

Venom characterization of the bark scorpion *Centruroides edwardsii* (Gervais 1843): Composition, biochemical activities and *in vivo* toxicity for potential prey

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ABSTRACT

In this study, we characterize the venom of *Centruroides edwardsii*, one of the most abundant scorpions in urban and rural areas of Costa Rica, in terms of its biochemical constituents and their biological activities. *C. edwardsii* venom is rich in peptides but also contains some higher molecular weight protein components. No phospholipase A₂, hemolytic or fibrinogenolytic activities were found, but the presence of proteolytic and hyaluronidase enzymes was evidenced by zymography.

Venom proteomic analysis indicates the presence of a hyaluronidase, several cysteine-rich secretory proteins, metalloproteinases and a peptidylglycine α -hydroxylating monooxygenase like-enzyme. It also includes peptides similar to the K⁺-channel blocker margatoxin, a dominant toxin in the venom of the related scorpion *C. margaritatus*. MS and N-terminal sequencing analysis also reveals the presence of Na⁺-channel-modulating peptides with sequence similarity to orthologs present in other scorpion species of the genera *Centruroides* and *Tityus*. We purified the hyaluronidase (which co-eluted with an allergen 5-like CRiSP) and sequenced ~60% of this enzyme. We also sequenced some venom gland transcripts that include other cysteine-containing peptides and a Non-Disulfide Bridged Peptide (NDBP).

Our *in vivo* experiments characterizing the effects on potential predators and prey show that *C. edwardsii* venom induces paralysis in several species of arthropods and geckos; crickets being the most sensitive and cockroaches and scorpions the most resistant organisms tested. Envenomation signs were also observed in mice, but no lethality was reached by intraperitoneal administration of this venom up to 120 μ g/g body weight.

1. Introduction

Among the Chelicerata, scorpions comprise a successful clade of arachnids with a long evolutionary trajectory, since they probably first appeared in terrestrial environments in the late-Devonian period (Mullen and Sissom, 2019). Several adaptations explain their evolutionary success; perhaps the most notable is the presence of venom-producing glands and a stinger in the last segment of the metasome (Hjelle and Polis, 1990). Although the evolution of complex venoms has occurred in independent lines in Arthropoda (Senji Laxme

et al., 2019), this effective inoculation mechanism is exclusive of scorpions and explains in part why this group dominates the statistics of arthropod envenoming in the World (Chippaux, 2012).

Currently, more than 2400 species of scorpions are recognized, more than half included in the large family Buthidae (Lourenço, 2018; Senji Laxme et al., 2019). This family also contains all the medically important species, some of which are capable of inducing severe envenomation, which relates to the potential sensitivity of mammals to scorpion neurotoxins. In humans, stings from buthids produce intense local pain and mild inflammation in the affected area; while some of the dangerous

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species may trigger systemic envenomation that could cause death in children and debilitated individuals (D'Suze et al., 1995; Borges et al., 2004; Chippaux, 2012). Overall, most effects are related to the presence of neurotoxic peptides that mainly affect sodium channels (Batista et al., 2002; Sunagar et al., 2013; Santibáñez-López et al., 2016).

As with other organisms, scorpions' venom plays an important role in immobilizing the prey but also to deter potential predators (Sunagar et al., 2013). Hence, it is not surprising that different components of the venom act unevenly in target species. For instance, several buthid scorpions have peptides with high affinities to ion channels of mammals, while others predominantly affect those of arthropods (Lourenço, 2018). In part, these differences explain the large intra- and inter-specific variation of peptide and protein components expressed in scorpion venoms.

Buthidae is represented by several genera in the New World (Espósito et al., 2018) and among them, *Centruroides* stands out as one of the most diverse with more than ninety recognized species. Members of this genus are mainly distributed in southern North America, Central America, and the Antilles, although a few species also reach northern South America (Armas et al., 2008; Sissom and Lourenço, 1987). Although the taxonomy of the genus *Centruroides* is relatively stable, recent reviews reveal some confusion regarding the identity of some species inhabiting South America (Armas et al., 2012), a situation that complicates our understanding of venom toxicity in the region. This is the case of the bark scorpion *Centruroides edwardsii* (Gervais, 1843), a widely distributed species that inhabits seasonal areas from Mexico to Colombia, with populations introduced in Cuba and Senegal (Armas et al., 2011). Until a few years ago, *C. edwardsii* was considered a junior synonym of *C. margaritatus*, although recently Armas et al. (2011, 2012) established it as a valid species based on some morphological characters.

As in other scorpions, members of the genus *Centruroides* are predated by several vertebrates, including lizards, snakes, birds, rodents and other mammals (Van der Meijden et al., 2013; Sunagar et al., 2013). In contrast, small vertebrates can become prey items to these scorpions. For example, some *Centruroides* species have the potential to paralyze and kill lizards (Miranda et al., 2015) and *C. edwardsii* predated on small reptiles, such as snakes (Acosta-Chaves and Villalobos-Chaves, 2015) and anolid lizards (Sasa, M., personal observation).

Even when vertebrates could be the target of scorpion venoms, it is evident that invertebrates, including some arachnids, are most of the time, their chosen prey. Miranda and collaborators (2015) have reported that in natural conditions, some species of *Centruroides* take advantage of small and middle-sized insect prey such as *Phyllophaga* adult beetles and *Atta* and *Camponotus* ants, which emerge with the May–June first rains in Central American countries.

In this study, we describe the major components and main *in vitro* and *in vivo* biological activities of the venom of *Centruroides edwardsii* from Costa Rica, aiming to increase the knowledge on the venoms of Central American scorpions and to characterize their toxic effects on potential prey.

2. Materials and methods

2.1. Specimens and venom extraction

The procedures for this study were approved by the Biodiversity Commission of Universidad de Costa Rica (No. 111–2018). During a five-year period we collected more than 100 adult *Centruroides edwardsii* specimens from both sexes (Fig. 1A) from two locations in the North Pacific of Costa Rica: Palo Verde National Park (10° 20'41.50 "N 85° 20'20.90" W) and Santa Rosa National Park (10° 50'2.75 "N, 85°



Fig. 1. *Centruroides edwardsii* specimen collected in Santa Rosa National Park (Costa Rica) and its venom protein pattern. **(A)** *C. edwardsii* specimen. **(B)** 15% SDS-PAGE under reducing conditions of *C. edwardsii* venom soluble fraction (after centrifugation at 10,000 g). Venom sample corresponds to a pool from different specimens (males and females) collected in two locations of Costa Rica and obtained by electric stimulation of telsons.

36/45.32" W). Some of the scorpions were individually kept at the LIAP-animal facility at Instituto Clodomiro Picado, in San José, Costa Rica, in plastic boxes, providing them with food (insect larvae) and water *ad libitum*.

We extracted the venom using electro-stimulation of the fifth meta-some segment (telson). Briefly, the segment was placed between two electrodes, and we applied a discharge of 40–50 V (0.7–0.8 A). We collected the venom in capillary tubes. Samples were transferred and pooled into a 1.5 mL plastic tube, lyophilized, and stored at -70°C until use.

2.2. Electrophoresis, fibrinogenolytic activity, and substrate zymography

Venom (20 μg) was separated by SDS-PAGE (15%) under reducing conditions (5% 2-mercaptoethanol, at 100°C for 5 min), in a Mini-Protein system (Bio-Rad) at 150–180 V and stained with Coomassie blue R-250.

We estimated fibrinogenolytic activity by incubating 20 μg of fibrinogen (Sigma Chemical Co.) with the venom (2 μg) in a final volume of 40 μL , for 3 h at 37°C . Then, the mixture was analyzed by SDS-PAGE (12%) as described, and the degradation of fibrinogen chains was determined by Coomassie blue R-250 staining of the gels (Delafontaine et al., 2017).

To determine proteolytic activity and the molecular weight of the proteases present in the venom, gelatin zymography was performed according to the procedure of Herron et al. (1986) with modifications of Rucavado et al. (1998). Briefly, unreduced venoms (15 μg) were separated by SDS-PAGE in 12% gels containing type-A gelatin (Sigma Chemical Co.) at a concentration of 0.25 mg/mL. After washing for 1 h with 1% Triton X-100, gels were incubated at 37°C for 24 h in 50 mM Tris-HCl, pH 8.0 buffer, containing 5 mM CaCl_2 , and stained with Coomassie Blue R-250. A similar experiment was carried out in the presence of 1 mM EDTA in the samples and incubation buffer, to discern the presence of metalloproteinases.

Hyaluronidase activity present in the venom (15 μg) and fractions (10 μg) was determined by the method reported by Cevallos et al. (1992) based on SDS-PAGE in a 12% gel containing 0.5 mg/mL hyaluronic acid from rooster comb (Sigma Chemical Co.). Incubation buffer (0.1 M NaCl, 0.1 M sodium phosphate) was adjusted to pH 6.6. Gels were stained with Alcian Blue 8GX (Sigma Chemical Co.).

2.3. Phospholipase activity and hemolysis

Phospholipase A_2 (PLA_2) activity was determined on micellar phosphatidylcholine (Sigma Chemical Co.) by the phenol red colorimetric method (De Araujo and Radvanyi, 1987). Twenty μg of venom were added to 1 mL of substrate (0.25% w/v sn-3-phosphatidylcholine, 0.4% v/v Triton X-100, 0.004% w/v phenol red) at 30°C , and the change in absorbance at 558 nm was monitored for 1 min. A purified PLA_2 from *Bothrops asper* snake venom was used as a positive control. PLA_2 activity was also assayed using the synthetic monodisperse substrate 4-nitro-3-octanoyloxy-benzoic acid (4-NOBA) following previously described methods (Mora-Obando et al., 2014). This enzymatic activity was also tested using egg yolk-erythrocyte containing agarose plates according to Gutiérrez et al. (1986).

Rabbit and human erythrocytes (from type O healthy volunteer donors) were obtained by centrifugation of citrated blood, washed three times with phosphate-buffered saline (PBS) and used for the hemolytic assay. Several concentrations of venom were tested on a 10% erythrocyte suspension for 30 min at 37°C , and absorbances were read at 545 nm after centrifugation.

