

EVALUATION OF THE NEUTRALIZING CAPACITY OF *Androctonus crassicauda* (OLIVIER, 1807) ANTIVENOM AGAINST *Leiurus quinquestriatus* (EHRENBERG, 1928) VENOM (SCORPIONES: BUTHIDAE)

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ABSTRACT: The two most venomous species of the family Buthidae, *Leiurus quinquestriatus* and *Androctonus crassicauda*, are found in Africa and in the Middle East. Potency and paraspecific activities of *A. crassicauda* antivenom (RSHC anti-Ac) were tested against *L. quinquestriatus* venom.

The sera produced by Refik Saydam Hygiene Center (RSHC) showed strong reactivity against the venoms of *A. crassicauda* and *L. quinquestriatus* in western blotting and dot-blot analysis. RSHC anti-Ac presents immunoactivity and neutralizing potential against *Leiurus quinquestriatus* venom. Neutralization capacity of antivenom was found to be 400 µL against 40 minimum lethal doses (MLD) of *A. crassicauda* scorpion venom and 10 MLD of *L. quinquestriatus* venom. This study indicates that the RSHC anti-Ac could be used for treating *L. quinquestriatus* stings.

KEY WORDS: scorpion, *Androctonus crassicauda*, *Leiurus quinquestriatus*, venom, monovalent antivenom, paraspecific activity.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Scorpions use their venom effectively for offensive and defensive purposes (16). The number of dangerous species in the Buthidae family is significantly higher than in other scorpion families (17, 35, 44). Among this family, the species belonging to the genera *Androctonus* and *Leiurus* are considered the most significant to humans, causing a large number of envenomations in Africa and in the Middle East (11, 12, 14, 36). In particular, *Leiurus quinquestriatus* (Ehrenberg, 1928) and *Androctonus crassicauda* (Olivier, 1807) are recognized as the most poisonous (11, 25, 44). Also known as the “yellow scorpion” (37) *L. quinquestriatus* is found in a large desert region spanning Algeria, Mali, Tunisia, Libya, Niger, Chad, Sudan, Ethiopia, Somalia, Egypt, Israel, Jordan, Lebanon, Syria, Turkey, Kuwait, Saudi Arabia, Qatar, United Arab Emirates, Oman and Yemen (5, 6, 8, 13, 21, 26, 35, 41, 42, 45). Likewise, *A. crassicauda* is a highly relevant species in the Middle East (11, 14, 18) and is encountered in Azerbaijan, Armenia, southeast Turkey, Syria, Iran, Iraq, Jordan, Israel, Egypt (Sinai Peninsula), Saudi Arabia and Yemen (25, 27, 33, 41, 42).

Apart from antivenom, there is still no specific treatment for envenomation by scorpions. However, the antivenom’s role in the treatment of scorpion stings remains controversial and its effectiveness depends on its potency (4, 11, 39). In Turkey, scorpion antivenom has been produced at the Refik Saydam Hygiene Center (RSHC) since 1942 by immunizing horses with macerated *A. crassicauda* telsons (27, 41, 42). In addition to its efficacy against *A. crassicauda* envenomations, RSHC anti-Ac has been reported to be useful following the stings of other scorpion species as well (28, 41, 43).

Paraspecific activity of antivenom has clear practical advantages (if species-specific antivenom is unavailable), particularly in remote regions. Therefore, identification of the factors that lead to effective antivenom production is crucial. In this study, the potency and paraspecific activities of RSHC anti-Ac will be presented against *L. quinquestriatus* venom.

MATERIALS AND METHODS

Scorpions

A. crassicauda scorpions were collected in the southeastern Anatolia region (Mardin); *L. quinquestriatus* scorpions were collected in northern Iraq. Animals were

kept in plastic boxes and transferred to RSHC. The scorpions received water daily and were fed crickets or cockroaches after each milking.

Experimental Animals

Female *Swiss albino* mice weighing 25 ± 1 g were employed to determine minimum lethal dose (MLD), and minimum effective dose (MED) (28). They were kept in the experimental room at ambient temperature with $60 \pm 10\%$ humidity and were fed commercial rodent pellets and water *ad libitum* throughout the experiment.

