Exploring venom diversity in *Mixcoatlus browni* and *Mixcoatlus barbouri*: A comparative analysis of two rare Mexican snake species with crotoxin-like presence

Edgar Neri-Castro [a, b, *], Vanessa Zarzosa [b], Bruno Lomonte [c], Fernando Zamudio [a], Lorena Hernandez-Orihuela [b], Alejandro Olvera-Rodríguez [b], Audrey Michelle Rodríguez-Solís [b], Miguel Borja [a], Uri O. García-Vázquez [d], Jason M. Jones [e], Chistopher L. Parkinson [f], Alejandro Alagón [b, **]

[a] Investigador por México, Facultad de Ciencias Biológicas, Universidad Juárez del Estado de Durango, Avenida Universidad s/n. Fracc. Filadelfia, C.P. 35010, Gómez Palacio, Dgo, Mexico
[b] Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Avenida Universidad 2001, Chamilpa, C.P. 62210, Cuernavaca, Mor, Mexico
[c] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, 11501, Costa Rica
[d] Laboratorio de Sistemática Molecular, Carrera de Biología, UMIEZ, Facultad de Estudios Superiores Zaragoza, Universidad Nacional Autónoma de México, Batalla 5 de Mayo s/n, Ejército de Oriente, Ciudad de México, 09230, Mexico
[e] Herp.mx A.C., Villa del Álvarez, Colima, Mexico
[f] Department of Biological Sciences and Department of Forestry, and Environmental Conservation, Clemson University, 190 Collings St. Clemson, SC, 29631, USA

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ABSTRACT

The genus *Mixcoatlus* is composed of three species: *Mixcoatlus barbouri*, *M. browni*, and *M. melanurus*, of which the venom composition of *M. melanurus*, the most common species of the three, has only recently been described. However, very little is known about the natural history of *M. barbouri* and *M. browni*, and the venom composition of these two species has remained thus far unexplored. In this study we characterize the proteomic profiles and the main biochemical and toxic activities of these two venoms. Proteomic data obtained by shotgun analysis of whole venom identified 12 protein families for *M. barbouri*, and 13 for *M. browni*. The latter venom was further characterized by using a quantitative 'venomics' protocol, which revealed that it is mainly composed of 51.1 % phospholipases A_2 (PLA₂), 25.5 % snake venom serine proteases (SVSP), 4.6 % ʟ-amino oxidases (LAO), and 3.6 % snake venom metalloproteases (SVMP), with lower percentages other six protein families. Both venoms contained homologs of the basic and acidic subunits of crotoxin. However, due to limitations in *M. barbouri* venom availability, we could only characterize the crotoxin-like protein of *M. browni* venom, which we have named Mixcoatlutoxin. It exhibited a lethal potency in mice like that described for classical rattlesnake crotoxins. These findings expand knowledge on the distribution of crotoxin-like heterodimeric proteins in viper snake species. Further investigation of the bioactivities of the venom of *M. barbouri*, on the other hand, remains necessary.

1. Introduction

Mexico ranks first in the New World in terms of the diversity of venomous snakes, boasting 89 species grouped into 13 genera. Two genera are endemic to Mexico, *Ophryacus* and *Mixcoatlus*. The latter is represented by three species: *M. melanurus*, *M. barbouri* and *M. browni*. Of the latter two, the composition of their venoms is

unknown and the information about their natural history is scarce. The taxonomic history of *Mixcoatlus* genus is complex. After its description, *Porthidium melanurum* was reassigned to *Ophryacus* by Gutberlet based on morphology [1], and *Agkistrodon browni* was considered a synonymy of *Cerrhopidion barbouri*. However, more recently, Jadin et al. (2011) described the genus *Mixcoatlus* and included *Ophryacus melanurus*, *Cerrophidion barbouri* and *Cerrophidion browni*. In terms of distribution, *M. barbouri* and *M. browni* are restricted to the pine-oak forests and cloud forests of the Sierra Madre del Sur in Guerrero. On the other hand, *M. melanurus* is found in arid tropical pine-oak scrub in southern Puebla and northern Oaxaca [2,3].

Few studies have been conducted on the venoms of Mexican viper species; however, significant efforts have been made in the last five years to characterize venoms of which little was previously known. Results of these studies have shown that there are several viper species with venoms that express neurotoxic components similar to crotoxin, referred to as crotoxin-like proteins, such as *Crotalus basiliscus* [4], *C. lepidus klauberi* [5], *C. s. scutulatus* [6,7], *C. tzabcan* [8], *C. mictlantecuhtli* [9], *C. simus* [8], *C. ehecatl* [8], *Ophryacus sphenophrys* [10], and *Mixcoatlus melanurus* [11]. Nevertheless, there are still numerous species awaiting analysis, so this list may be even longer. These descriptions are highly significant from both an evolutionary and clinical viewpoint. The latter is particularly important for medical professionals, as understanding that there are species capable of causing neurotoxic envenomations is crucial for predicting clinical presentations and thus improving treatments.