2.4. RP-HPLC and SDS-PAGE

Four mg of *C. edwardsii* venom was dissolved in 200 μL of 0.1% trifluoroacetic acid (TFA; solution A), centrifuged for 5 min at 10,000 g to

remove insoluble material, and separated by reverse-phase HPLC on a C_{18} column (250×4.6 mm, 5 μm particle size; Phenomenex) using an Agilent 1220 chromatograph with monitoring at 215 nm. Elution was performed at 1 mL/min by applying the following gradient with solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–70% B over 65 min. A variant method was performed for separation of some proteins (using the same column), that consisted on a gradient with solution B: 0% B for 5 min, 0–45% B over 45 min, and 45–70% B over 15 min. Fractions were collected manually, dried in a Speed-Vac (Thermo), dissolved in water, and further separated by SDS-PAGE (15%) under reducing conditions. Protein bands were visualized by Coomassie blue R-250 staining, and selected bands were submitted to mass spectrometry, as described below.

2.5. Mass spectrometry (MS)

Major protein bands selected from SDS-PAGE of RP-HPLC venom fractions were excised from gels and subjected to reduction with dithiothreitol (10 mM) and alkylation with iodoacetamide (50 mM), followed by overnight in-gel digestion with sequencing grade bovine trypsin or chymotrypsin (Sigma Chemical Co.), as described (Rey-Suárez et al., 2016). Some RP-HPLC fractions were directly reduced, alkylated, and digested in solution. The resulting proteolytic peptides were analyzed by MALDI-TOF-TOF on an Applied Biosystems instrument (ABSciex 4800 Plus). Mixtures of 0.5 μL of α -cyano-4-hydroxycinnamic acid and 0.5 μL of each sample were spotted onto an Opti-TOF 384 plate, dried and analyzed in positive reflector mode. Spectra were acquired after 500 shots at a laser intensity of 3000, using as external standards CalMix-5 (ABSciex) spotted on the same plate. Up to 10 precursor ions from each MS spectrum were selected for automatic collision-induced dissociation MS/MS spectra acquisition at 2 kV, in positive mode (500 shots/spectrum, laser intensity of 3900). The resulting spectra were searched using ProteinPilot v.4 and the Paragon® algorithm (ABSciex) against the UniProt/SwissProt Scorpiones database, at a confidence level of $\geq 95\%$, for protein identification or protein family assignment by similarity. Fragmentation spectra of sufficient quality were also manually interpreted to obtain *de novo* sequences or sequence tags. The obtained sequences were searched using BLAST (<https://blast.ncbi.nlm.nih.gov/>) to find the most similar proteins included in the database.

Venom hyaluronidase, separated by RP-HPLC, was reduced, alkylated, and digested overnight with trypsin or for 3 h with chymotrypsin, at 37° . The reaction was stopped by adding 1 μL of TFA. Peptides were then separated by RP-HPLC in a C_{18} column (2.1×150 mm, 3 μm particle; Phenomenex) at 0.3 mL/min, monitored at 215 nm, using an acetonitrile gradient of 60 min: 1% B for 12 min, 1–10 %B for 5 min, 10–25% B for 30 min, 25–35% B for 3 min, 35–70% B for 5 min, and 70% B for 5 min. Peptides were finally analyzed by MALDI-TOF-TOF as described.

2.6. Size-exclusion HPLC, RP-HPLC and N-terminal peptide sequencing

Venom (2 mg) was separated by size-exclusion chromatography (SEC-HPLC) in a S2000 BioSepSEC column (10×300 mm), equilibrated with 0.06 M NaCl, 0.02 M sodium phosphate buffer (pH 7.2), at 1 mL/min, in an Agilent 1220 chromatograph, monitoring at 215 nm. Fractions were further separated by RP-HPLC (see above) to perform automated N-terminal Edman sequencing of some individual peptides on a Shimadzu Biotech PPSQ 33A Sequencer instrument according to the manufacturer's instructions.

2.7. cDNA library construction and analysis of the transcript sequences

cDNA libraries were constructed from total mRNA extracted from one male specimen of *C. edwardsii* scorpion venom glands (telson), following the SMARTer cDNA Library Construction kit (Clontech, Takara) protocol. A random screening of the colonies (210 colonies)

using the non-amplified cDNA library was performed. cDNA was amplified (with vector primers) and clones that displayed a correct PCR amplification band were selected. The plasmid DNA of such colonies was obtained by mini-prep and the inserts were sequenced from both ends (GATC Biotech Sequencing Service, Germany). Eighty-nine Sanger sequences (high-quality ESTs) were identified. In order to confirm the precursors deduced from our singleton and clustering DNA and deposit the sequences in the GenBank database, we performed additional DNA sequencing of the obtained plasmids (58 plasmids).

Quality values of the sequenced DNA data produced by Phred were used in the CAP3 sequence assembly program for finding overlaps between reads, removal of false overlaps and construction of clusters. Multiple alignments were generated, and consensus sequences were compared against databases (Blastx and GenBank). To perform a functional classification and annotation of these sequences, we identified putative precursor sequences by the corresponding open reading frames. The deduced amino acid sequences were analyzed and compared against different databases (SignalP 4.1, ProP 1.0, Blast, Kallisto and UniProtKB).

2.8. Target species

We tested the following target species (Order, mean $\pm$ SD of the weight). Arthropods: seventh instar of the crickets *Acheta assimilis* (Orthoptera, 0.40 ± 0.05 g) and *A. domesticus* (0.30 ± 0.04 g); adult cockroaches *Periplaneta australasiae* (Blattaria, 1.0 ± 0.5 g); last instar of the mealworm *Tenebrio molitor* (Coleoptera, 0.13 ± 0.02 g); and the fly *Peckia intermutans* larvae of 4 days of age (Diptera, 0.25 ± 0.03 g). Since it has been observed that scorpions display cannibalism and prey on other scorpions (Miranda et al., 2015), we injected the venom in adult *C. edwardsii* and *Didymocentrus krausi* (Diplocentridae) specimens and determine lethality at 24 h.

We also tested mice (CD-1, $16.0\text{--}18.0 \pm 2.0$ g) and house geckos *Hemidactylus frenatus* (Gekkonidae, $1.0\text{--}4.0$ g) as vertebrate target species. We raised all target specimens at the ICP facility, with the exception of the scorpions that were collected as described (Rojas-Azofeifa et al., 2019). The choice of each of these model species was not random since *C. edwardsii* is a generalist predator, which includes a great variety of arthropods in its diet, but also small invertebrates such as lizards and snakes (Acosta-Chaves and Villalobos-Chaves, 2015). The use of a murine model allowed us to compare and extrapolate the possible effects of this venom in other mammals.

2.9. Bioassays

For each target species, we randomly selected previously weighed individuals to create homogenous doses-groups. *C. edwardsii* venom was re-dissolved in insect saline solution (154 mM NaCl, 2.68 mM KCl, 2.25 mM CaCl_2 , pH 6.85; Friedel and Nentwig, 1989) and injected into each group according to doses and species. Injections into the arthropods were performed using Hamilton® glass syringes of 10 or 50 μL . The injection site varied among target species: the intersegmental membrane of the ventral side (*A. assimilis*, *A. domesticus*, *P. australasiae* and scorpions), the dorsolateral side of the thorax (*T. molitor* and *P. intermutans* larvae), or ventral region (*H. frenatus* and mice). We performed two replicates of each trial.

From our assays, we evaluated the degree of paralysis and lethality, and other signs associated with the toxicity. For all species with legs, we define paralysis as the inability of an individual to return to his ventral position once turned on its back; and we recorded if the movement of appendages persisted when a tactile stimulus was applied. In the case of limbless models (*T. molitor* and *P. intermutans* larvae), we recorded paralysis as the absence of mobility and retraction of the cuticle. We evaluated paralysis at 3, 6, and 12 h after the injection of venom.

2.10. Statistical analysis

We estimated the median lethal dose (LD_{50}) and ninety-five percent lethal dose (LD_{95}) at 24 h after venom injection, using the Probit model (Finney, 1971; Trevors, 1986). We used the χ^2 goodness-of-fit test to evaluate the fit of our data to the probit model. We compared the relative toxicity of *C. edwardsii* venom between pairs of target species, using the 95% confidence interval of the ratio between paired lethality estimates, assessed using Polo Plus® v.2.0 (LeOra Software, Robertson et al., 2007). This interval is calculated from the intercept and slope estimates of the probit models as well as the variance-covariance matrix (Payton et al., 2003). If the confidence interval of the ratio of two lethality estimates includes 1, then the LD values were not considered significantly different (Robertson et al., 2007). The determination of the ratio is a powerful comparison method for the analysis of confidence interval overlap (Payton et al., 2003).

3. Results

3.1. Venom composition and enzymatic activities

The soluble part of *C. edwardsii* (Fig. 1A) venom (a pool from male and female specimens), obtained after solubilization in distilled water and centrifugation to remove the insoluble fraction, was separated by 15% SDS-PAGE under reducing conditions (Fig. 1B). The protein pattern shows the presence of some bands in a wide range of molecular weights, and a band containing peptides around or under 10 kDa.

By substrate zymography in the presence of hyaluronic acid (Fig. 2A) or gelatin (Fig. 2B), we detected hyaluronidase activity at 40–50 kDa, and at least two bands with proteolytic activity close to 23 and 40 kDa. When gelatin zymography was performed in the presence of EDTA the proteolytic activity of these two bands was inhibited (not shown), suggesting their metalloproteinase nature. However, their molecular mass did not correspond with other metalloproteinases identified from digested SDS-PAGE bands by MS (see below), indicating that there are several proteolytic enzymes in this venom which are probably below the limit of detection of gelatin zymography.

No PLA_2 activity was detected in the venom when measured by three different techniques, which include phenol red colorimetric method, synthetic monodisperse substrate and egg yolk-erythrocyte plates. We also did not find *in vitro* fibrinogenolytic activity by SDS-PAGE or direct hemolytic activity on human or rabbit erythrocytes after 30 min of incubation with the venom (results not shown).