Venom

Venom was milked from mature scorpions by telson electrical stimulation (28). It was diluted with equal volume of sterile double-distilled water and then centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant was removed and immediately stored at -20°C until use.

Antivenom

Antivenom was prepared from the same pool of hyperimmune serum obtained from horses immunized against *A. crassicauda* venom, according to an immunization protocol described by Ozkan *et al.* (27). Increasing venom doses, mixed half-and-half with adjuvants, were injected subcutaneously into horses on the 1st, 14th, 21st, 28th, 35th and 42nd days. On the 45th, 48th and 51st days, blood samples were collected three times from the jugular vein of each animal and stored in containers with 10% sodium citrate. After plasma separation, antivenom was obtained, from combined plasma, by the digestive method and kept in the dark at 4°C (27). One dose of RSHC anti-Ac was normalized to neutralize 2 MLD of *A. crassicauda* venom in rats when tested subcutaneously (1, 27).

Lethality Assay

The MLD for *A. crassicauda* and *L. quinquestriatus* toxins was determined by subcutaneous (SC) administration of venom, diluted in physiological saline solution (PSS) [0.9% (w/v) NaCl], to mice as described previously (28). Groups of five mice received *A. crassicauda* and *L. quinquestriatus* venoms at doses ranging from 1 to 12.5 μ L/mouse (for *L. quinquestriatus*) and 2.5 to 15.0 μ L/mouse (for *A.*

crassicauda). The injected venom volume was kept constant at 500 µL/mouse. The control group received PSS. Following treatment, the animals were monitored for 48 hours and lethality was recorded at the end of the experiment (28). The minimum dose that kills 100% of animals is considered the MLD (Table 1).

Table 1. The MLD was determined for *L. quinquestriatus* and *A. crassicauda* venoms by SC injection. Neutralization capacity of antivenom was assayed for both venoms in mice

MLD of scorpion venoms							
<i>A. crassicauda</i> scorpion				<i>L. quinquestriatus</i> scorpion			
Venom (µL/mouse)	Time (h)	Mice (death/total)		Venom (µL/mouse)	Time (h)	Mice (death/total)	
2.5	48	0/5		1.0	48	0/5	
5.0	48	1/5		3.0	48	2/5	
7.5	48	2/5		5.0*	48	5/5	
10.0*	48	5/5*		7.0	48	5/5	
12.5	48	5/5		9.0	48	5/5	
*MLD: 10 µL/mouse				*MLD: 5 µL/mouse			
MED of RSHC anti-Ac							
<i>A. crassicauda</i> scorpion				<i>L. quinquestriatus</i> scorpion			
Venom (X MLD)	Time (h)	Antivenom (µL)	Mice (surviving/total)	Venom (X MLD)	Time (h)	Antivenom (µL)	Mice (surviving/total)
40	48	100	0/6	10	48	100	0/6
40	48	400*	6/6	10	48	400*	6/6
40	48	700	6/6	10	48	700	6/6
40	48	1000	6/6	10	48	1000	6/6
*MED: 400 µL							

* MLD: minimum lethal dose; MED: minimum effective dose

Serum-neutralization Assays

To a series of tubes containing equal amounts of each individual venom, four different quantities of *A. crassicauda* antivenom were added. A 2.5 mL solution containing 40 MLD from *A. crassicauda* and 10 MLD from *L. quinquestriatus* venom were prepared in PSS, mixed with the antivenom and incubated at 37°C for 60 minutes. Groups of six mice were injected SC with these mixtures (Table 1). The injection volumes were kept constant at 0.5 mL/mouse in all groups. The control group was injected with PSS-venom solutions. The number of surviving mice was recorded for 48 hours after injections and the antivenom dose that protected mice was considered efficacious. The effective dose of horse serum was expressed as MED (28).