Regarding the composition of *Mixcoatlus* spp. venoms, only that of *M. melanurus* is currently known [11], while no information is available on the other two species. An intriguing aspect of *M. melanurus* venom is its possession of a potent neurotoxin, Melanurotoxin, which bears amino acid sequence similarity to Crotoxin, a thoroughly characterized toxin found in many rattlesnake venoms. It is therefore of interest to determine whether the other two species also possess this neurotoxin. The primary objective of this study is to comprehensively characterize the venom composition, biochemical, and toxic activities of the venoms from *M. barbouri* and *M. browni*.

2. Methods

2.1. Ethical and animal collection permits

The animal experiments were approved by the Bioethics Committee of the Institute of Biotechnology at the National Autonomous University of Mexico, IBt, UNAM (project 345). All specimens were collected under permits issued to U.O.G.V. (permit number FAUT-0246) by the Secretariat of Environment and Natural Resources of the Mexican government.

2.2. Venoms and antivenom

Obtention of the venoms of *M. browni* and *M. barbouri* pose significant challenges due to their status as rare animals, and their occurrence in socially conflicted areas. *Mixcoatlus browni* venom was obtained by pooling samples from three adult specimens collected in Guerrero, while the venom of *M. barbouri* was sourced from a single adult specimen also found in Guerrero. Antivenom cross-recognition and neutralization studies were performed with Antivipmyn®, which is produced by immunizing horses with a mixture of the venoms of *Bothrops asper* and *C. simus*. Batch B-OJ-32, with a total protein concentration of 7 mg/mL, was used before its expiration date (August 2022) for all experiments.

2.3. Protein quantification

Protein quantification was estimated by using absorbance at 280 nm, considering one absorbance unit equals 1 mg/mL.

2.4. SDS-PAGE

Electrophoretic profiles of venoms and fractions were generated using 12.5 % SDS-PAGE gels under reducing conditions. Fractions obtained through RP-HPLC were analyzed, with 10 μg of each fraction loaded onto the gels. Pre-stained protein markers from Maestrogen (cat. No. 02102-250) were included for reference. Proteins were stained with Coomassie Blue for 1 h and subsequently destained using a solution consisting of 10 % acetic acid and 10 % isopropanol.

2.5. Chromatographic profiles

Each venom (0.7–1.5 mg) was dissolved in 500 μL of solvent A (0.1 % trifluoroacetic acid; TFA), followed by centrifugation at 15,000 rpm for 5 min. The samples were then separated using RP-HPLC on a C_{18} column (4.6 × 250 mm; Vydac®) and monitored at 214 nm. Elution was carried out at a flow rate of 1 mL/min, applying a gradient towards solvent B (100 % acetonitrile, containing 0.1 % TFA) according to the following program: 0 % B for 5 min, 0–15 % B over 10 min, 15–45 % B over 60 min, 45–70 % B over 10 min, and 70 % B over 9 min [12].

2.6. Lethality

The median lethal dose (LD_{50}) of individual or pooled venoms was determined by intravenous (i.v.) injection of various doses into groups of three mice (of either sex) of the CD-1 strain weighing between 18 and 20 g [13]. Deaths were recorded after 24 h and the LD_{50} was estimated by a non-linear regression method using Prism v.8.0.2 software (GraphPad Software).

2.7. Mass spectrometry (ESI-MS) and N-terminal amino acid sequencing

Intact masses of particular venom components were determined in a Finnigan LCQFleet mass spectrometer (Thermo) with electrospray ionization (ESI-MS). Samples were analyzed in positive ionization mode by direct infusion at 10 μL/min and spray voltage of 1.9 kV, using 50 % acetonitrile and 0.1 % acetic acid as solvent. Spectra were acquired using the Tune Plus software (Thermo) and deconvolution was performed with Xtract software (Walzthoeni et al., 2015) to obtain isotope-averaged intact molecular masses. N-terminal amino acid sequencing of some venom fractions was performed in a PPSQ-33A Protein Sequencer (Shimadzu), according to the manufacturer.

2.8. Shotgun-based whole venom proteomic profiling by nLC-MS/MS

Whole venom samples (15 μg) were subjected to shotgun proteomic profiling. Proteins were reduced with 10 mM dithiothreitol for 30 min at 56 °C, alkylated with 50 mM iodoacetamide for 20 min in the dark, and digested overnight at 37 °C using sequencing-grade trypsin in 25 mM ammonium bicarbonate, in a total volume of 40 μL. Digestion was stopped by adding 0.4 μL of formic acid, and the resulting tryptic peptides were separated by RP-HPLC on a nano-Easy 1200 chromatograph (Thermo) in-line with a Q-Exactive Plus mass spectrometer (Thermo). A total of 10 μL of the peptide mixture, containing 0.8 μg, was loaded onto a

C_{18} trap column (75 μm × 2 cm, 3 μm particle size; PepMap, Thermo). The trap column was washed with 0.1 % formic acid (solution A), and the peptides were subsequently eluted at 200 nL/min on a C_{18} Easyspray column (75 μm × 15 cm, 3 μm particle size; PepMap, Thermo) using a gradient towards solution B (80 % acetonitrile, 0.1 % formic acid) over 120 min (1−5% B in 1 min, 5−26 % B in 84 min, 26−80 % B in 30 min, 80−99 % B in 1 min, and 99 % B for 4 min). MS and MS/MS spectra were acquired using Excalibur control software (Thermo) and then processed using PEAKS X (Bioinformatic Solutions) against the Uniprot Serpentes database, as previously described [14]. Matches were assigned to known protein families by similarity. Cysteine carbamidomethylation was set as a fixed modification, while deamidation of asparagine or glutamine and methionine oxidation were set as variable modifications, allowing up to 3 missed cleavages by trypsin. Parameters for match acceptance were set to FDR<0.1 %, detection of at least one unique peptide, and −10lgP protein score ≥50.

