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Bothrops atrox from Ecuadorian Amazon: Initial analyses of venoms from individuals

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ABSTRACT

Bothrops atrox is the most clinically relevant snake species within the Amazon region, which includes Ecuadorian territories. It comprises a large distribution, which could contribute to the genetic and venomic variation identified in the species. The high variability and protein isoform diversity of its venom are of medical interest, since it can influence the clinical manifestations caused by envenomation and its treatment. However, in Ecuador there is insufficient information on the diversity of venomic phenotypes, even of relevant species such as *B. atrox*. Here, we characterized the biochemical and toxicological profiles of the venom of six *B. atrox* individuals from the Ecuadorian Amazon. Differences in catalytic activities of toxins, elution profiles in liquid chromatography, electrophoretic patterns, and toxic effects among the analyzed samples were identified. Nonetheless, in the preclinical testing of antivenom, two samples from Mera (Pastaza) required a higher dose to achieve total neutralization of lethality and hemorrhage. Taken together, these data highlight the importance of analyzing individual venoms in studies focused on the outcomes of envenoming.

1. Introduction

Snake venoms are complex cocktails of bioactive compounds, mainly proteins and peptides, which are molecular determinants of clinical and physiopathological aspects reported in patients bitten by snakes (Markland, 1997; Lu et al., 2005; Almeida et al., 2017; Casewell et al., 2020). These accidents are frequently associated with serious socio-economic repercussions mainly due to permanent disability or death (Williams et al., 2019). This is common within rural areas, where there is limited access to the health system and scarce functional and clinical infrastructure (Harrison et al., 2009; Chaves et al., 2015; Brenes-Chacón et al., 2019). In this context, in 2017 the World Health Organization (WHO) categorized again snake envenoming as a priority neglected disease (Chippaux, 2017; Kurtovi et al., 2020).

This life-threatening disease represents a relevant public health problem in tropical areas such as Ecuador (Gutiérrez et al., 2017). In this country, *Bothrops atrox* and *Bothrops asper* cause more than 70% of all envenoming accidents (Ministerio de Salud Pública, 2017). *B. atrox* inhabits the Ecuadorian Amazon and is the species with the highest clinical significance in the region (Yañez-Arenas et al., 2018), an epidemiological pattern that occurs throughout the Amazon basin (Calvete et al., 2011). Genetic studies have identified the high degree of molecular variability in populations of *B. atrox* in Brazil, which is probably related to its wide geographical distribution (Monteiro et al., 2020). Furthermore, venom phenotype variation has also been reported by proteomic approaches (Calvete et al., 2011; Mora-Obando et al., 2014; Sousa et al., 2017). These results have been related to several factors including age, sex, geographic distribution and environmental

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characteristics (Guercio et al., 2006; Nunez et al., 2009; Moretto Del-Rei et al., 2019; Sanz et al., 2020). Recently, Moretto Del-Rei (2019) demonstrated biochemical and functional variabilities of venom samples from this species belonging to three regions in northern Brazil. These differences are of therapeutic interest, since they may have consequences on the clinical outcomes of the victim and in the neutralization efficiency of antivenoms, which are the primary medical therapy to treat snakebite envenoming (Fry et al., 2003; Sousa et al., 2017; Casewell et al., 2020).

The polyvalent immunoglobulins manufactured by Clodomiro Picado Institute (ICP), Costa Rica, are the official treatment used in Ecuador (Fan et al., 2019). This medication is developed using a mixture of venoms from *B. asper*, *Crotalus simus* and *Lachesis stenophrys* as determinants of immunogenic response (Arroyo et al., 2017). Due to its biochemical and immunological properties, this biological product has shown a high cross reactivity against the toxic effects induced by several viperid venoms (Bogarín et al., 2000; Gutierrez et al., 2014). However, because of interspecific and intraspecific variation it is essential to evaluate the action of heterologous immunoglobulins in the region where they will be used, against the main toxic effects caused by venoms (Gutiérrez et al., 2011; World Health Organization, 2017). In previous studies, the ICP antivenom demonstrated to be efficient in neutralizing the action of *B. atrox* venom pools from different regions across the Amazon (Calvete et al., 2011; Laines et al., 2014; Monteiro et al., 2020; Resiere et al., 2020).