3.2. Partial proteomic and transcriptomic sequence analysis and identification of venom proteins and peptides

By MALDI-TOF MS we determined the presence of several peptides (Fig. 3). We detected some very small peptides under 3 kDa (Fig. 3A) and two groups of peptides with higher molecular mass (between 3 and 8 kDa) (Fig. 3B), most of which probably correspond to K^+ - and Na^+ -channel toxins. We cannot rule out the presence of other types of peptides such as Non-Disulfide Bridged Peptides (NDBP), for instance, since a transcript precursor was found in the venom gland.

C. edwardsii venom peptides identified by MS after separation by SEC-HPLC (from SDS-PAGE digested bands), named Ce.1, Ce.2 and Ce.3, showed some similarity with Na^+ - and K^+ -channel toxins present in other venoms, e.g. margatoxin (a K^+ -channel blocker) and *Tityus bahiensis* Tb2-II (a Na^+ -channel toxin). A small sequence with similarity to scorpine-like peptides was also found. In SEC-HPLC fraction 5, a Cys-rich secretory peptide was also present (Fig. 4, Table 1).

Some venom fractions separated by SEC-HPLC were further purified by RP-HPLC (Supplementary material, Fig. S1) and individual peptides were obtained (CE.N1, CE.N5 and CE.N6) (Fig. 4). Homogeneity was corroborated by running 15% SDS-PAGE (under reducing conditions) and by mass spectrometry (data not shown). By Edman degradation, we

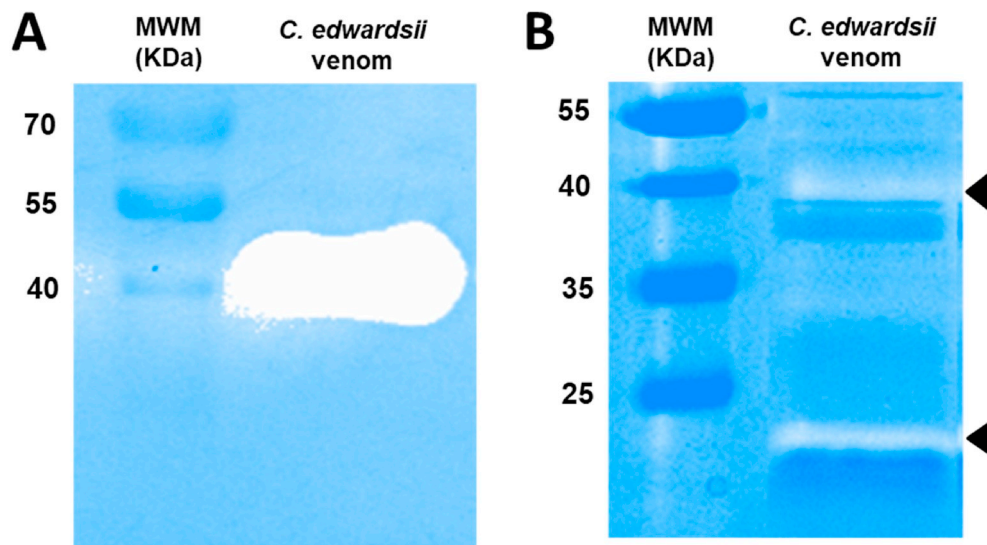


Fig. 2. *Centruroides edwardsii* venom hyaluronidase and proteolytic activity determined by zymography. (A) Venom was separated in hyaluronic acid or (B) gelatin-containing 12% SDS-PAGE (under non-reducing conditions) and after removing SDS by incubation with Triton X-100 (for 1 h), the enzymatic reaction was carried out overnight at 37 °C. Arrows indicate the main proteins with proteolytic activity on gelatin.

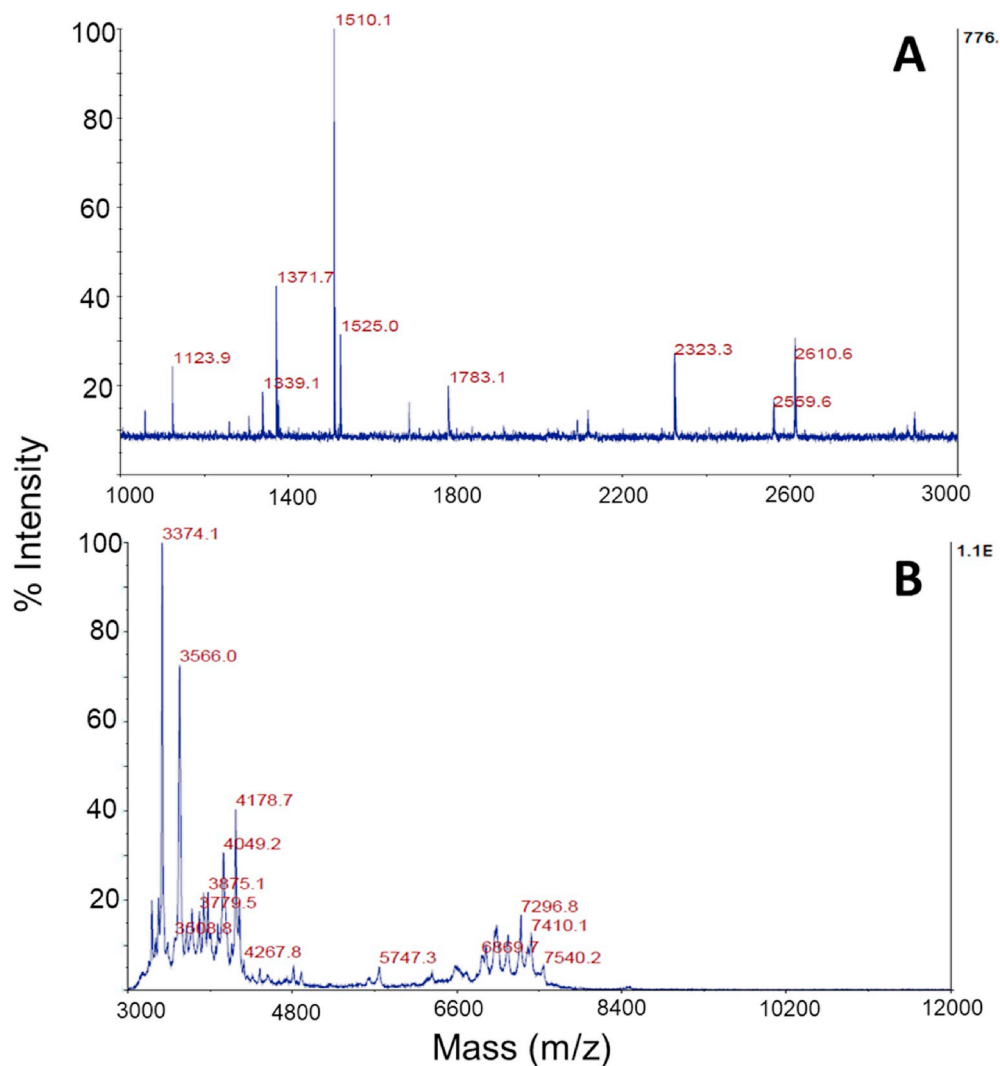


Fig. 3. MALDI-TOF mass spectra of *Centruroides edwardsii* venom peptides. (A, B) Venom was analyzed in linear positive mode, in the m/z range (1000 to 3000 and 3000 to 12,000 Da), in the presence of saturated α -cyano-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA.

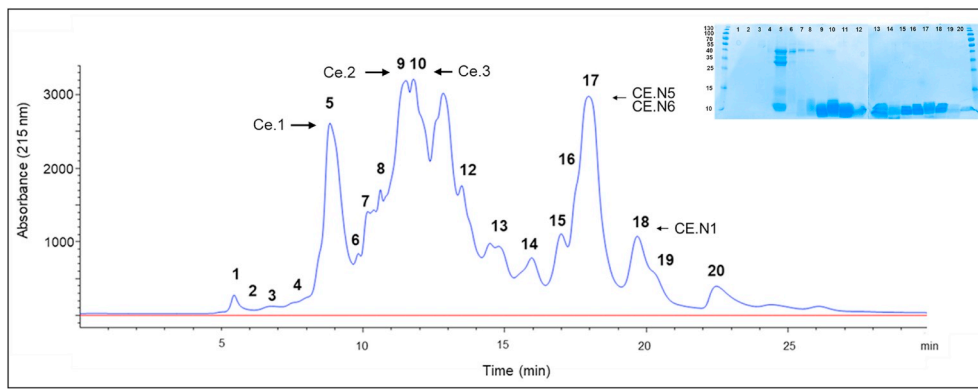


Fig. 4. Separation of *Centruroides edwardsii* venom by size exclusion-HPLC and SDS-PAGE of the main fractions. Venom was separated by SEC-HPLC in an S2000 Bio-SepSEC column, equilibrated with 0.06 M NaCl, 0.02 M sodium phosphate buffer (pH 7.2) at 1 mL/min, and some peptides were re-purified by RP-HPLC and identified by Edman degradation or MALDI-TOF/TOF after trypsin digestion. The identified peptide sequences are shown in Table 1 (Ce.1, Ce.2 and Ce.3) and in the text of the Results section (CE.N1, CE.N5 and CE.N6). Inset at the upper right side shows an SDS-PAGE (under reducing conditions) with the main fractions of the SEC-HPLC.

Table 1

Summary of the *Centruroides edwardsii* venom protein assignments by MALDI-TOF/TOF MS of tryptic peptides obtained after SEC-HPLC, RP-HPLC and SDS-PAGE according to Figs. 4 and 5.

