lyophilized.

Assessment of protein fractions: Using Bradford method to measure the protein concentration in the isolated fractions, we analyzed the collected fractions by SDS Polyacrylamide Gel Electrophoresis (PAGE), and visualized the components by Coomassie blue staining.

Determination of LD50: To determine the LD50, we injected the Venom Intravenously (IV) into albino mice (average weight 18-20g). To ensure safety, mice were chosen over rats for the determination of LD50 due to their high sensitivity to the venom. After the mice were injected with the venom, they were monitored for 24 hours. The Spearman-Kaerber method [11] was then used to determine the LD50.

Animals: A total of 72 healthy male Wistar rats weighing 200±20 grams were obtained from the laboratory animal house at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. They were kept at constant room temperature (25°C) under a 12 hour dark-light cycle. The rats had free access to standard pellet food and tap water. They were then separated randomly into eight groups of nine rats each, and were injected intraperitoneally with normal saline, the crude venom, or the fractions of H. lepturus venom. The dose of each fraction was selected based on an effective percentage of the fraction venom as listed below:

- Group 1: Controls = 0.5ml (normal saline)
- Group 2: 1000 µg/kg, crude venom
- Group 3: 120 µg/kg, fraction I
- Group 4: 430 µg/kg, fraction II
- Group 5: 80 µg/kg, fraction III
- Group 6: 180 µg/kg, fraction IV
- Group 7: 60 µg/kg, fraction V
- Group 8: 130 µg/kg, fraction VI

**Blood sample collection:** Before the blood collection, an intraperitoneal injection of a 60 mg/kg dose of pentobarbital sodium was used to anesthetize the animals. Heparinized blood samples were then collected by cardiac punctures at 1, 3 and 24 hours after the injection of the crude venom or the fractions. The blood samples were centrifuged at 4000g for 10 min and the plasma was separated for later biochemical analyses.

**Biochemical analyses:** The serum levels of aspartate aminotransferase (AST), Lactate Dehydrogenase (LDH), Creatine Phosphokinase (CPK) and CK-MB enzyme were determined, using Pars-Azmoon Diagnostic kits (Tehran, Iran) and a biochemical automated analyzer (BT-1500, Italy). The serum cardiac troponin-I was measured, using Mybiosource, an ELISA kit for rats (Catalog #: MBS727624; San Diego, CA, USA).

**Histopathological studies:** The heart tissues, pre-dissected and fixed in 10% formalin solution, were embedded in paraffin blocks for sectioning. The block were then sectioned at five micron and stained with Haematoxylin and Eosin (H&E). Subsequently, Phosphotungstic Acid-Hematoxylin (PTAH) staining was used to visualize the degenerate and damaged cardiac myofibrils. These sections were then examined histopathologically under light microscopy (Nikon-BH2, Japan).

**Statistical analyses:** The data were reported as Means±SEM and subjected to a One-way Analysis of Variance (ANOVA). The LSD test was used to examine the post-hoc differences among the group means, and the P values lower than 0.05 were considered as significant. The computer software SPSS V. 16.0 was used to perform the statistical analyses of the data.

**Results**

**Determination of LD50:** The LD50 values were determined to be 6.2mg/kg, based on its IV injection into the albino mice.

**Protein purification:** The gel chromatography of the crude venom provided six peaks, represented by the fractions F-I to F-VI. The optical density values of these peaks are shown in Figure 1. The protein concentrations in fractions F-I to F-VI were estimated to be 8.04, 28.81, 5.36, 12.06, 4.02 and 8.71mg, respectively. As shown in Table 1, the yield rates of 100mg of the crude venom in the six fractions were 12%, 43%, 8%, 18%, 6%, and 13%, respectively.

**SDS-PAGE electrophoresis:** The six fractions collected from the gel chromatography were subjected to 12% SDS-PAGE, and the protein profiles of the venom were identified (Figure 2). The molecular weights of the venom proteins were between ≥160 and ≤5 kDa. The molecular weights of the proteins in F-I fraction ranged between 160 to 25 kDa. The proteins isolated from the F-II fraction had a molecular weight of 25-28 kDa. The fraction II produced detectable bands with molecular weights of 25, 22.14 and 8 kDa while the molecular weights of F-III bands were about 14 and 8 kDa, respectively. Bands less than 5 kDa were observed in three tracts represented by F-IV, F-V and F-VI fractions.