Recent proteomic and functional studies have identified significant differences at the individual level in *B. atrox* venoms. Amazonas et al. (2019) observed the variability in biochemical profiles, whilst Moura-da-Silva et al. (2020) evidenced a high divergence of toxin isoforms with impact on clinical manifestations of envenoming. Likewise, recent findings about viperid venoms demonstrate how individual variations can cause biochemical and immunological changes (Aguiar et al., 2019; Oliveira et al., 2019). According to Alape-Giron et al. (2008) and Sousa et al. (2017), these functional changes of venoms at the individual level should not go unnoticed, taking into account their impact on clinical evolution and response to antivenom administration. To our knowledge, this variance at biochemical and toxicological levels of individual *B. atrox* venom has never been studied in Ecuador.

Therefore, the present work describes the biochemical and toxicological characterization, as well as the comparison of venoms of *B. atrox* individuals from the Ecuadorian Amazon region. Likewise, we report the neutralization evaluation of polyvalent antivenom ICP against the toxicological effects of this highly venomous snake.

2. Materials and methods

2.1. Bothrops atrox *venoms*

Six *B. atrox* individuals from three different geographic sites in the Ecuadorian Amazon were captured in order to obtain the venoms (Fig. 1). Each individual species was identified and verified by a herpetologist. Additionally, a code was assigned to maintain the specimen at the Museum of Zoology of the Universidad Tecnológica Indoamérica (MZUTI) (Table 1). The life stage was identified according to Guercio et al. (2006). Snakes were secured with the use of transparent telescoping plastic tubes and induced to bite into a Parafilm®-covered glass beaker. Venom samples were collected within a short period of time after the adult snakes were captured. The secretion was pipetted into a 1.5 ml eppendorf tube, in a vacuum container containing anhydrous calcium sulfate, to store the venoms as dried samples (Salazar-Valenzuela et al., 2014). All six samples were stored at −35 °C until laboratory use. A code was assigned to each venom to facilitate its identification during the study (Table 1).

2.2. *Biochemical profile of* B. atrox *venoms*

Two analytical approaches were used in this study to evaluate each venom sample: reverse phase high-efficiency liquid chromatography