Band	MW* (kDa)	m/z	z	Matching peptide sequence ^a	Conf	Sco	% cov	Best UniProtKB match	Code	Protein family
Ce.1	~10	1408.5	1	QGSYGHSQVIW	99.0	8	5.7	Cys-rich secretory peptide <i>Hotentotta judaicus</i>	ADY39606	Cys-rich secretory peptide
		1424.5	1	KEGYAMDHEGCK	99.0	8	18.8	Toxin Tb2-II NaTx5.3 <i>Tityus bahiensis</i>	P56609.1	Sodium channel inhibitor
Ce.2	≤10	964.4	1	AQFGQSAGAK	98.2	5	22.6	K + -channel toxin α-KTx 2.2 <i>Centruroides margaritatus</i>	P40755	Potassium channel inhibitor
Ce.3	≤10	1494.8	1	KLLSLVVPDNQLR	man	man	man	scorpine-like peptide Tco 41.46–2 <i>Centruroides sculpturatus</i>	XP_023220230	Scorpine-like peptides
Ce.8	40	1198.5	1	YVTAVFNAGEK	99.0	14	15.5	CRISP <i>Centruroides hentzi</i>	A0A2I9LNW3	Cys-rich secretory protein
		1661.7	1	FQHKDGLCAFLIGR	99.0	10	10.3	CRISP <i>Tityus obscurus</i>	A0A1E1WVY9	Cys-rich secretory protein
		1509.6	1	VGCGVAGYEENGVK ^{pa}	98.9	8				
		977.4	1	Q ^{py} SCPAIYR	96.5	9				
		1121.5	1	DGLCAFLIGR	99.0	11				
		1834.8	1	G ^{cm} AP ^{ox} QYFEFKEYGVK	99.0	15				
		1449.6	1	NYGHFSQVIWAK	99.0	16				
Ce.9	38	2256.0	1	LIGKHDPLEPQMFYVPEDK	99.0	11	38.9	Peptidylglycine α-amidating monooxygenase <i>Centruroides hentzi</i>	A0A2I9LP74	Copper type II ascorbate-dependent monooxygenase
		1385.5	1	FWWNCGEMAGSK	99.0	16				
		1493.7	1	AGVYLLGTSGFALPK	99.0	21				
		1454.6	1	DAPP ^{py} LN ^{da} LPDGVGFK	99.0	15				
		2004.9	1	SGGINLVLVQVHYADVTR	99.0	14				
		1552.8	1	IDKPLT ^{di} LHPFAFR	98.6	11				
		2686.2	1	FINGGTDNSGIITLLPGTDPSVTGR	88.0	9				
		991.3	1	CTMYNFR	87.5	7				
Ce.10	42	1402.6	1	QKETYLCAVAYK	63.3	8				
		1198.5	1	YVTAVFNAGEK	99.0	12	5.3	CRISP <i>Centruroides hentzi</i>	A0A2I9LNW3	Cys-rich secretory protein
		1121.5	1	DGLCAFLIGR	68.9	6	5.5	CRISP <i>Tityus obscurus</i>	A0A1E1WVY9	Cys-rich secretory protein
		1449.6	1	NYGHFSQVIWAK	99.0	12				
Ce.11	37	1263.6	1	CAK ^{ca} YLLSLPR	99.0	11	2.4	Metalloproteinase <i>Tityus obscurus</i>	A0A1E1WW34	Metalloproteinase
Ce.12	31	1277.6	1	DADMIMLLVTR	99.0	13	3.0	Metalloproteinase <i>Centruroides hentzi</i>	A0A2I9LP68	Metalloproteinase
		1277.6	1	DADMIMLLVTR	99.0	16				
Ce.14	30	1405.6	1	DADMIMLLVTRK	99.0	10	4.8	Metalloproteinase <i>Centruroides hentzi</i>	A0A2I9LP68	Metalloproteinase
		1981.9	1	YATGLAKDADMIMLLVTR	93.9	7				

▼: Reduced SDS-PAGE apparent mass, in kDa.

** Confidence (Conf) and Score (Sco) values are calculated by the Paragon™ algorithm of ProteinPilot™ v.4.0. Few matches with Conf values < 95%, but manually verified, are indicated by italics. Sequences obtained from spectra that were manually resolved (*de novo*) are indicated by “man”.

^a Cysteine residues are carbamidomethylated. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown by superscripts, with the following abbreviations: pa: propionamide; py: pyroglutamic acid; ox: oxidation; cm: carbamidomethyl; da: deamidated; di: didehydro; ca: carbamyl; do: deoxidation; dh: dehydrated; py: pyroglutamic; hk: hydroxykynurenine; ck: catK+.

obtained some N-terminal sequences that matched to database *Centruroides* and *Tityus* venom peptides such as CeI18 from *C. elegans* (CE.N1 sequence: KKDGYVPDSEECRY), Neurotoxin-3 (CseV1) from *C. sculpturatus* (CE.N5 sequence: KDGYPDLSRGCKVACLFDSQ), and TbIT-1 from *T. bahiensis* (CE.N6 sequence: KDGVLVKKSDGCK-YGCVMLPG). All the identified N-terminal sequences belong to voltage-dependent Na⁺-channel binding-like peptides.

Main SDS-PAGE protein bands from fractions obtained from the last

part of the RP-HPLC venom chromatogram were digested with trypsin and analyzed by MS (Fig. 5A; Table 1). Some CRISP-like proteins were detected, as well as two different metalloproteinases: one with similarity to metalloserrulase-9 (Metzincin family) from *Tityus obscurus*, and the other to *C. hentzi* antarease-like Zn²⁺-metalloprotease. Both proteases display molecular masses under 40 kDa (Fig. 5B, Table 1).

We also found a peptidylglycine α-hydroxylating monooxygenase-like (PAM) enzyme, such as the one predicted from *C. sculpturatus*

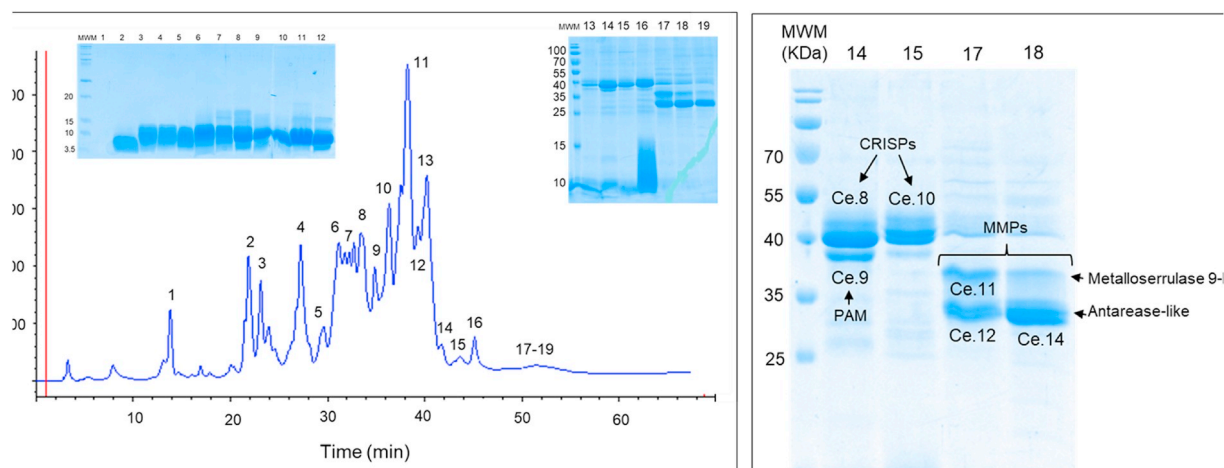


Fig. 5. *Centruroides edwardsii* complete venom RP-HPLC separation, SDS-PAGE and identification of some of the proteins present in the venom. (A) Venom was separated into its main compounds by RP-HPLC using a C₁₈ column and elution was performed at 1 mL/min by applying an acetonitrile gradient. Inset gel shows an SDS-PAGE (under reducing conditions) with the main fractions of the RP-HPLC (B) Venom protein identification by proteomic analysis of the RP-HPLC late-eluting fractions. Trypsin-digested peptide sequences are presented in Table 1. Elution was performed at 1 mL/min by applying an acetonitrile gradient. The applied gradient consisted in solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–45% B over 45 min, and 45–70% B over 15 min. Fractions were dried in a SpeedVac, re-dissolved in distilled water and further separated by 15% SDS-PAGE under reducing conditions. MS analysis was carried out as described in Methods. PAM: Peptidylglycine α -hydroxylating monooxygenase like-enzyme; CRiSPs: Cysteine-rich secretory proteins; MMPs: Metalloproteinases.

cDNA and identified in the proteome of the striped scorpion *C. hentzi* (Fig. 5B; Table 1). This is an enzyme responsible for post-translational modification of venom peptides (C-terminal amidation), a process that has been well characterized in gastropod venom peptide maturation (Ul-Hasan et al., 2013), and recently, in arthropod venom glands (Delgado-Prudencio et al., 2019).

After identifying the RP-HPLC fraction with higher hyaluronidase activity (Fig. 6A), we analyzed it by MALDI-TOF MS which evidenced a broadly dispersed ion peak with an apex at 48,642.8 Da, together with its corresponding doubly-protonated ion at 24,418.4 Da (Fig. 6B). SDS-PAGE of this fraction suggested the presence of two proteins with very close molecular weights (Fig. 6C). In addition, hyaluronic acid

zymography indicated the presence of some aggregates that display enzymatic activity, near to 100–130 kDa (Fig. 6D). We digested the protein with either trypsin or chymotrypsin and identified several peptides corresponding to two different protein families, namely hyaluronidase and cysteine-rich secretory proteins (CRiSP), in both cases with similarity to proteins found in *C. hentzi* or predicted for *C. sculpturatus* (Supplementary material, Fig. S2).