SDS-PAGE, Western Blotting and Dot Blot Analysis

Venoms were initially analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (23). Venom samples of *A. crassicauda* and *L. quinquestriatus* were separated on precast NuPAGE® 12% Bis-Tris gels (Invitrogen Corporation, USA) and were electrophoretically transferred to a nitrocellulose membrane (NCM) and divided into two sections. The membranes were incubated in blocking buffer – 3% bovine serum albumin (BSA) in Tris-Buffered Saline Tween-20 (TBST – 0.1% Tween 20, 150 mM NaCl, 10 mM Tris-Cl, pH 7.4) (Sigma-Aldrich, USA) – for 60 minutes.

The membranes were washed three times with TBST and then their strips were exposed to RSHC anti-Ac (1:4000 concentration) for one hour. Membranes were again washed three times with TBST and subsequently incubated with horseradish peroxidase (HRP) conjugated anti-horse antibody (1:5000) for 60 minutes. The membranes were washed with TBST for 10 minutes and antigens were visualized using Immun-Star® HRP Chemiluminescent substrate (BioRad, USA). Membranes were exposed to an X-ray film in a darkroom and developed.

For dot blot analysis NC membranes were divided into 1 cm² sections and samples of diluted venom (2 µL, 3 µL and 4 µL) were applied to the center of the grid by using a narrow-mouth pipette tip. The membranes were dried for 180 minutes at room temperature and a procedure similar to western blotting was followed from this point onward. Briefly, the membranes were blocked for one hour using BSA and later

incubated with the RSHC anti-Ac (1:1000) for one hour. The membranes were then washed and incubated with HRP-conjugated anti-horse antibody (1:5000) for 60 minutes. Afterwards, they were rewashed before reacting with Immun-Star® HRP Chemiluminescent substrate. Finally, the membranes were exposed to an X-ray film and developed in a darkroom.

RESULTS

The minimum lethal doses of the scorpion venoms were found to be 5 and 10 µL by SC injection, respectively, for *L. quinquestriatus* and *A. crassicauda* (Table 1). The potency of RSHC anti-Ac (500 µL) has previously been determined to neutralize 2 MLD in 150 g rats according to instructions. Neutralization capacity of RSHC anti-Ac was observed to be 400 µL against 40 MLD of *A. crassicauda* venom and 10 MLD of *L. quinquestriatus* venom, while all mice died in the control groups.

The antivenom reacted strongly with *A. crassicauda* venom. Western blotting also showed the presence of *L. quinquestriatus* venom components recognized by RSHC anti-Ac. Western blotting indicated proteins between 39,000 and 191,000 Da in both venoms (Figure 1).

Different protein concentrations in venoms were also estimated by dot blot. Figure 2 shows the strong reaction of *L. quinquestriatus* venom protein with RSHC anti-Ac.

High-performance liquid chromatography (HPLC) was employed to obtain the chromatographic profile of *A. crassicauda* venom, from which about 25 compounds were discretely separated as shown in Figure 3 (unpublished data).

Venom analysis by mass spectrometry (LC-ESI-HRMS) of [MH]⁺ detected 42 major components within 437 to 44.737 Da, as shown in Table 2 (unpublished data).

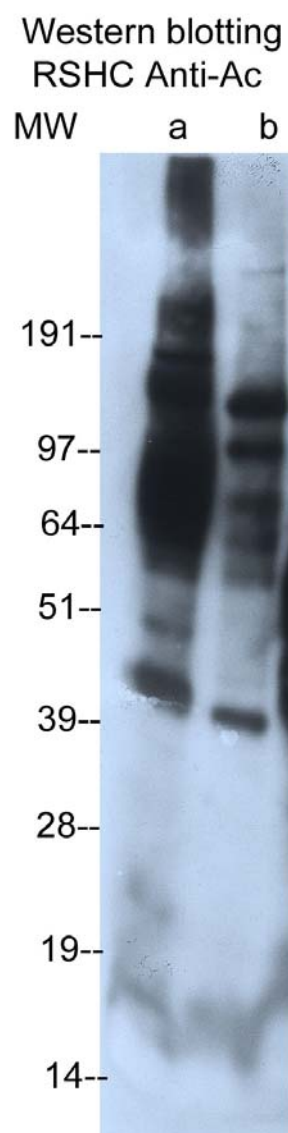


Figure 1. The venoms from *L. quinquestriatus* (**A**) and *A. crassicauda* (**B**) were separated and transferred to membranes. Bands are detected using the RSHC anti-Ac (1:4,000). Molecular weight (MW): SeeBlue® Plus2 (Invitrogen Corporation, USA).