**Biochemical analysis:** Biochemical markers, such as LDH and AST can be used to detect tissue injury resulting from the scorpion envenomation. The plasma levels for CPK and its cardiac isoenzyme and CPK-MB in combination with cardiac troponin were measured in the plasma of rats, envenomated with the crude venom and the fractions. The plasma AST (Figure 3) and LDH (Figure 4) levels increased significantly in groups 2, 4 and 8 (P<0.05) at the 1st, 3rd and 24th hours after the injection of the crude venom, fractions F-II and F-VI compared with those of the control and other groups, indicative of tissue damage.

**Table 1.** Protein contents and yields of H. lepturus venom fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>F I</th>
<th>F II</th>
<th>F III</th>
<th>F IV</th>
<th>F V</th>
<th>F VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg)</td>
<td>8.04</td>
<td>28.81</td>
<td>5.36</td>
<td>12.06</td>
<td>4.02</td>
<td>8.71</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>12%</td>
<td>43%</td>
<td>8%</td>
<td>18%</td>
<td>6%</td>
<td>13%</td>
</tr>
</tbody>
</table>
Significant increases in the plasma CPK concentration (P<0.05) were observed for groups 2, 4, 6 and 8 at the 1st, 3rd and 24th hours post-injection of the crude venom, and its fractions FII, F-IV and F-VI compared to the control and the remaining groups (Figure 5). In groups 2 and 8, the levels of CK-MB (Figure 6) and cardiac troponin-I (Figure 7) significantly increased (P<0.05) at 1st, 3rd and 24th hour’s post-injection of the crude venom and its fraction VI compared to the controls and other groups.

**Histopathological examinations:** The microscopic examination of the heart tissue in the groups receiving the crude venom revealed remarkable alterations, shown as multifocal fragmentations of the myocardial fibers as compared to the controls (Figures 8A & 8C). Moreover, hemorrhage was detected among the degenerated fibers (Figure 8C). Likewise, the histopathological study of the venom fractions in various groups showed degeneration and hemorrhage in F-VI group (Figure 8E). In PTAH stained groups, the striations of the cardiac muscles disappeared as compared to those in the controls (Figures 8B, 8D & 8F).

![Figure 1. Sephadex G-50 gel filtration curve of H. lepturus venom](image1)

![Figure 2. SDS-P gel electrophoresis of H. lepturus venom fractions](image2)

![Figure 3. Mean±SE of plasma AST in various experimental groups over three collection times](image3)
Cardiac damage is one of the most life threatening complications of scorpion envenomation in the victims [6, 12, 13]. This study investigated the toxicity of various fractions of H. lepturus scorpion venom. We compared the cardiotoxic effects of the crude venom versus those of its fractions in rats, testing for specific biochemical markers. The components of the venom are mostly peptides with various biological activities [7]. These include peptides that inhibit potassium (Hemitoxin) [5] and calcium channels (Hemicalsin) [4]. Other toxic proteins have also been identified, such as Heminecrolysin, with dermonecrotic activity [14, 15] and Hemilipin that inhibits angiogenesis [16, 17].

**Discussion**

Cardiac damage is one of the most life threatening complications of scorpion envenomation in the victims [6, 12, 13]. This study investigated the toxicity of various fractions of H. lepturus scorpion venom. We compared the cardiotoxic effects of the crude venom versus those of its fractions in rats, testing for specific biochemical markers. The components of the venom are mostly peptides with various biological activities [7]. These include peptides that inhibit potassium (Hemitoxin) [5] and calcium channels (Hemicalsin) [4]. Other toxic proteins have also been identified, such as Heminecrolysin, with dermonecrotic activity [14, 15] and Hemilipin that inhibits angiogenesis [16, 17].

**Cardiotoxicity effects:** In our study, the biomarkers of cardiac injury including troponin-I and CK-MB showed the highest increase in the groups receiving crude ven-

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**Figure 4.** Mean±SE of plasma LDH in various groups over three collection times

*, ** & *** Significant deference compared to other groups 1, 3 and 24 hours, respectively, after injection (P<0.05).