Additionally, some venom fractions were subjected to in-solution digestion with trypsin, as described above, and the resulting peptides were analyzed by nLC-MS/MS after desalting in ZipTip C_{18} columns (Millipore). Peptides were separated using an Ultimate 3000 Dionex chromatograph in-line with an LTQ-Orbitrap Velos (Thermo), using a C_{18} capillary column (Acclaim PepMap; Thermo) and a gradient from 4 % to 85 % solvent B (where solvent A consisted of water, and solvent B consisted of acetonitrile, both containing 0.1 % formic acid). Separation was carried out over 120 min at a flow rate of 300 nL/min. All spectra were acquired in positive mode, with dynamic exclusion set at a maximum of 0.5 nL/min.

2.9. Phospholipase activity

The synthetic substrate 4-nitro-3-(octanoyloxy)-benzoic acid (4-NOBA) was used to determine the PLA_2 activity of the venoms and of fractions with a molecular mass (~14 kDa) expected for PLA_2. Microplate wells were filled with 200 μL of reaction buffer (10 mM Tris, 10 mM $CaCl_2$, 0.1 M NaCl, pH 8.0) and 25 μL of the substrate (4-NOBA; 1 mg/mL acetonitrile). The reaction buffer and substrate were placed in the microplate at 37 °C for 10 min and then 20 μL of the venoms (1 mg/mL) or fractions were added. The mixtures were incubated for 60 min at 37 °C, and absorbances were measured at 450 nm [15]. Raw values were multiplied by 100 (arbitrary value) for better visualization. Each sample was analyzed in triplicate wells.

2.10. Hemorrhagic activity

Twenty μg of each venom dissolved in 50 μL PBS was injected intradermally in the shaved backs of five ICR-CD1 mice weighing between 28 and 30 g. After 3 h, euthanasia was performed via CO_2 inhalation, and the dorsal skin was removed and stretched over a glass plate. Subsequently, the extent of hemorrhagic halo induced by each venom was measured to determine the average hemorrhagic area produced [7].

2.11. Fibrinogenolytic activity

Ten μg of each venom was incubated with 50 μg of human fibrinogen in a final volume of 50 μL for 30 min at 37 °C. Next, 5 μL of each sample was mixed with 3 μL of SDS-PAGE sample buffer containing 2-mercaptoethanol. Samples were boiled at 100 °C for 5 min, centrifuged at 13,000 rpm for 3 min, and analyzed by SDS-PAGE in 12.5 % gels [16]. To determine if the activity was caused by metalloproteases present in the venoms, their inhibition with ethylenediaminetetraacetic acid (EDTA) was performed. Venoms were pre-incubated with 5 mM EDTA for 30 min at 37 °C, then the steps described above were performed. The same procedure was carried out, but using the PMSF inhibitor to inhibit the serine proteases. Finally, both inhibitors were used together as previously indicated.

2.12. Purification and heterodimer formation of Mixcoatlutoxin

The acidic and basic subunits of Mixcoatlutoxin were purified using RP-HPLC under the conditions outlined in section 2.5. The collected fractions underwent analysis by mass spectrometry and partial sequencing. Subsequently, fractions were dried by vacuum centrifugation, redissolved in 100 μL of water, and quantified by absorbance at 280 nm using a Nanodrop spectrophotometer. Heterodimer formation was tested by mixing the two subunits at a 2:1 M ratio (acidic:basic) and incubating for 30 min at 37 °C.

2.13. Cross-recognition of Mixcoatlutoxin subunit B by anti-crotoxin and Antivipmyn polyclonal antibodies

Purified B subunit of Mixcoatlutoxin (0.5 μg/100 μL) was coated onto 96-well plates, by overnight incubation. The same amount of Crotoxin B subunit was coated for comparison. Antibody titers against both antigens were assessed by ELISA, using rabbit anti-crotoxin (50 μg/mL) or Antivipmyn (700 μg/mL) [17]. Serial dilutions of both antibodies, with a factor of 1:3, were incubated with the antigens for 1 h, washed, and detected by their corresponding anti-immunoglobulin/enzyme conjugates (goat anti-rabbit IgG or rabbit anti-horse IgG, conjugated to alkaline phosphatase, both at 1:4000 dilution). Titers were defined as the antibody dilution resulting in half-maximal binding signal, using GraphPad Prism, version 9.5. In the case of Antivipmyn, antibody binding was also evaluated comparatively against wells coated with crude venoms of *M. browni* or *Crotalus mictlantecuhtli* (0.5 μg/100 μL), as described for the purified toxins.