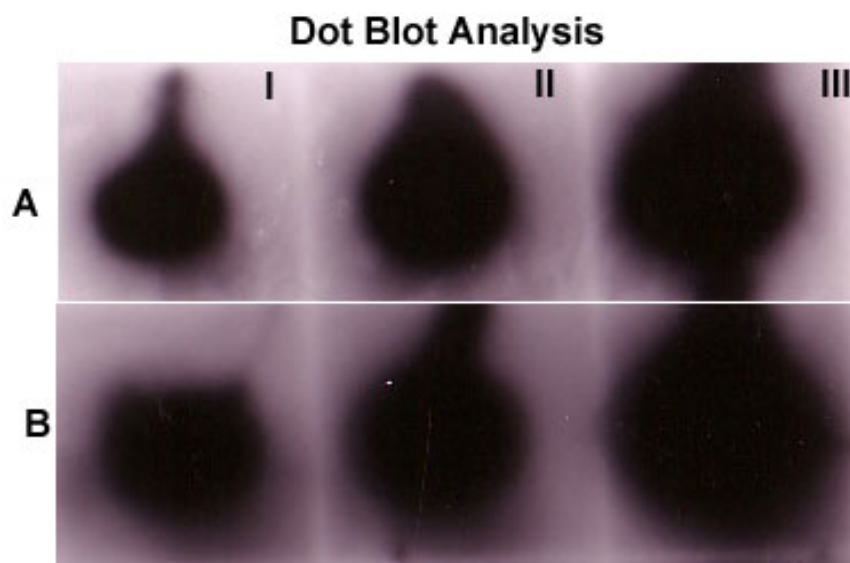


Figure 2. *L. quinquestriatus* (A) and *A. crassicauda* (B) venoms were incubated in membranes 2 μ L (I), 3 μ L (II) and 4 μ L (III); venom proteins reacted strongly with RSHC anti-Ac (1:1000).