**Figure 5.** Mean±SE of plasma CPK in various groups over three collection times

*, ** & ***Significant deference compared to other groups 1, 3 and 24 hours, respectively, after injection (P<0.05).

**Figure 6.** Mean±SE of plasma CK-MB in various groups over three collection times

*, ** & ***Significant deference compared to other groups 1, 3 and 24 hours, respectively, after injection (P<0.05).
om and the fraction VI after 24h of exposure. The rise in the serum troponin-I and CK-MB enzymes has been suggested as a consequence of heart muscle damage [18, 19]. Together with a rise in both serum LDH and CPK levels [13, 20], the early increase in the serum troponin-I has been suggested to be a specific and sensitive measure of cardiac muscle injury in response to scorpion envenomation. Therefore, the early assay of troponin-I after the envenomation may serve as a helpful method of detecting the risk of myocardial injury in the victims [21]. Consistently, Ait Laaradia et al [22] have shown that Bothus lieniardi scorpion sting has significantly increased the serum levels of LDH and CPK enzymes due lesions developing in the myocardial and pulmonary tissues. The rise in the serum levels of these enzymes is also likely to be due to damages in tissues other than the heart [22].

**Microscopic findings:** Various theories have been suggested to account for the cardiac muscle injury following scorpion envenomation. In this study, the cardiac muscles demonstrated considerable degradation after exposure to the crude venom or its fraction VI compared to those in other groups. Microscopically, the myofibrils developed degeneration and hemorrhage 24 hours after

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Figure 7. Mean±SE of plasma Troponin-I in various groups over three collection time

*, ** & ***Significant deference compared to other groups 1, 3 and 24 hours, respectively, after injection (P<0.05).

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Figure 8. Photomicrographs of the heart tissue

A & B: Control group; C & D: Crude venom; E & F: F VI fraction;
A: Note normal structure of cardiac muscle fibers; B: Striations of fibers (sarcomers) are obvious (white arrow);
C & E: Note splitting of fibers (black arrows) and erythrocytes between fragmented fibers (hemorrhage);
E & F: Tearing fibers (black arrows) and destroying the striation are obvious;
A, C and E: H & E, Bar: 20 µm; B, D and F: PTAH staining, Bar: 10 µm).
the envenomation with the crude venom or the fraction VI. These findings are consistent with those reported by a number of previous studies. For instance, Heidarpour et al [13] observed that the administration of *H. lepturnus* venom produced severe myocytolysis plus interstitial necrosis within 3-6 hours of the envenomation [13].

Likewise, El Hidjan et al [23] reported cardiac tissue degeneration three hours after the injection of *Hottentota gentilis* venom into laboratory animals [23]. Besides, Sifi et al [24] found massive alterations in myocardial tissue after the envenomation with *Androctonus australis* hector. The detrimental effects consisted of hemorrhage, necrosis, interstitial edema, and hypertrophy of the myocardial fibers, massively infiltrated with leukocytes [24]. In another study, focal fragmentation of myocardial fibers and hemorrhage were reported in response to *Butthus lienihardii* venom exposure [22].

Clinical manifestations: The clinical manifestations of the toxins, mainly cardiogenic shock and pulmonary edema, are the leading causes of death after exposure to the scorpion venom [25, 26]. There have been studies reporting fatal cases of cardiac dysfunction due to myocardial lesions after scorpion envenomation. For instance, Benvenuti [27] observed coagulative myocytolysis and necrosis in the victims after envenomation by *Tityus serrulatus* scorpion [27]. In another study, Aboumaad et al [9] reported that three severe cases of patients who had been stung by *Androctonus mauritanicus* scorpion developed severe complications of the myocardium. [9]

In the current study, Group 8 that received fraction VI of the venom, a rise in the cardiac biomarkers was observed. It is likely that the component of fraction VI reached the cardiac muscle rapidly because it had a molecular weight of less than 5 KDa, leading to cardiotoxic damages manifested earlier than the toxic components of other fractions.