Partial transcriptomic analyses provided additional information on peptides potentially present in this venom, by using cDNA libraries obtained from venom gland transcripts. This strategy revealed sequences with similarity to a Na⁺-channel toxin, a putative K⁺-channel blocker and an NDBP (Fig. 7). Precursor transcript for Na⁺-channel toxin

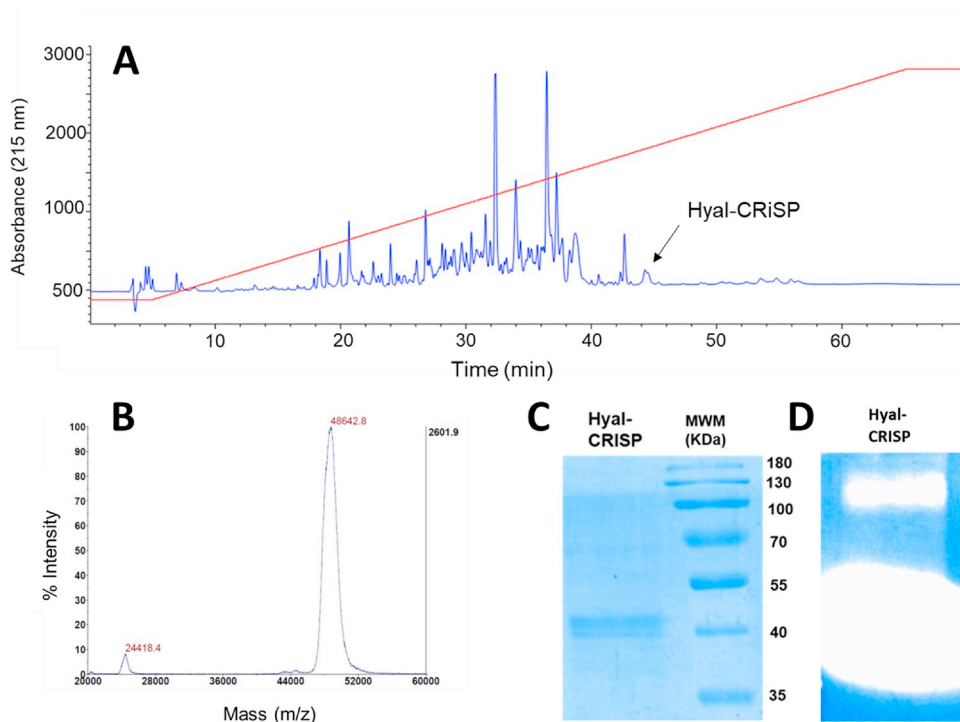


Fig. 6. RP-HPLC *Centruroides edwardsii* venom separation and MALDI-TOF mass spectra, SDS-PAGE and hyaluronic acid zymography of the RP-HPLC fraction containing the highest hyaluronidase activity. (A) Venom was separated into its main compounds by RP-HPLC using a C₁₈ column and elution was performed at 1 mL/min by applying a gradient that consisted in solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–70% B over 65 min. The fractions after minute 38 were tested for hyaluronidase activity by hyaluronic acid zymography and the one with the highest activity was purified and characterized. (B) MALDI-TOF MS analysis of the hyaluronidase activity-containing fraction in the presence of saturated sinapinic acid in 50% acetonitrile/0.1% TFA, in positive linear mode. The peak at ~24 kDa corresponds to the doubly-protonated ion of ~48 kDa. (C) 15% SDS-PAGE of RP-HPLC separated hyaluronidase. (D) Hyaluronic acid zymography of RP-HPLC purified hyaluronidase.

has 63% sequence identity to *C. noxious* toxin Cn11 (Fig. 7A), which is active on invertebrates (Ramírez-Domínguez et al., 2002). Precursor transcript sequence presented in Fig. 7B has a sequence related to pMeKTx21–1, a K^+ -channel blocker present in *Mesobuthus eupeus* venom (Kuzmenkov et al., 2015). This transcript is also related to defensin-1 from *Androctonus bicolor*. The NDBP partial transcript shows homology to a precursor peptide from the subfamily 6 (Fig. 7C). A 4-Cys containing additional sequence obtained from a transcript from the gland, but with no match to databases, was also obtained (Table SI), which could correspond to a new toxic peptide.

3.3. In vivo effects on target species

The different target species evaluated here vary in their sensitivity to *C. edwardsii* venom. Among the arthropods, crickets were more susceptible while the cockroach and the mealworm were the most resistant (Table 2). In scorpions, the venom did not induce any effect, at least at 24 h (data not shown). For vertebrates, *C. edwardsii* venom was lethal to geckos, but in the murine model, we could only demonstrate sub-lethal effects even at considerable doses. Also, differences in the observed estimates for lethality were statistically significant among susceptible and resistant species, as evidenced by our lethal dose ratio analysis (Table 2). In all our trials the controls showed no effects after the injection of saline solution and remained alive after 24 h.

Acheta domesticus showed the lowest capacity to resist venom toxicity (Table 2), and doses lower than 10 $\mu\text{g/g}$ paralyzed a significant percentage of individuals. This proportion increased as the observation period extended (Fig. 8B). In contrast, during the following 10 min after venom injection, *A. assimilis* displayed extreme activity characterized by sudden and uncoordinated jumps from one side to the other of the cage.

After this time, we observed that some individuals recover their normal mobility, but others remained immobile. Three hours after injection only individuals inoculated with the highest dose (163 $\mu\text{g/g}$), showed signs of paralysis while more than 80% of individuals of this species were paralyzed after 6 h from inoculum (Fig. 8A).

Both, *Tenebrio molitor* and *Peckia intermutans*, showed body flaccidity as the most visible sign of toxicity, although we recorded substantial differences in the proportion of affected individuals at different doses. In *T. molitor*, only a low proportion of individuals injected with a high dose (500 $\mu\text{g/g}$) showed signs of early (3 h) intoxication, although lower doses triggered effects at 6 and 12 h (Fig. 8E). In contrast, flaccid paralysis in *P. intermutans* was achieved at much lower concentrations (Fig. 8D).

On the other hand, the cockroach *Periplaneta australasiae* showed high resistance to *C. edwardsii* venom, with total paralysis of some individuals recorded only after 12 h or at doses above 380 $\mu\text{g/g}$ (Fig. 8C). However, for this species, we also observed the loss of balance, erratic movements, and difficulties to return to the ventral position even at lower doses.

In the gecko *H. frenatus*, signs of toxicity were present at doses of 170 $\mu\text{g/g}$, although early paralysis was evident at higher doses (Fig. 8F). Evidence of pain or discomfort in the area of the inoculum was deduced by animal behavior (licking and biting of the area). Ataxia, loss of balance and uncoordinated movements of legs and body, were observed. The lethality of the venom to this species was higher than that observed for the cockroach and the mealworm (Table 2).

We were unable to determine the LD_{50} of *C. edwardsii* venom in the murine model, even after injecting 120.0 $\mu\text{g/g}$ by the intraperitoneal route. However, we did observe several envenomation signs at lower doses. Thus, injection of 10.8 $\mu\text{g/g}$ caused an excitement phase and individuals repeatedly licked the area of the inoculum, which we interpret

A

AccNum name	signal peptide	MATURE PEPTIDE	aa	I%	e-value
Ced MK932018 CR13_114		...GYLLDKKGKIVFVN-SEWNSLKGKSGKYGY--KALAYTESAPDNVVKVWTSGTNT---	58	100	-----
Cno P58296 ToxCn11		-----ARDGYPVDEKGGKLSLIND-KWNSAHSRGGKYGYTYTGGGLAYTEAVPDNVKVTYETNT---	63	63	1e-18
Tcl AEQ18792 Tc12		-----igvrvvesKEGYLMDREGKLSLIRPSGYGREELKGGSSGYRW--LACYTYGLPDRVKVWSYATNTgkk	71	54	3e-13
Tse O77463 ToxTs4		mkrmlfiscclliidivvgGREGYPADSKGKITFLTAAGYNTETLKKGSSGYLAWP--ACYTYGLPDSVKIWTSETNkgk	84	54	3e-11
Csc XP023223307 ToxIm2		mksfllficclmvidvvesKDGYPMDNKGKXVWVINS-KSDNTKAMKGGKSGYTYFLGLACWTEGLPSNAKWEKATNkgk	85	51	5e-11

B

AccNum name	signal peptide	MATURE PEPTIDE	aa	I%	e-value
Ced MK932019 CR13_165		mkaavlfclafictteimpveaQYTD--ANNRACKKDGRRGGSLLGFVN-VQFFH--	58	100	-----
Abi AIX87626 defensin-1		mktivllfvlalvfctlemgvveaGFGCPFNQGRHHRHSIGRRGGYRGIFK-QTAYRK	62	43	1e-06
Meu AIL4897 pMeKTx21-1		mkiptvilllafilcsqeiaveaSGGVF--WRNSDQIRRRYRGGIQGILNRYVYLS-	59	43	0.001

C

AccNum name	signal peptide	MATURE PEPTIDE	aa	I%	e-value
Ced MK932020 CR13_25		-----PLRRLAAGLAVVSTQSTWDDFDALDDMDLDDLLDLEPADLVLLDMWANMLENSDFEDDFE...	63	100	-----
Csc XP023227050 AniPep		mvrkslivlllvsvlvstflttadaYPASFDDDFDALDDMDLDDLLDLEPADLVLLDMWANMLENSDFEDDFE-	75	100	0.004
Mgi AHZ63125 NDBP6		mssktllvlllvglvstffftadaYPASMDFDNDLEELNLDLDDYFDLEPADFVLLDMWANMLENSDFEDDFEY	76	78	4e-12
Odo ALY33539 AMP5		mssktllvlllvglvstffftadaYPASMDNYDDALEELNLDLDDYFDLEPADFVLLDMWANMLENSDFEDDFE-	75	76	5e-12
Abi AIX87589 Aba-2		mssktllvlllvglvptffftadaYPASMDNYDDALEELNLDLDDYFDLEPADFVLLDMWANMLENSDFEDDFE-	75	76	3e-11
Boc ACJ23162 Tx65		-----MDNYDDALEELNLDLDDYFDLEPADFVLLDMWANMLENSDFEDDFE-	47	76	8e-11

Fig. 7. Multiple alignments of *Centruroides edwardsii* peptide precursors and deduced sequences. (A) Precursor sequences related to scorpion toxin Cn11 that affects Na^+ -channels including *C. edwardsii* CR13_114. (B) Precursor sequence from *C. edwardsii* (CR13_165) and related scorpion defensin-peptides. (C) Non-disulfide bridged peptide (NDBP) precursors and *C. edwardsii* CR13_25 partial sequence. Signal peptides are indicated in lower case letters; sequences in bold and capital letters correspond to mature peptides. Identical residues are highlighted in grey color and Cys residues in red. The sequence number of residues, identity (%) and E-values are shown on the right columns. Identity percentages and e-values correspond to the complete precursor sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Lethality expressed as LD₅₀ and LD₉₅ (µg/g) (upper panel) and lethal dose ratio LD₅₀ and LD₉₅ (lower panel) of *Centruroides edwardsii* venom on different potential prey after 24 h.