3. Results

3.1. Chromatographic profiles by RP-HPLC and intact masses

Separation of *M. browni* venom resulted in a total of 32 fractions, major ones eluting between 50 and 70 min. Fractions F1 to F4 could not be analyzed on the gel due to their low proportion in the venom. Fractions F5 to F11 did not exhibit observable proteins on the gel, although ESI-MS analysis of F6 and F7 showed molecular masses corresponding to disintegrins, with 6675 (medium-sized disintegrin) and 9202 Da (large-sized disintegrin), respectively. The fraction 10 was not analyzed due to its low proportion Conversely, fractions F16 to F22 displayed masses and electrophoretic profiles consistent with PLA_2 enzymes (Fig. 1). On the other hand, for the *M. barbouri* venom, 17 fractions were separated. Fractions F1 and F2 were excluded from analysis due to their limited yield, while fractions F4 and F5 exhibited no detectable protein bands on SDS-PAGE. Fractions F6 and F7 showed bands at approximately 25 kDa, whereas F10 through F16 revealed bands ranging from 20 to 75 kDa, potentially indicating the presence of Snake Venom Metalloproteinases (SVMP's) (Fig. 2).

3.2. Shotgun proteomic profiling of venoms

Utilizing a whole venom shotgun proteomics approach, the PEAKS algorithm matched 88 Protein Groups in the venom of *M. barbouri* (Supplementary Table S1) and 77 in the venom of *M. browni* (Supplementary Table S2). A manual alignment of the unique peptides found for each protein family was used to calculate
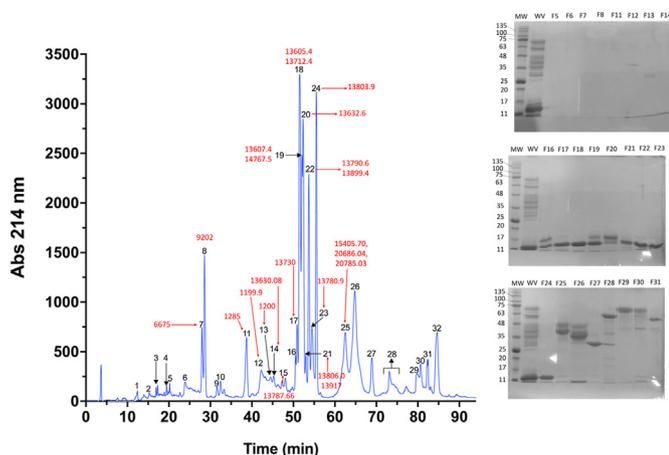
Fig. 1. RP-HPLC profile of *M. browni* venom (1.5 mg). The numbers in red indicate the intact mass for each fraction. In the right panel, each fraction (10 μg) was analyzed by 12.5 % SDS-PAGE under reducing conditions.
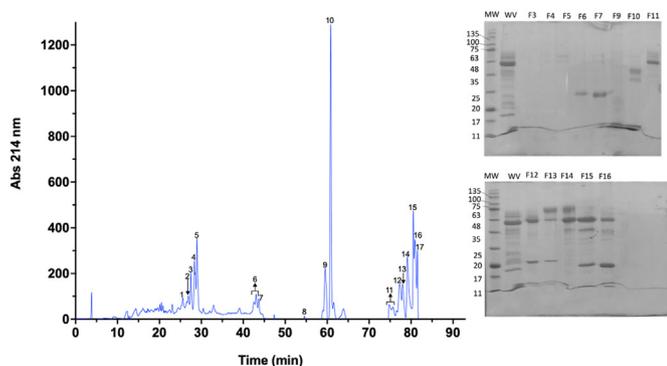
Fig. 2. RP-HPLC profile of *Mixcoatlus barbouri* venom (0.7 mg). In the right panel, each fraction (10 μg) was analyzed by 12.5 % SDS-PAGE under reducing conditions.

the minimum number of distinct proteins in the venoms. In the case of *M. barbouri*, these numbers corresponded to a minimum of 7 Snake venom metalloproteinases (SVMP), 2 snake venom serine proteinases (SVSP), 6 phospholipases A (PLA2), 3 C-type lectins (CTL), 1 cysteine-rich secretory protein (CRISP), 2 nerve growth factor (NGF), and one each for phospholipase B (PLB), ʟ-amino acid oxidase (LAAO), vascular endothelial growth factor (VEGF), phosphodiesterase (PDE), glutaminyl cyclase (GCY), and nucleotidase (NUC) proteins. For *M. browni,* a minimum of 2 SVMP, 3 SVSP, 8 PLA2, 3 CTL, and one each for LAO, VEGF, NGF, PDF, GCY, NUC, PDGF, PLB and HYAL was calculated (Suppl. Tables S1 and S2). Our analysis revealed that *M. barbouri* venom exhibited a greater diversity of SVMP proteins, whereas *M. browni* venom displayed a higher diversity of PLA2 proteins. In total, we identified 13 and 12 protein families in the venoms of *M. browni* and *M. barbouri*, respectively. Among the most notable findings is the discovery of short peptide sequences that match the acidic and basic chains of Crotoxin, a major β-neurotoxin described for neurotoxic vipers. In the case of *M. barbouri*, we were unable to confirm its lethal activity due to the insufficient amount of venom obtained; however, for the venom of *M. browni*, characterization could proceed.