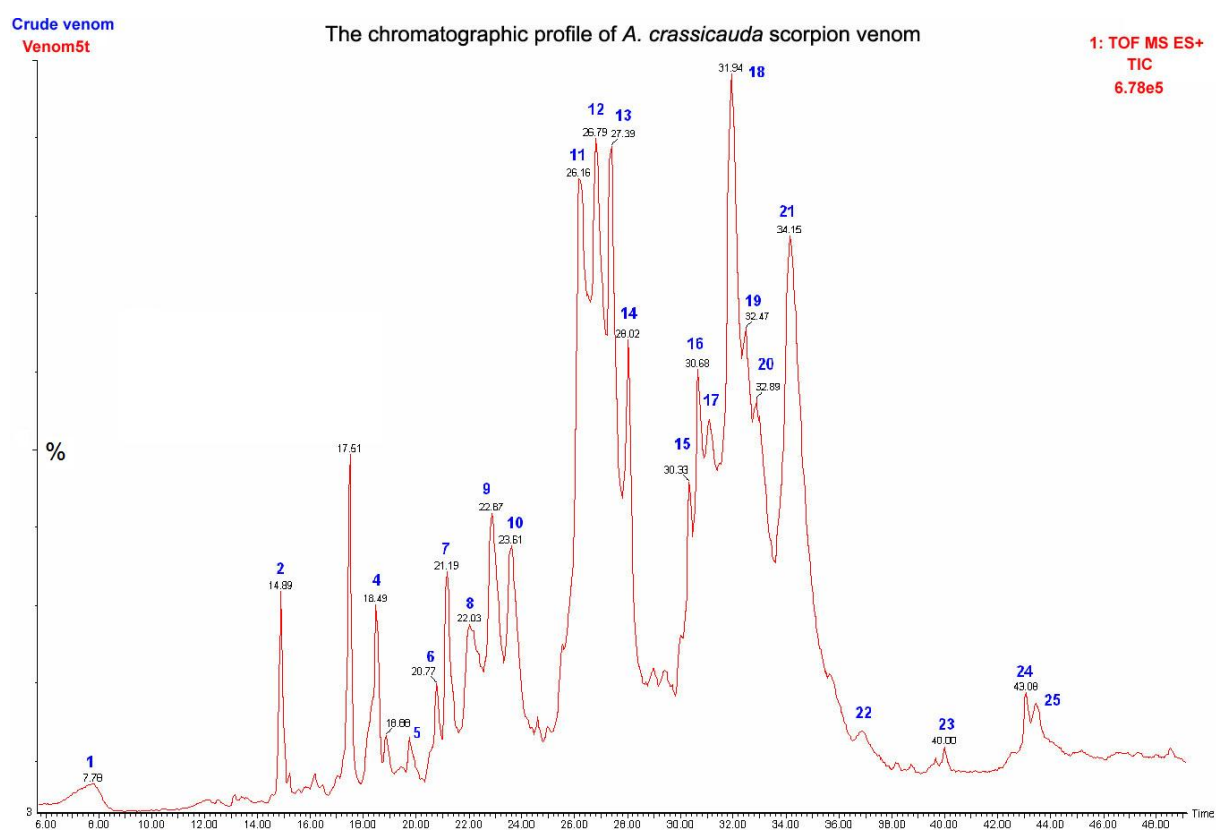


Figure 3. Mass spectrum of total ion chromatogram and UV trace of the crude venom. Total ion chromatography of *A. crassicauda* venom between 6 and 49 minutes, with peak identifications.

Table 2. Molecular masses of components from *A. crassicauda* venom

Peak number	Retention time (min)	Molecular weight (Da)
1	7.7-7.8	437.0; 494.0
2	14.9	4,095.3; 4,102.9; 4,107.6; 4,113.2 4,120.4;4,136.6; 4,200.6; 4,218.8
3	17.5	3,178.0
4	18.5	1,486.9
5	19.8	1,430.3
6	20.8	3,866.7
7	21.2	3,878.8
8	22.0	7,207.6; 7,178.9; 5,305.8; 7,248.9
9	22.9	7,178.4
10	23.6	7,179.3
11	26.2	6,908.2
12	26.8	6,908.2; 7,149.6
13	27.4	6,779.9
14	28.0	6,779.9
15	30.3	7,193.0
16	30.7	13,257.1; 7,189.1
17	31.2	8070.2
18	31.9	16,917.8; 7,418.1; 8,458.6
19	32.5	6,945.3
20	32.9	16,365.2; 16,412.7; 16,341.4
21	34.2	16,243.8
22	36.9	14,269.4
23	40.0	44,737.0
24	43.1	13,896.8
25	43.5	13,807.0

DISCUSSION

The two most venomous scorpion species in Turkey are from the Buthidae family, *L. quinquestriatus* and *A. crassicauda* (10, 29, 41, 42). *L. quinquestriatus* was first reported in Adiyaman, Turkey, by Tulga (41) and later observed in Gaziantep, Hatay, Kilis and Mardin provinces in the same country (9, 10, 19, 20). Scorpion sting cases followed by antivenom treatment have been reported in Adiyaman (1150/1191); Mardin (1104/1104); Hatay (895/895); Gaziantep (883/871) and Kilis (92/92) provinces, according to Health Ministry recording for 2006 (unpublished data). Scorpion stings are primarily due to accidents and in most cases the scorpion is neither seen nor identified (24). In the Middle East, it is known that common scorpion stings were caused by *A. crassicauda*, *L. quinquestriatus* and the remainder were caused by unidentified scorpions (17). Antivenoms are still widely used, although their role in scorpion sting treatment remains controversial (15, 17, 39). In Turkey, the monovalent *A. crassicauda* antivenom has been used to treat all scorpion stings since 1942 (1). Numerous epidemiological and clinical studies indicate that the antivenom has been successfully used for scorpionism cases (1, 3, 38, 40).