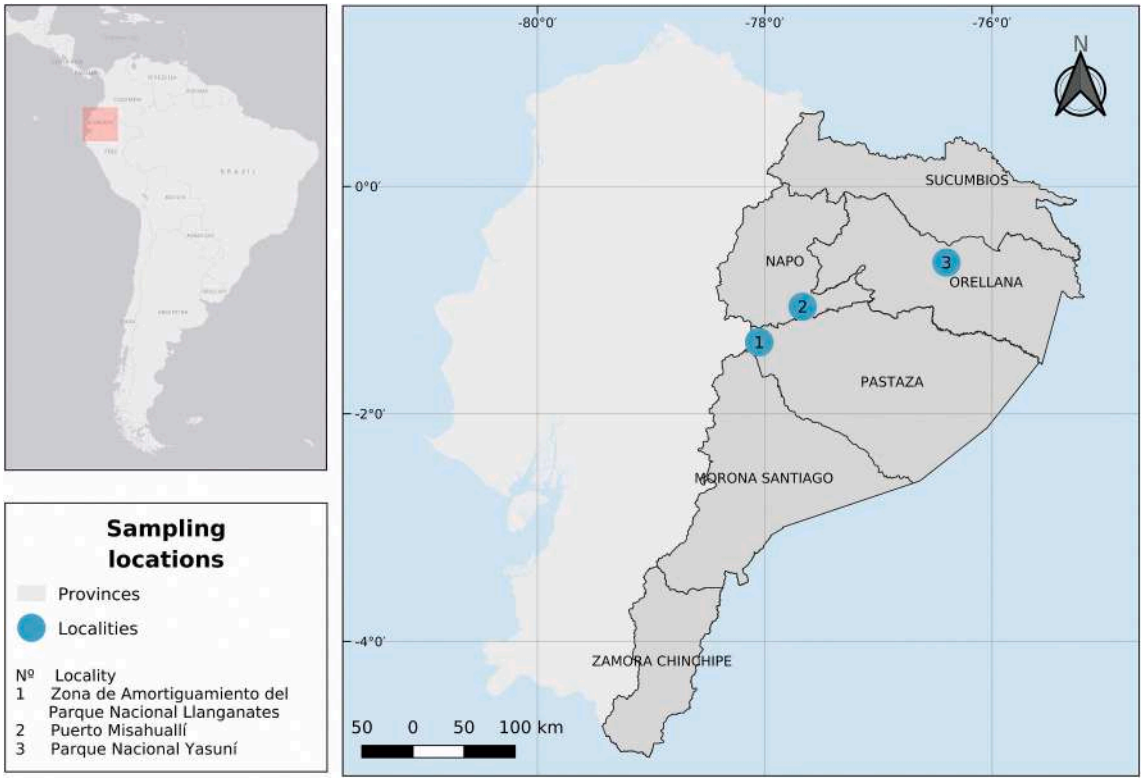

Fig. 1. Geographic origin sites of *B. atrox* individuals from which venom samples were collected. Provinces of the Ecuadorian Amazon are shown in gray in the map. The geographic points where the adult individuals were collected are reported.

Table 1
Data of *Bothrops atrox* individuals collected. Individuals were identified before venom extraction. Geographic origin: Locations in which individuals were collected according to geographic points showed in the map (Fig. 1). Venom Id: code assigned in the present study to identify the origin of each venom sample. Sex: F: Female; M: Male. Stage: Life stage of each individual collected. Length (SVL) (cm): *Snout-vent-length* of collected individuals. Specimen number (MZUTI): code assigned after species identification to preserve specimens in the Museum of Zoology of Universidad Tecnológica Indoamérica.

Geographic origin	Venom Id.	Sex	Stage	Length (SVL) (cm)	Specimen number (MZUTI)
(1) Mera - Pastaza	MR1	F	Adult	120.1	5675
(1) Mera - Pastaza	MR2	F	Adult	122.8	5674
(1) Mera - Pastaza	MR3	F	Adult	85.8	5676
(1) Mera - Pastaza	MR4	F	Adult	118.4	5634
(2) Tena - Napo	TN	M	Adult	81.7	5683
(3) Yasuní - Orellana	YN	F	Adult	90.3	5536

(RP-HPLC) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. For chromatographic separation, a solution of 60 μl of trifluoroacetic acid (0.1%) and 40 μl of ammonium bicarbonate (1 M) was used to dissolve 0.5 mg of venom. The sample was homogenized for 2 min in Vortex and centrifuged for 3 min at 8000 rpm and room temperature, to remove insoluble material. Afterwards, 100 μl of the sample were injected into the HPLC (PREP 150 LC, Waters) equipped with the 2489 UV/Vis detector, pumps and 2545 injector and fraction III collector. The venom components were separated in a C18 column (250 × 4.6 mm, 5 μm particle size) eluting at 1 ml/min with a linear gradient of trifluoroacetic acid 0.1% (mobile phase A) and acetonitrile, and 0.1% trifluoroacetic acid in a 66:34 ratio (mobile phase B). This was monitored at 214 nm. Selected venom fractions were collected manually and lyophilized (Benchtop Pro 9 L, Omnitronics).

Biochemical analysis were performed by SDS-PAGE, according to Laemmli (1970). Briefly, 0.1 mg of dried venom were dissolved in 100 μl of loading buffer (0.075 M Tris-HCl, pH 6.8; 10% (v: v) glycerol, 4% (m: v) SDS, 0.001% (m: v) bromophenol blue). Electrophoresis was performed using a stacking gel with a concentration of 5% and a 12.5% concentration for the running gel, under reducing conditions (in the presence of 1 M DTT) at 120 V and a temperature of 4 °C. For this analysis, 20 μl of each sample were used. Molecular markers (Rainbow ™ Molecular Weight Markers, GE Healthcare) were employed to identify the molecular mass of venom proteins. Gels were subjected to silver staining as described by Almeida et al. (2016). The fractions from RP-HPLC were separated with the same protocol. Gel images were digitized for analysis.

2.3. *Enzymatic profile of* B. atrox *venoms*

Enzymatic activity of individual venoms was evaluated through spectrophotometric methods (Glomax Discover System, Promega) using chromogenic substrates (azocasein, Nα-BenzoylDL-arginine 4-nitroanilide and 4-nitro-3-octanoyloxybenzoic acid) in triplicate. The results were expressed as specific activities as the mean ± standard deviation and are representative of three independent experiments.