3.3. Estimation of protein family abundances for Mixcoatlus browni venom

As summarized in Supplementary Tables S3 and S4, the

proteomic analysis of specific venom fractions by *de novo* sequencing, along with N-terminal amino acid sequencing analysis, allowed us to reconstruct quantitatively the venom composition of *M. browni* (Fig. 3). Predominant protein families were PLA2 (51.2 %) and SVSP (25.5 %), followed by LAO (4.2 %), disintegrins (3.6 %), SVMPs (3.5 %), and low percentages other six protein families. The first peptides suggesting the presence of neurotoxin in the venom was obtained from the shotgun analyses (Suppl. Tables S1 and S2). Considering the elution times of the reported crotoxin-like peptides and the molecular weights of PLA2, 2 μg/g of fractions F18 to F24 were injected in mice, of which only F22 was lethal and generated neurotoxic effects. F22 showed the N-terminal amino acid sequence NLLQFNKMIKEETGKNAIPFYA-FYGCYCGWGGRGRPKDAT, which shares 90 % similarity with *Crotalus d. terrificus* Crotoxin, and 92 % with *Ophryacus sphenophrys* Sphenotoxin. On the other hand, manual *de novo* peptide sequencing of F12, 13, and 14, revealed sequence similarity to Crotoxin subunit A (Suppl. Table S3). Thus, we inferred that *M. browni* venom contains a new crotoxin-like heterodimeric protein, representing 9.4 % of the venom proteins (our estimation reflecting the relative protein amounts that correspond, by protein sequence homology, to components of crotoxin), here named Mixcoatlutoxin.

3.4. Phospholipase A2 and hemorrhagic activity

Both *M. browni* and *M. barbouri* showed PLA2 activity on the 4-NOBA substrate, with values of 5.8 ± 1.0 and 4.3 ± 0.5 Units, respectively. As well, hemorrhage was induced in mice by the i.d. injection both venoms (20 μg), resulting in areas of 14.9 ± 4.3 mm^2 and 25.0 ± 1.5 mm^2 for *M. browni* and *M. barbouri* venoms, respectively.

3.5. Lethal activity

The median lethal dose (LD_{50}) for the venom of *M. barbouri* could not be determined due to the unavailability of venom. However, i.v. injection of a single dose of 1.30 μg/g did not result in death of the three injected mice. In contrast, the i.v. LD_{50} for *M. browni* was estimated at 2.0 μg/g (1.9–2.2 μg/g, 95 % confidence interval). Throughout these experiments, mice injected with the venom displayed neurotoxic signs, initially characterized by hind limb paralysis, followed by noticeable contractions in the intercostal muscles.

3.6. Fibrinogenolityc activity

Both *M. barbouri* and *M. browni* caused complete degradation of the α and β chains of human fibrinogen. When venoms were pre-incubated with EDTA to inhibit metalloproteases, the α chain was completely spared from degradation, while the β chain was only partially degraded. For both venoms, when PMSF was used to inhibit serine proteases, digestion of the β chain was prevented, while the α chain disappeared (Fig. 4).

3.7. Lethality of Mixcoatlutoxin

The i.v. LD_{50} of the basic subunit of Mixcoatlutoxin was 1.52 μg/g (1.51–1.54 μg/g), while the acidic subunit alone was non-lethal at 3.15 μg/g. However, when the acidic subunit was mixed with the basic subunit in a 2:1 ratio, respectively, to reconstitute the complex, the lethal potency increased two-fold, with an estimated LD_{50} of 0.7 μg/g (0.69–0.72 μg/g).
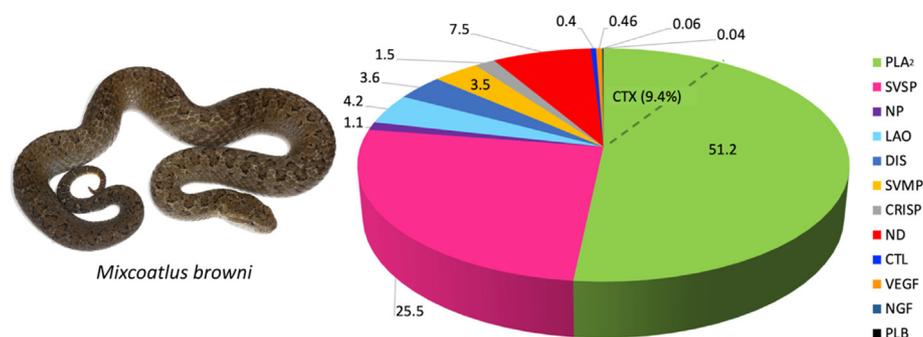
Fig. 3. Venomics of *Mixcoatlus browni*. Numbers refer to protein family relative abundances, in percentage. PLA₂: Phospholipases; SVSP: Snake Venom Serine Proteases; NP: Non-Proteinaceous Compound; Dis: Disintegrin; SVMP: Snake Venom Metalloproteases; CRISP: Cysteine-Rich Secretory Proteins; ND: Not Determined; CTL: C-Type Lectins; VEGF: Snake Venom Vascular Endothelial Growth Factors; NGF: Nerve Growth Factors; PLB: Phospholipase B.
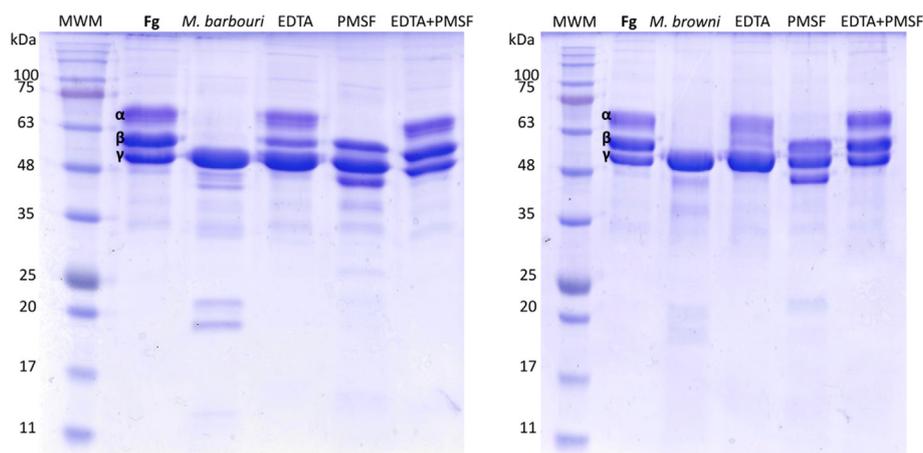
Fig. 4. Fibrinogenolytic activity of *Mixcoatlus* venoms. Fibrinogenolytic activity was evaluated on 12.5 % SDS-PAGE. Molecular weight markers (MWM) are shown at the left, followed by untreated human fibrinogen as a control (Fg). The lane labeled *M. barbouri* shows the venom incubated with fibrinogen, while the next lanes show venom pre-incubation with EDTA, PMSF, or EDTA and PMSF simultaneously. The figure on the right shows the same arrangement but using *M. browni* venom.