<i>C. edwardsii</i> venom (µg/g)	<i>Archeta assimilis</i>	<i>Archeta domesticus</i>	<i>Periplaneta australasiae</i>	<i>Tenebrio molitor</i>	<i>Peckia intermutans</i>	<i>Hemidactylus frenatus</i>
LD ₅₀	47.0 (26.3–84.1)	3.6 (2.1–5.5)	212.6 (114.1–351.2)	147.5 (86.9–236.1)	97.5 (59.6–174.9)	124.3 (68.5–200.3)
LD ₉₅	27.6 (140.6–167.4)	10.9 (7.0–33.6)	950.9 (534.4–4752.0)	465.0 (289.0–1695.7)	307.2 (185.6–1458.8)	ND
Potential prey	<i>A. domesticus</i>	<i>P. australasiae</i>	<i>P. intermutans</i>	<i>T. molitor</i>	<i>H. frenatus</i>	
<i>A. assimilis</i>	LDR ₅₀ = 12.74	LDR ₅₀ = 0.22	LDR ₅₀ = 0.47	LDR ₅₀ = 0.30	LDR ₅₀ = 0.37	
	(6.92-23.45)	(0.11–0.41)*	(0.25–0.85)*	(0.16–0.55)*	(0.21–0.64)*	
	LDR ₉₅ = 0.29 (0.08–1.5)	LDR ₉₅ = 0.29 (0.08–1.05)	LDR ₉₅ = 0.90 (0.26–3.6)	LDR ₉₅ = 0.59 (0.18–1.94)	LDR ₉₅ = 1.28 (0.43–3.84)	
<i>A. domesticus</i>		LDR ₅₀ = 0.01	LDR ₅₀ = 0.03	LDR ₅₀ = 0.02	LDR ₅₀ = 0.02	
		(0.01–0.03)*	(0.02–0.06)*	(0.01–0.04)*	(0.01–0.04)*	
		LDR ₉₅ = 0.01 (0.004–0.03)*	LDR ₉₅ = 0.03 (0.01–0.09)*	0.02 (0.009–0.05)*	LDR ₉₅ = 0.05 (0.02–0.11)*	
<i>P. australasiae</i>			LDR ₅₀ = 2.12	LDR ₅₀ = 1.36	LDR ₅₀ = 1.68	
			(1.18–3.79)*	(0.76–2.44)	(1.0–2.83)*	
			LDR ₉₅ = 3.09 (1.01–9.44)*	LDR ₉₅ = 2.04 (0.69–5.97)	LDR ₉₅ = 4.43 (1.68–11.68)*	
<i>P. intermutans</i>				LDR ₅₀ = 0.64	LDR ₅₀ = 0.79	
				(0.36–1.12)	(0.48–1.3)	
				LDR ₉₅ = 0.66 (0.24–1.79)	LDR ₉₅ = 1.43 (0.58–3.48)	
<i>T. molitor</i>					LDR ₅₀ = 1.23	
					(0.75–2.02)	
					LDR ₉₅ = 2.17 (0.94–5.0)	

*Significant differences ($p < 0.05$). ND=Not determined. Confidence intervals are in between parenthesis.

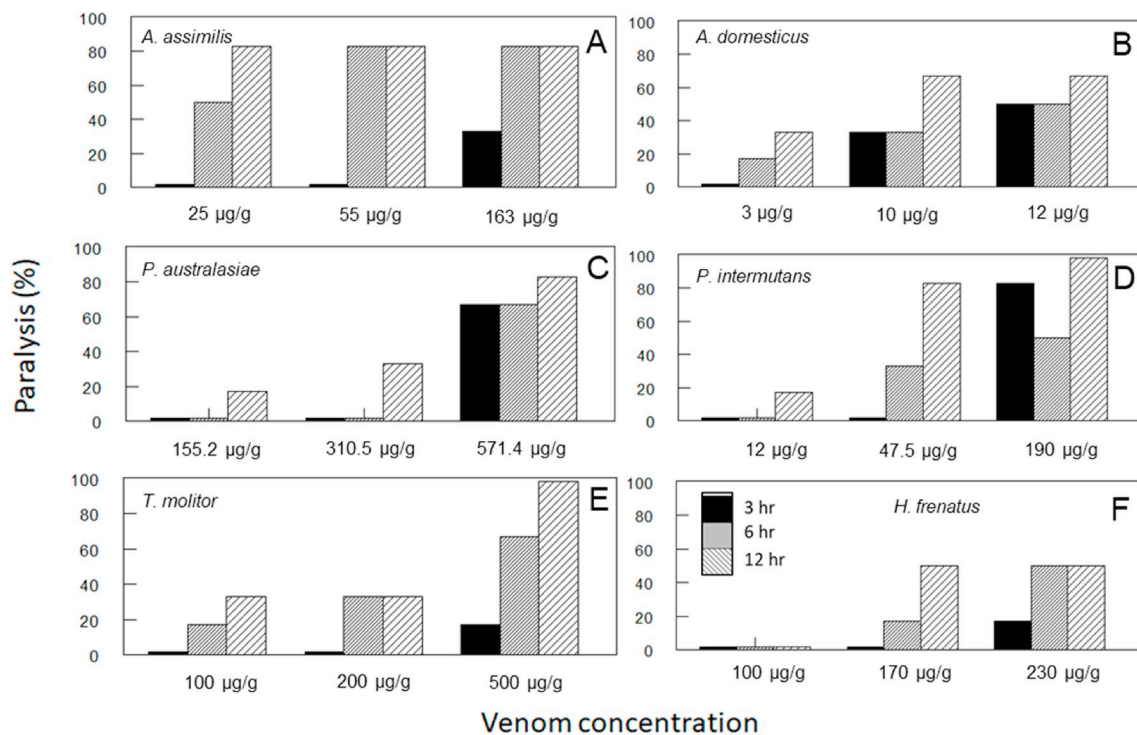


Fig. 8. Paralysis induced by *Centruroides edwardsii* venom on different potential prey. Groups of five animals were injected with different doses of venom dissolved in saline solution and observations were recorded at three different times (3, 6 and 12 h) after venom injection. The percentage of paralyzed individuals was determined in each observation. Injections were applied with a Hamilton® syringe, in the case of invertebrates (volumes between 10 and 50 µL) in different parts of the body, depending on the prey, as described in Methods. Paralysis was defined as the inability of the animal to return to its position, even when some extremities still conserve some movements when a stimulus is applied. In the case of larvae, paralysis is evidenced by retraction of cuticula and the absence of movement.

as an indication of discomfort or pain. Doubling this dose caused an alteration in movement activity, an increase in heart rate, and mice entered an excitation phase preceded by erratic and uncoordinated movements. We also observed an increase in salivation and a decrease in

respiratory rate that led to an ataxia phase lasting for approximately 20 min. After this period the individuals managed to recover. Other observed signs are summarized in Table 3. During our trials, we did not observe mice paralysis at any dose.

4. Discussion

Our analysis on the protein composition of *C. edwardsii* venom reveals the presence of peptides with masses compatible with neurotoxins that modulate Na⁺- (7–8 kDa) and K⁺-channels (3–5 kDa). By MS and Edman degradation, we identified fragments similar to several peptides also present in venoms from other New World buthids. Among them, a margatoxin-like peptide, a Na⁺-channel toxin with similarity to Tb2-II from the Brazilian scorpion *T. bahiensis* (Pimenta et al., 2001), and a scorpine-like peptide. Margatoxin is a 39 amino acid voltage-dependent K⁺-channel inhibitor of K_v1.3 channels (García-Calvo et al., 1993), that was originally identified in sister species *C. margaritatus*, and scorpine-like peptides are also K⁺-channel binding molecules with antimicrobial properties, present in several scorpion venoms (Luna-Ramírez et al., 2016).

C. edwardsii venom also contains other peptides with sequences similar to Na⁺-channel-modulating toxins. Two of them were similar to TbIT-I (from *T. bahiensis*) and neurotoxin 3 (CsEv3) toxins, which are reported to be more toxic for invertebrates than vertebrates (Jablonsky et al., 1999; Pimenta et al., 2001). Na⁺-channel β-toxins shift the voltage of activation of these channels towards a more negative one due to a reduction of the Na⁺ current peak amplitude (Pedraza-Escalona and Possani, 2013). In this way, these toxins induce some of the common neurotoxic alterations observed in the different target animals assessed in our study, including paralysis and death (Pimenta et al., 2001).

When we sequenced the N-terminal of some *C. edwardsii* venom purified peptides, one of these sequences was KKDGYPDSEECRY, identified in Na⁺-channel β-toxin CeII8, from the Mexican scorpion *Centruroides elegans* (Vandendriessche et al., 2010). CeII8 is a toxin that binds to voltage-gated Na_v1.7 mammal channels, and despite the fact that it is not lethal to mice, it induces pain and is also insect depressant (Vandendriessche et al., 2010). Na_v1.7 voltage-channels are present in neurons of the dorsal root ganglion from the Central Nervous System (CNS) and are responsible for the initiation phase of pain sensations. Therefore, toxins that bind to these channels can cause hypersensitivity in mammals (Jami et al., 2018; Saez et al., 2010). Ataxia and other CNS effects are signs of toxicity induced by some analgesics that act on this specific Na⁺-channel (Saez et al., 2010), suggesting that some motor alterations observed in vertebrates envenomed by scorpions could be associated to the activities of neurotoxins that bind Na_v1.7 channels.

Our partial analyses on *C. edwardsii* venom gland transcripts allowed us to identify a few other potentially toxic components: a defensin-like peptide, a Na⁺-channel toxin similar to Cn11 from the Mexican scorpion *C. noxious* (Ramírez-Domínguez et al., 2002), and an NDBP. Interestingly, a toxin similar to Cn11, named Cm38, has been previously found in *C. margaritatus* venom, which depolarizes rat dorsal root ganglion neurons and induces firing of multiple action potentials (Dueñas-Cuellar et al., 2015).