The lethal dose of scorpion venom can diverge given the variability among scorpion venom toxicities due to genus, species, sex, body weight, geographic origin, venom extraction procedures, structure and amount of venom.

In toxicology, the median lethal dose (LD₅₀) is the quantity of venom required to kill 50% of the animals in a controlled test. Although it is a standard measure of toxin potency, it is a misleading indicator due to scorpion venom variability (22, 26, 29, 30, 38). In the present study, we employed MLD and MED the for antivenom potency test instead of LD₅₀ and ED₅₀. We used a toxic or lethal unit of scorpion venom as the corresponding volume and demonstrated the RSHC anti-Ac minimum capacity to neutralize MLD of *L. quinquestriatus* venom. Tulga (41) emphasized that *L. quinquestriatus* venom is four to five times more toxic than *A. crassicauda* venom. Ismail (17) highlighted that smaller values of *L. quinquestriatus* venom are 26 to 28 times more lethal than the largest value of *L. quinquestriatus* scorpion venom. According to our results, *L. quinquestriatus* venom has double the toxicity of *A. crassicauda* scorpion venom.

Four decades ago, *A. crassicauda* antivenom was found more effective against venoms from some dangerous scorpion species – *A. australis*, *Buthus occitanus*

(Algeria), *Tityus serrulatus*, *T. bahiensis* (Brazil), *Parabuthus* spp. (South Africa), *Centruroides sculpturatus* and *C. vittatus* (USA) – and was more efficient than their respective homologous antivenoms (43). Recently, Ozkan *et al.* (28) stated that *A. crassicauda* antivenom was capable of neutralizing 20 MLD of *Mesobuthus gibbosus* venom in mice. In addition, Tulga (41) produced homologous antivenom from *L. quinquestriatus* venom and performed cross-protection test in rats, weighing 150 g each. Two-hundred microliters of *L. quinquestriatus* antivenom neutralized only 2 MLD of homologous venom but not 2.5 MLD of *A. crassicauda* venom. However, an equal amount of *A. crassicauda* antivenom (0.2 mL) neutralized 2 MLD of *L. quinquestriatus* venom (41). In our current study, bioassays showed similar results and we demonstrated that 0.4 mL of RSHC anti-Ac neutralized 10 MLD of *L. quinquestriatus* venom in mice.

Scorpion venoms can be classified into two groups according to their molecular sizes, long-chain and short-chain neurotoxins. Among these more studied groups are the short-chain neurotoxins that present 3,000 to 4,400 Da and act on potassium or chloride channels. Long-chain neurotoxins, that have 6,500 to 7,800 Da, act mostly on sodium channels (16, 31, 32, 34).

Recently, *A. crassicauda* scorpion venom from Turkey has been studied and five toxins from it were described by Caliskan *et al.* (7). This animal, known as one of the Old World scorpions, presents a more complex venom composition (despite its lesser toxicity) than *L. quinquestriatus* (41, 42). In parallel bioassays, western blotting and dot blot of poisons from *A. crassicauda* and *L. quinquestriatus* with the antivenom showed that both venoms reacted with RSHC anti-Ac serum. Western blotting indicated between 39,000 and 191,000 Da proteins for both. Following a scorpion sting, the treatment with species-specific antivenom is recommended. If this is unavailable, antivenom against *A. crassicauda* should be used for Old World scorpion stings (2).

The present study showed that RSHC anti-Ac was effective in neutralizing *L. quinquestriatus* venom in both *in vivo* assay and *in vitro* analysis. This indicates the efficacy of RSHC anti-Ac in preventing *L. quinquestriatus* scorpionism. Future studies should be conducted on *L. quinquestriatus* stings to determine the clinical benefits of RSHC anti-Ac. Therefore, the effects of the antivenom on stings from this species should be better established with epidemiological and clinical studies.

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