Toxic effects on liver and kidneys: Studies have demonstrated complications, such as hemolytic, nephrotoxic, and hepatotoxic effects, following exposure to the venom of this scorpion [2, 28]. Jalali et al [3] have reported that significant rises in the serum levels of AST, LDH, ALT and CPK occurred in their laboratory animals after envenomation with *H. lepturnus* sting, likely due to direct tissue damages [3]. In pediatric cases, another study has reported significant rises in the serum levels of CPK, LDH, ALT and AST enzymes observed in over 200 children who were stung by *H. lepturnus* scorpion [13].

**Toxic effects on other organ tissues:** In this study, all serum biochemical markers increased in the groups that received the crude venom. Correa et al [29] have suggested that the in vivo enzymatic and histopathological changes are the results of tissue damages induced by the whole venom and/or its isolated fractions [29]. Our findings demonstrated that the serum levels of AST, CPK and LDH increased in all groups that were exposed to the venom, suggesting that liver, lungs, heart, and muscles were considered the potential targets of the toxicity [30]. The cytotoxicity has also been reported previously in the intestine, stomach and kidneys of mice [13]. The rises in the LDH and CPK levels serve as useful diagnostic and prognostic tests in humans [3]. The direct stimulatory effect of the venom on the adrenals and sympathetic nervous system is known to induce a significant release of catecholamines [20, 22]. The excess catecholamines can cause the accumulation of endothelins, leading to major vasoconstriction. Endothelins are vasoconstrictor peptides released primarily from the endothelium and have a key role in vascular homeostasis [31].

The unchallenged effects of α-receptors’ stimulation can result in the suppression of insulin secretion, hyperkalemia, hyperglycemia, and the accumulation of fatty acids and free radicals that are detrimental to bodily tissues including myocardium [21]. However, it is believed that most of the symptoms caused by the scorpion venom are due to large amounts of neurotransmitters released due to the autonomic storm [3]. The massive tissue injury following *H. lepturnus* stings is likely caused by the toxic effects of some proteins with gelatinolytic, casinolytic, and hyaluronidase activities [13]. The low molecular weight peptides present in *H. lepturnus* venom may quickly reach critical target organs, such as the heart and kidneys [32]. Lastly, one cannot rule out the indirect effect of the venom on the stimulation of acetylcholine and catecholamine release from the postganglionic nerve endings [29].

Limitations: Due to the presence of little amounts of the venom in each scorpion, a large number of them were needed for the venom extraction (1500 to 2000 scorpions for 100 mg). Because of the importance of the environmental conditions on the isolation of the venom fractions, the extraction process should be completed in one continuous step. Observing the required experimental temperature is essential during all extraction phases in order to avoid damages to the structure and function of the protein constituents.

Recommendations for Future Studies: For optimal evaluation the mechanism of action of each venom fraction on different organs, detailed future studies are warranted.
In this context, evaluation of the effects of each fraction may be considered in the presence of polyvalent antivenom agents. Identification of the toxic fractions will be a prelude to the production of antidote for each fraction.

Conclusions

The total peptides in the H. lepturus scorpion venom consist of polypeptides that are typically divided into various groups based on their structures, target sites and toxicities in mammals. We isolated six fractions from the venom, among which fraction VI had the most cardiotoxicity effects and contained components with the lowest molecular weights. Indeed, the release and distribution of proteins with low molecular weights in vital organs, such as the heart can be associated with subsequent disorders and complications. Some of the side effects of fractions VI were likely due to the compounds present in that fraction. The histopathological injuries observed in this study were supported by the biochemical analyses of the popular and reliable biomarkers.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Ethics Committee of Shahid Chamran University of Ahvaz (Ethics Code: EE/98.3.02.88218/SCU.AC.IR).

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Author's contributions

Investigation, writing original draft, data analysis: Moein Yazdkhasti; Study design, project administration: Mohammad Razi Jalali; Resources, Validation: Mohammad Razi Jalali, Gholam Hosein Khadjeh; Visualization: Gholam Hosein Khadjeh; Writing, review & editing: Mohammad Razi Jalali, Hedieh Jafari, Anmahita Rezaie; Conceptualization: Hedieh Jafari; Methodology, reading, final approve of the manuscript: All authors.

Conflict of interest

The authors declared no conflict of interest.

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References


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