Venom peptide maturation from precursor sequences requires post-translational modifications including proteolytic processing and C-terminal amidation. In *C. edwardsii* venom, we identified an enzyme potentially responsible for peptide amidation, corresponding to a copper type-II ascorbate-dependent enzyme named peptidylglycine α-hydroxylating monooxygenase, with similarity to monoO, an enzyme found in the transcriptome and proteome of the striped scorpion *C. hentzi* from Florida (Ward et al., 2018). In the cone snail *Conus*, venom duct conotoxins undergo amidation at the C-terminus, which is associated with increased stability and affinity for the target ion channels (Ul-Hasan et al., 2013). The same maturation process is most likely occurring in arthropod venoms since it is known that their peptides can be also amidated (Delgado-Prudencio et al., 2019; Guerrero-Vargas et al., 2012; Rates et al., 2008) and that this event significantly increases the affinity for targets (Coelho et al., 2014).

Another important step in neurotoxin maturation is the proteolysis of precursors into peptides with different length. Regarding the proteolytic processing of peptides, in the case of the spider *Cupiennius salei*, for instance, a 28 kDa-heterodimer chymotrypsin-like serine protease has been considered responsible for the maturation of venom peptides (Langenegger et al., 2018). We did not find any serine proteinases in *C. edwardsii* venom, but we identified sequences from two different proteins similar to metalloproteinases (metalloserpinase- and antitrypsin-like proteases). Purification of these enzymes in arthropod venoms is difficult because their concentration could be even 1000-fold lower than that of main neurotoxins (Kuhn-Nentwig et al., 2004). Another factor that contributes to making it difficult to identify proteases is the loss of activity during purification, for instance by the effect of solvents used in RP-HPLC.

Despite we could not identify any serine proteinases, we cannot rule out their presence in *C. edwardsii* venom, as they have been widely reported in other *Centruroides* species. For instance, *C. hentzi*, another “mammal-harmless” member of this group, expresses nine proteases in its transcriptome, three of which are serine proteinases (Ward et al., 2018). Gelatin zymography showed that other metalloproteinases (whose activity was inhibited by EDTA) are also present in this venom since their molecular weights did not correspond to the ones identified by MS. In other New World buthids, such as some *Tityus* species, proteases are also expressed, but their activity is very low, requiring a high concentration to produce activities such as fibrinogenolytic (Carmo et al., 2014), for instance. Estrada-Gómez and collaborators (2014) reported that the venom of *C. edwardsii* from Colombia lacks coagulant and proteolytic activity on azocasein. In partial agreement to that result, in our study, we did not find any proteolytic activity on fibrinogen (at 3 h), but gelatin was a substrate for some of these enzymes.

In addition, *C. edwardsii* venom, like the venom of *C. hentzi* and other scorpions, contains some CRiSPs and a hyaluronidase, even though the latter, in the case of *C. hentzi*, was found only in the transcriptome (Ward

Table 3

Parameters associated with the toxicity of *Centruroides edwardsii* venom injected in different invertebrates and vertebrates.

Signs	<i>Archeta domesticus</i>	<i>Archeta assimilis</i>	<i>Periplaneta australasiae</i>	<i>Tebebrio molitor</i>	<i>Peckia intermutans</i>	<i>Hemidactylus frenatus</i>	<i>Mus musculus</i>
Walking impairment	X	X	X				
Loss of balance	X	X	X			X	X
Tendency to stay on the dorsal side	X	X	X				
Involuntary movements (abdomen)	X	X	X		X	X	X
Ataxia						X	X
Overexcitement	X	X	X	X		X	X
Body rigidity						X	X
Involuntary movements (limbs)							X
Flaccidity				X	X		
Increased salivation							X
Piloerection							X
Defecation						X	X

et al., 2018). Hyaluronidases are a group of interesting and poorly characterized enzymes found in venoms and animal fluids, arthropod secretions not being the exception. Usually, hyaluronidases are in such a low concentration that they can be missed in proteomic analyses (Bordon et al., 2015). Several studies have shown that these enzymes potentiate the toxicity of scorpion (Horta et al., 2014) and snake (Girish and Kemparaju, 2006; Kemparaju and Girish, 2006) venoms, altering the extracellular matrix of different tissues, especially blood vessel walls (Mahadeswaraswamy et al., 2011). In *Tityus serrulatus*, Oliveira-Mendes et al. (2019) recently showed that the neutralization of venom hyaluronidase prevents venom components to reach organs such as heart, lungs, liver and spleen of mice, remaining at the injection site or the bloodstream.

Venom hyaluronidases from several arthropods, especially from insects such as wasps, have been characterized as highly allergenic proteins, together with some CRiSPs such as allergen-5 (Bordon et al., 2015; Rungsa et al., 2016). Working with the venom of *Acanthoscurria natalensis* spider, Barth et al. (2019) recently showed that a hyaluronidase and a CRiSP form a non-covalently bound complex. Similarly, in the venom of the wasp *Vespa magnifica*, antigen-5 (a CRiSP with allergenic properties) and the hyaluronidase, elute together when purified by size-exclusion chromatographic techniques (An et al., 2012). Through our RP-HPLC separation, we obtained two proteins that elute together, a hyaluronidase and a CRiSP similar to allergen-5. We were unable to establish conclusively whether both proteins formed a complex, but it is an interesting possibility that should be followed in future studies.

According to Lourenço (2018), New World scorpion genera such as *Centruroides* and *Tityus* produce toxins that affect both mammals and insects, in contrast to Old World buthids which are more prone to express separated toxins. In bark scorpions such as *C. edwardsii*, venom probably affects vertebrates fundamentally inducing pain and discomfort, not paralysis or death; even when insect voltage-regulated Na^+ -channels share around 60% of identity with mammalian membrane proteins (King et al., 2008). Venom components in scorpions must be subject to pressures imposed by both, the arrest of potential predators and the ability to immobilize prey. Often, lethality is not the main goal of predatory venom (Harris and Jenner, 2019) since, in the case of catching and eating prey, paralysis could be enough for trophic purposes. To avoid predators, on the other hand, the purpose could be to induce pain and uncomfortable sensations that would increase the possibility of escaping (Baptista-Dias et al., 2018; Sunagar et al., 2013). In any case, regulated use of the venom should be important, especially if other energy-less costly strategies to capture prey or evade predators could be employed (Lourenço, 2018).

A preliminary observation reported that *C. edwardsii* venom is not toxic for excitable and non-excitable mammalian cells in culture (Rojas-Azofeifa et al., 2019). This suggests that the presence of peptides that bind to Na^+ and K^+ channels, as the ones identified here, is not sufficient to induce significant cellular toxicity in mammals. Accordingly, *C. edwardsii* whole venom was not lethal for mice as evidenced in our assay. In contrast, Brenes and Gómez (2016) reported high venom lethality (LD_{50} 4.4 $\mu\text{g/g}$) towards mice, from *C. edwardsii* collected in the same localities analyzed here. Their report, however, did not specify how the lethality analyses were conducted or the route of injection.

Nonetheless, *C. edwardsii* venom induces several envenomation signs in mice, some associated with pain (paw licking), some mild neurologic events including increased salivation, piloerection, and involuntary movements of abdomen and limbs. Lizards were also affected by this venom, with symptomatology including ataxia, involuntary movements on the abdomen and loss of balance. For these reptiles, paralysis and death can occur at high doses. All of these signs are probably associated with the presence of neurotoxins like the ones identified in *C. edwardsii* venom.

Variations in the susceptibility of target species to the scorpion venoms have been reported in other studies (Manzoli-Palma et al., 2003). Our assays evidenced that insects show differences in the

sensitivity to *C. edwardsii* venom. *T. mollitor* larvae were very resistant, more than larvae from the *P. intermutans* flies, but the most resistant insects tested were cockroaches. Crickets, on the other hand, were the most sensitive, and interestingly, we found differences between the two species tested. This differential effect could be based on the substitution of some of the amino acids from the target molecular sequences (voltage-gated Na^+ channels, for instance), as it has been observed for other insects when arthropod toxins were tested (Bende et al., 2014). Salazar et al. (2018) showed that the venom of other *Centruroides* species from Panama induces paralysis in crickets but only one peptide purified from the species tested (from *C. bicolor*) was lethal to mice when injected intraperitoneally. We also tested the potential effect of this venom in two species of scorpions (including *C. edwardsii* itself), but they were completely resistant to the venom, showing no signs of envenomation.

In the present study, we characterized the venom of a common species of buthid scorpion that inhabits Latin American countries and that, as well as other studied species, is more toxic for invertebrates than vertebrates. However, this venom can be lethal to some small reptiles and cause some signs of toxicity in mammals. As well as for other *Centruroides* species, the venom is an important source of neurotoxins (including several Na^+ -channel binding toxins), proteases, CRiSPs and a hyaluronidase, which probably makes the venom more effective, at least for vertebrates. Possible similarities between *C. edwardsii* and its sister species *C. margaritatus*, in terms of their venoms, is pending on a better characterization of the latter, since, even when they have been separated as species, they share very similar phenotypes, and therefore it is possible that they could produce venoms with comparable biochemical and biological properties.