3.8. Neutralization by Antivipmyn

We conducted the neutralization of Antivipmyn's lethal activity towards *M. browni* venom, which required 57.9 μL of antivenom to neutralize 3 LD_{50}, meaning that 1 vial of antivenom is able to neutralize 518 LD_{50}. When expressing these results as the mass of antivenom required to neutralize 1 mg of venom, 10.66 mgAV/mgV are needed.

3.9. Cross-recognition of Mixcoatlutoxin by anti-crotoxin and Antivipmyn antibodies

Antivipmyn was able to recognize *C. mictlantecuhtli* and *M. browni* whole venoms very similarly; titers were 0.24 μg/mL (C.I. 0.0016 to 0.003) and 0.23 μg/mL (C.I. 0.0017 to 0.003), respectively. However, for the basic subunits of both venoms, a titer could not be obtained since we did not reach the asymptote. It is evident, though, that subunit B of *C. mictlantecuhtli* was better recognized than that of *M. browni* (Fig. 5). On the other hand, the polyclonal anti-crotoxin antibodies cross-recognized both *C. mictlantecuhtli* and *M. browni* purified B subunits, although recognition was significantly stronger for the former, being 28 times higher than the latter.

4. Discussion

4.1. Chromatographic profiles and venomics

Mexico ranks first in venomous snake diversity in the American continent [18]; however, it is one of the most lagging countries in snake venom research. Scant information has been gathered on the venoms of *Mixcoatlus* species, primarily focusing on *M. melanurus* [11], while *M. browni* and *M. barbouri* have remained unstudied until now. Unfortunately, we had little venom available from *M. barbouri*, so our present characterization is only partial, while for *M. browni*, we were able to carry out a more comprehensive characterization.

The chromatographic profiles showed that the venom of *M. browni* contains a greater number of fractions than *M. barbouri*. These profiles are not typical of other viperids, where often fractions from minute 75 onwards (usually metalloproteinases) tend to be the most abundant. Indeed, the proteome of *M. browni* venom showed that PLA₂s are most abundant (51 %), followed by SVSPs (25.5 %). This venom has a similar profile to that of *M. melanurus* in terms of relative abundance of protein families, in which SVMPs are not very abundant. On the other hand, *M. barbouri* showed a more simple chromatographic profile, relatively similar to those of some viperids; however, by shotgun proteomics it presented a higher
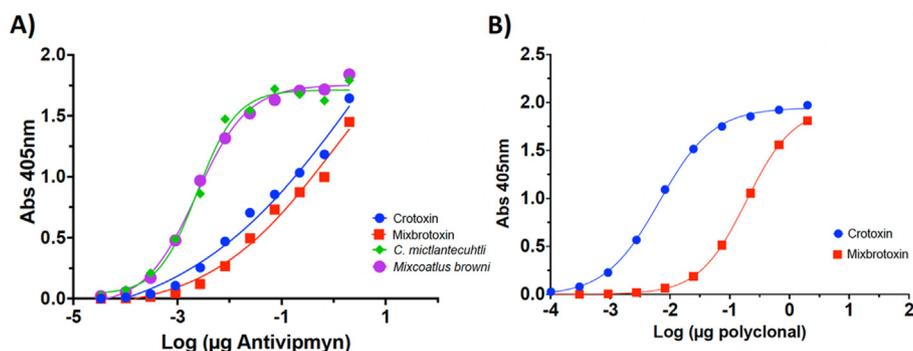
Fig. 5. Recognition by ELISA. A) of Antivipmyn towards the components of the venom of *M. browni*, *C. mictlantecuhtli*, Sub B of Mixcoatlutoxin, and sub B of crotoxin. B) Recognition of polyclonal antibodies from rabbits immunized with sub B of crotoxin towards the Sub B of Mixcoatlutoxin and sub B of crotoxin.

number of proteins, explained by the very high number of SVMP variants compared to *M. browni*. Among the most noteworthy findings is the identification of Crotoxin-like components in both venoms, as further discussed below.