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Appendix A. Supplementary data

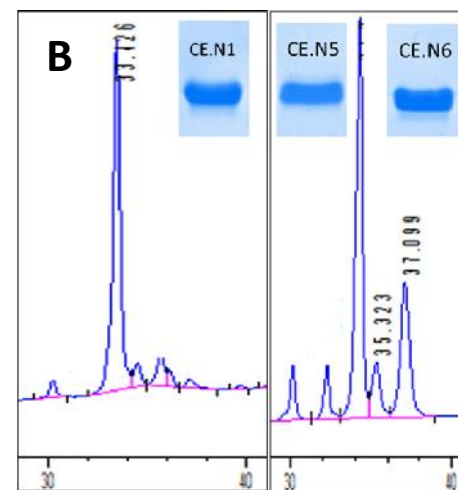
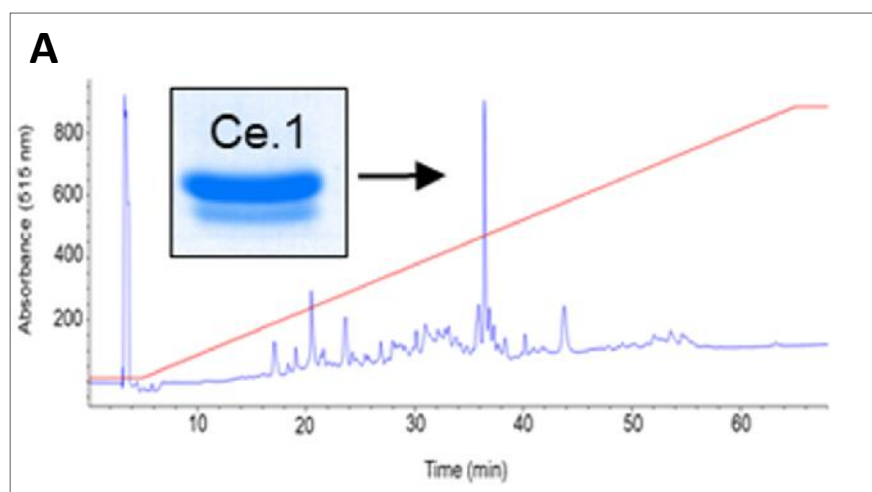
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxicon.2019.09.021>.

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A Hyaluronidase

XP_023226974 C.sculpturatus MHSISIFSIFISIIYSAQADFVKYWEVPSF
tr|A0A2I9LP21 C.hentzi MHSISIFSIIISIIYSAQADFVKYWEVPSF
----- C.edwardsii XXXXXXXXXXXXXXXXXXXXXVYWEVPSF

LC SKKYKINITQDLTSHKVLVNQGEGFNGDKIVIFYENQLGKYPYIDPTKGDVNGGLLQV
LC SKKYKINITQDLTSHKVLVNSGEGFNGDKIVIFYENQLGKYPYIDSTKGDVNGGLLQV
LC SKXXXXXXXXXXXXXXXXVLVNQGEGFNGDKXXXXXXXXENQLGKYPYXXXXXXXXXXXXXXXX

ADLKEHLKVS KDDITKFI PNPKFDGIGVIDWESWRPSWDFNWGKMKVYRERSIDL VKSKH
ADLKEHLKVS KDNITKFI PNPKFDGIGVIDWESWRPSWDFNWGKMKVYRERSIDL VKSKH
XXXXXXXXKVS KDDITKFI PNPKFDGIGVIDWESWRPSWDFNWGKMXXXXXXXXXXXXSKH

PDWSSKKIEEAAIKEWEDSAKEWMLKTLKLAEDMRPDAGWCYYYFPDCYNYNGKDQPSQF
PDWSSKKIEEAAIKEWEE SAKEWMLKTLKLAEDMRPDAGWCYYYFPDCYNYNGKDQPSQF
PDWSSKKIEEAAIKEWEE SAKEWMLKXXKLAEDMRPDAGWXXYYFPDCYNYNGKDQPSQF

TCNARVREQNSRLSWLWNQSTALCPSIYTQESH IKKYNMSQRAWWIDARLRETM RVANPN
TCNARVREQNSRLSWLWNQSTALCPSIYTQESH IKKYNMSQRAWWIDARLRETM RIANPN
TCNARVREQNSRLSWXXXXXXXXXXXXXTQESH IKKYXXXXXAWWIDARLRETMXXXXXX

TPIYPYINYVLPGTNETIPSMDFKRMLGQIASLGLDGAIIWGSSYHVLTKSQCELTATYV
TPIYPYINYILPGTNDTIPSMDFKRMLGQIASLGLDGAIIWGSSYHVLTKSQCELTATYV
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXHVLTKSQCELTATYV

KDVMAPTIATVVLNTNRC SQVICKGRGH CYWPDEPFTSWKY LIDPKLPVFKPTNISCKCK
KDVMAPTIATVVLNTNRC SQLICKGRGH CYWPDESFTSWKY LIDPKMPVFKPTNISCKCK
KDVMAPTIATVVLNTNRC SQLICKXXXXXXXXXXXXXXXXXXLIDPKMPVFKPTNISCKCK

401
GYTG RYCQIAP
GYTG RYCQIAN
GYTG RYCQIAN

B Venom allergen 5-like (CRiSP)

XP_023241578 C.sculpturatus MKGTLFFDIPPKLIFVLIYLCSSVLSDCPA
tr|A0A2I9LNV7 C.hentzi MKGTLFFDITPKLIFLLIYLCSSVLSDCPA
----- C.edwardsii XXXXXXXXXXXXXXXXXXXXXXXDCPA

LYK RYSHEHTFCKTKNQKCHVKKWGVSEDDREIIIDLHNKVRNNIAIGKDQSGRLPAAGD
LYK RYSHEHTFCKAKNQKCLIKKWGVSEDDQEIIIDLHNKVRNNIAVGKDQSGRLPAAGD
LYK RYSHEHTFCKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

MLEMEWDDELAQIAQKLADQCVFKHDCDDCRKVENFDVGQNIYTSTITAVKPPESFWDA
MLEMEWDDELAQIAQKLADQCVFKHDCDDCRKVENFDVGQNIYTSTITAVKPPPEFWDA
XXXXXXXXXXXXXXXXXXLADQCVFKHDCDDCRKVENFDVGQNIYXXXXXXXXXXXX

IRSWYSEIYRFTPDIKPF TSDHATGHFTQLAWSTTWRVGC GYVLYEKRRDSWTQLYVCN
VRSWYSEIYRFTPDIKPF TSDHATGHFTQLAWSTTWRVGC GYVLYEKRRDSWTQLYVCN
XXSWYSEIYRFTPDIKPFXXXXXXXXXXXXXXXXXXXXXVGC GYVLYEKRRDSWTQLYXXX

YGPAGNIDDSEMYKVGKPCDKCPGNTCCGAHCKTRKPSSYLGLCKVLNGRGPDFDETDFG
YGPAGNIDDSEMYKVGKPCDKCPGNTCCGEHCKTRKPSSYFGLCKVLNGRGPDFDETDFG
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXNGRGPDFDETDFG

NFIFNCDFRPESSSDCNSKVEGSNKWQTRQIISDVYKTVVLNGGESSLK FSSNIQSKNG
NFIFDCDFRPESSSDCNSKVEGVNKNWQTRQIISDVYKTVVLNGGESSCLK FSSNIQSKNG
NFXXXXXXXXXXXXXXXXXXXXXXQTRQIISDVYXXXXXXXXXXXXXKFSSNIQSKNG

FCLTVSFRKGPNVAGTKSDSKFDLQLERRGPPLSFELDSEGNQWLPYSMGIPMNQPMQI
FCLTVSFRKGPNVAGTKSDSKFDVELDRKGAAPLSFELDSEGNQWLPYSMGIPMNRPMQI
FXXXXXXRKGPNVAGTKSDSKFXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX

418
NLKFSVPKGS PAQYLDVNYVRARPGVCK
NLKFSVPKGS PAQYLDIN YVRARPGVCE
XXXXSVPKGSPAQYLDISYVRARPGVCE

Table SI. Precursor sequences deduced from the cDNA library of venom glands (telson) from *Centruroides edwardsii*.

ID GenBank	Name	Sequence	similarity / e-value
		Toxins-like	
MK932018	CR13_114	GYLLDKKGCKIVCFVNSEWCNSLCKGKSGKYGYCKALACY CESAPDNVKVWTSNTNTC	Toxin Cn11, partial [<i>Centruroides noxius</i>], 1e-18 (63%). 8 Cys.
MK932019	CR13_165	MKAAVLFFCLAFIFCTTEIMPVEAQYTCDDANCNRACKKDG YRGGSCLGFINVQCFCH	Defensin-1 [<i>Androctonus bicolor</i>], 9e-07 (43%). 6 Cys
MK932020	CR13_25	PLRRLAAGLAVVSTQSTWDDFDALDDMNDLDDLDDLEP ADLVLLDMWANMLENSDFEDDFE	Antimicrobial non-disulfide-bridged peptide 6, partial [<i>Mesobuthus gibbosus</i>]. 4e-12 (79%); anionic peptide, partial [<i>Centruroides sculpturatus</i>], 5.7 (94%).
		Other venom components	
MK932021	CR13_54	MSKVTFKITQTADPKLPYKVLSPVEQTPFTAVLKFAAEFEKV PPATSAITDDGVGINPSQTAGDIFLKHGSELRLIPRDRVGNK IE	Ubiquitin-fold modifier 1 [<i>Limulus polyphemus</i>], 2e-49 (94%).
MK932022	CR13_66	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLL IFAGKQLEDGRTLSDYNIQKESTLHLVLRRLRGGAKKRKKKN YTTPKENKHKKKKVKLAVLKFKYKVDENGKITRLRRECPNDE CGAGVFMASHFDRQYCGKCCCLTFVYNKPPEA	60S ribosomal protein L40A, partial [<i>Latrodectus hesperus</i>], 1e-77 (97%).
Not submitted ¹	CR13_C2	ISSIAQERGQGVCSYKYNFHDLSNRCKPGWFL	Hypothetical protein, partial [<i>Tityus discrepans</i>], 2e-14 65%
Not submitted ¹	CR13_95	GRAGTGVDNIDCNAATRNGVLVINAPGGNAISAAELTCAMII TLRHLPPQACTSLKN	D-3-phosphoglycerate dehydrogenase, partial [<i>Stegodyphus mimosarum</i>], 4e-22 (77%).
Not submitted ¹	CR13_13	MGRNLEVVGDIEELLKWFEEVEKQLIECEPLVPELDTLVTL KEQKSLNEEVSSQKKKK	Uncharacterized protein (Cytoskeleton), [<i>Centruroides sculpturatus</i>], 7e-25 (95%)
		No match	
Not submitted ¹	CR13_138	KISTFVFLIEVKYCNFFWQAEKFKFIRQIYLKINNCGLVFIFT FRFCLCLQFF	4 Cys.

Predicted amino acid sequences of putative peptides (DNA sequences under 200 bp).