4.2. Biochemical and toxic activities of venoms

Regarding PLA$_2$ activity, the venom of *M. browni* showed slightly higher activity compared to that of *M. barbouri* using 4-NOBA as a substrate, a finding that correlates with the high percentage of PLA$_2$ present in the venom of *M. browni*. Although the quantitative protein composition of *M. barbouri* venom was not established, the magnitude of chromatographic peaks eluting at the known position for PLA$_2$s suggests a lower relative proportion of these enzymes in comparison to *M. browni* venom. On the other hand, *M. barbouri* venom exhibited higher hemorrhagic activity, generating a halo with a diameter 1.7-fold larger than *M. browni* venom. This also correlates with the presumed higher proportion of metalloproteinases present in the venom of *M. barbouri*, as suggested by its RP-HPLC profile As for lethality, only the LD$_{50}$ of *M. browni* venom could be obtained (2.2 μg/g), a potency within the range of some Mexican species such as *Crotalus basiliscus* (1.7, 2.0, 2.4 μg/g in different individual specimens) [4] and *C. lannomi* [19] (1.7 μg/g). A significant observation is that after 45 min, mice began to show flaccid paralysis of their limbs, particularly noticeable in the hind limbs. These signs are caused by venoms from species that possess crotoxin or crotoxin-like toxins, as described in *C. mictlantecuhtli* [9,20], *C. basiliscus* [4], *C. s. scutulatus* [7,21], among others. *Mixcoatlus browni* venom does not fit strictly into either the type 1 or type 2 classification [22] since it is not potent enough to belong to type 2 (where LD$_{50}$ < 1 μg/g is considered), and neither does it fit into type 1 due to its low percentage of metalloproteinases. Therefore, it would be interesting to correlate this venom with the ecology and natural history of this species. Unfortunately, there is no information regarding the diet of *M. browni*; in captivity, we have observed that these snakes readily accept lizards and mice. However, data on their feeding habits in the wild will be of great relevance to try to rationalize its venom composition.

Regarding fibrinogenolytic activity, both venoms cleave the α and β chains when using EDTA as a zinc chelator, thus rendering metalloproteinases inactive, or PMSF as a serine protease inhibitor. Results showed that in the case of *M. barbouri* venom, the α chain is degraded by SVMPs, while the β chain is degraded by both SVMPs and SVSPs. For *M. browni* venom, the α chain is predominantly degraded by SVMPs, although SVSPs also partially degrade it, while the β chain is mainly degraded by SVSPs. Finally, the δ chain is not degraded, a finding commonly observed in many snake venoms.

The antivenom Antivipmyn neutralized the venom of *M. browni*,

requiring 57.9 μL under our experimental conditions, which is equivalent to neutralizing 518 LD$_{50}$ per vial. Generally, for rattlesnake venoms, no less than 780 LD$_{50}$ per vial is neutralized by this antivenom. However, this neutralization appears to be adequate given that the amounts of venom that can be injected by this species are low. In our experience, a venom yield of 5–10 mg per extraction is obtained. The immunization mixture for Antivipmyn production includes the venom of *C. simus*. This venom contains a Crotoxin-like protein, which leads to cross-recognition towards newly described proteins of this kind, as found in the present study. The venoms of *C. mictlantecuhtli* and *M. browni* were similarly recognized by Antivipmyn. This is not surprising since antibodies generated against the main protein families (SVMP, SVSP, and PLA$_2$) have broad cross-reactivity between viperid species. In the case of the basic subunit of the crotoxin-like component of both these species, the large (six-fold) difference observed in their immunorecognition suggests a closer structural homology between the B subunit of *C. mictlatecuhtli* and crotoxin, as compared with the B subunit of *M. browni*.

4.3. Mixcoatlutoxin

The presence of acidic and basic subunits of a crotoxin-like heterodimer in both *Mixcoatlus* venoms was initially identified by the shotgun proteomics strategy, and subsequently confirmed in RP-HPLC fractions which were sequenced for identification (Suppl. Table S1). In the case of the B subunits, N-terminal analysis further provided certainty that the venoms contain crotoxin-like components. In lethality assays, the *M. browni* basic subunit induced the classical signs of flaccid paralysis, starting in the hind limbs and progressing to the forelimbs, causing intercostal muscles to contract rapidly to compensate for the lack of oxygen. The LD$_{50}$ of this subunit (1.52 μg/g) represents a lower lethal potency than those reported for other species such as *C. durissus terrificus*, in the range of 0.48–0.70 μg/g [23]; (Faure et al., 1993), or for *O. sphenophrys* (0.49 μg/g [10]; and *M. melanurus* (0.58 μg/g; [11]). However, it is more lethal than that of nigroviriditoxin B, which was reported to be 2.9 μg/g [24]. When the LD$_{50}$ of the reconstituted A + B heterodimer was calculated, the lethal potency increased by two-fold. It has been reported that there are different isoforms of the two subunits, and depending on the combinations, the LD$_{50}$ may increase or decrease. The LD$_{50}$ of reconstituted Mixcoatlutoxin (0.7 μg/g) compares closely to those reported for other species, for example, *C. d. terrificus* with 0.08–0.65 μg/g [23], *Gloydius intermedius* (0.45 μg/g; [25], *B. nigroviridis* (2.2 μg/g; [24,26], *O. sphenophrys* (0.16 μg/g; [10], and *M. melanurus* (0.31 μg/g; [11]).

Crotoxin is composed of subunit A and subunit B in a 1:1 M ratio; however, the expression of these two varies significantly

between one and the other [6–8,27,28]. In the case of *M. browni*, if we consider the molar ratio using the relative abundance of the fractions as a reference, we estimate that out of that 9.4 %, only 3.5 % forms the heterodimer; therefore, the rest of subunit B is free. Although this subunit is free, it still maintains significant lethality, as demonstrated in *O. sphenophrys* [10], where its LD_{50} decreases threefold compared to when it is in the heterodimeric form, and in the case of *M. melanurus* [11], its lethal activity decreases by 1.9 times. While this percentage of neurotoxic components may seem small, they are actually clinically relevant quantities. In patients who are not promptly treated, these venoms can lead to neurotoxic problems that, if not properly addressed, can result in respiratory failure.

In some rattlesnake species of Mexico, the crotoxin/crotoxin-like neurotoxin is polymorphic. For example, in *C. basiliscus* [4], in the northern part of its distribution, venoms lack such neurotoxin, while in Michoacan, positives and negatives for Crotoxin are found. Something similar occurs with *C. tzabcan* [8]. In the genus *Ophryacus*, of the three species encompassed within this genus, only *O. sphenophrys* possesses this toxin [10]. Although the venom of *Cerrophidium* has not been biologically characterized, there are reports of its transcriptomes in which only crotoxin was found in a *C. godmani*, while in the rest of the species, no transcripts were found [29].

In recent years, several species of vipers with neurotoxic components similar to Crotoxin (crotoxin-like) have been described, including various rattlesnakes, some of which interestingly may or may not contain Crotoxin, such as *C. s. scutulatus* and *C. basiliscus*, while in genera such as *Ophryacus*, *Mixcoatlus*, and *Cerrophidion* (at least by transcriptomics), which reinforces the idea that the vipers that arrived in the New World contained crotoxin-like proteins that were subsequently lost at the gene level in some species [25,30]. It is important to mention that there are other types of regulations such as miRNAs that regulate gene expression at the RNA level, particularly in Mesoamerican type II venom phenotypes [20]. For Mexican species, it has been demonstrated that miRNAs regulate the percentages of crotoxin in the venom of *C. simus* from Veracruz (now named *C. mictlantecuhtli*) and *C. tzabcan,* where juvenile specimens present a higher amount of crotoxin and adults lower percentages of crotoxin, and it was demonstrated that in the latter, miRNAs for both subunits increased [28]. However, these findings raise intriguing questions. Why is there polymorphism for Crotoxin in some rattlesnake species? Why do some genera still maintain the crotoxin-like neurotoxin? Is there any relationship between geographical or dietary conditions that influence the presence of these molecules? These are undoubtedly questions that have been raised for some time; however, with the new findings, they are being revisited and shown to be of interest from an evolutionary perspective. From a clinical point of view, these newly discovered crotoxin-like venom components appear to be effectively cross-neutralized by the antivenoms used in Mexico.

5. Conclusions

Mixcoatlus browni is a species whose venom has a crotoxin-like component, which in mouse assays causes neurotoxicity and lethality. A commercially available therapeutic antivenom (Antivipmyn) effectively neutralizes the lethal activity of this venom. Unlike most viper venoms, that of *M. browni* contains a very low proportion of SVMP. In the case of *M. barbouri* venom, we could only conduct a partial characterization, and showed the presence of acidic and basic subunits with similarity to crotoxin, although due to the scarcity of venom we were not able to confirm its neurotoxic activity.

CRediT authorship contribution statement

Edgar Neri-Castro: Writing − review & editing, Writing − original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Vanessa Zarzosa:** Writing − review & editing, Visualization, Investigation, Formal analysis, Data curation. **Bruno Lomonte:** Writing − review & editing, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation. **Fernando Zamudio:** Methodology, Formal analysis. **Lorena Hernandez-Orihuela:** Writing − review & editing, Software, Investigation, Formal analysis. **Alejandro Olvera-Rodríguez:** Writing − review & editing, Methodology, Investigation. **Audrey Michelle Rodríguez-Solís:** Methodology, Investigation. **Miguel Borja:** Methodology, Investigation. **Uri O. García-Vázquez:** Methodology, Investigation. **Jason M. Jones:** Methodology, Investigation. **Chistopher L. Parkinson:** Writing − review & editing, Methodology, Investigation. **Alejandro Alagón:** Writing − review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2024.05.015.

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