2.3.1. *Caseinolytic activity*

Caseinolytic activity was determined using azocasein (Lomonte and Gutiérrez, 1983). A solution was prepared with 5 mg of substrate (Sigma-Aldrich) diluted in 1 ml of buffer A (50 mM Tris-HCl, pH 8.0). Each venom was dissolved in buffer A, with a concentration of 1 mg/ml. To determine the activity, 10 μl of venom were mixed with 90 μl of substrate solution. After incubating for 90 min at 37 °C, 200 μl of TFA 5% were added to each sample and centrifuged for 5 min at 8000 rpm. The supernatant (150 μl) was placed in a 96-well microplate, where it was mixed with 150 μl of NaOH (0.5 M). The plate reader was set at 440 nm in order to measure the absorbance. A blank was prepared with 10 μl of buffer A and 90 μl of substrate to correct the absorbance. One unit of activity was defined as the amount of snake venom sample responsible for a change of 0.005 units of absorbance (Hatakeyama et al., 2020).

2.3.2. *Phospholipase A_2 activity*

The phospholipase A_2 (PLA_2) activity of the venoms was determined as described by Holzer and Mackessy (1996), with some modifications defined below. The substrate solution was prepared by dissolving 3.1 mg of 4-nitro-3 [octanoyloxy] benzoic acid (NOBA) in 1 ml of acetonitrile and 10 ml of buffer B (10 mM Tris -HCl, 1 M CaCl2, 10 mM NaCl, pH 7.8). Each sample was dissolved in buffer A at a concentration of 1 mg/ml. Later, 220 μl of substrate, 20 μl of venom, and 20 μl of distilled water were mixed in a 96-well microplate. After 20 min incubation at 37 °C, the absorbances were read at 410 nm. Furthermore, 40 μl of distilled water with 200 μl of substrate solution were used as blank to make absorbance corrections. A change in absorbance of 0.10 units was considered equivalent to the production of 25.8 nmoles of chromophore (Aguiar et al., 2019).

2.3.3. *Serine proteinase activity*

Serine proteinase activity was measured using the benzoyl-arginyl-p-nitroanilide (L-BapNa) substrate (Sigma-Aldrich) (Munekiyo and Mackessy, 1998; Ghorbanpur et al., 2010). The substrate solution was prepared by dissolving 2.18 mg of BapNa in 50 μl of dimethyl sulfoxide (DMSO) and 5 ml in buffer B. Each sample was dissolved in buffer A at a concentration of 1 mg/ml. Activity was measured by mixing 20 μl of venom with 200 μl of substrate and 50 μl of buffer A in a 96-well microplate at 410 nm and 37 °C after 30 min of incubation. A blank with 70 μl of buffer B and 200 μl of substrate solution was used for absorbance corrections. Specific activity was determined according to Vilca-Quispe et al. (2010).

2.4. *Toxicological profile of* B. atrox *venom*

Toxicological activities and neutralization assays were performed using male CD-1 mice (18–20 g), obtained from the National Institute for Public Health Research (INSPI). Animal experimentation and handling of CD-1 mice were carried out following the Guide for the Care and Use of Laboratory Animals protocols, with previous authorization from the Research Coordination at Ikiam University. The lyophilized polyvalent antivenom manufactured by ICP (BATCH: 5991117POLF; Expiration Date: 11/2022) was used in neutralization assays. The antivenom was donated by José María Velasco Ibarra Hospital, Tena. Venom doses applied in different trials were selected based on the investigation performed by Laines et al. (2014) and Moretto Del-Rei et al. (2019). The results of the hemorrhage, inflammation and myotoxicity activities were expressed as the mean ± the standard deviation.

2.4.1. *Evaluation of hemorrhagic effect*

The ability of *B. atrox* to produce blood escape into the surrounding skin was carried out according to the protocol described in Kondo et al. (1960) with modifications. A group of five CD-1 anesthetized mice per sample received an of 10 μg injection of venom, dissolved in 50 μl of PBS, in the dorsal area. After 3 h, the mice were euthanized with halothane to proceed with the removal of the dorsal skin in order to measure the hemorrhage area (mm^2). This was recorded with photographs that were later digitized for analysis with ImageJ. Three mice that received an injection of PBS instead of venom were used as control.

2.4.2. *Evaluation of edema-forming effect*

The local inflammatory response induced by *B. atrox* samples was

analyzed following the protocol by Lomonte et al. (1993). A group of five mice per sample was injected into the left hind footpad with 50 μg of venom dissolved in 50 μl of PBS and into the right hind foot with 50 μl of PBS. The increase in dimension of each foot caused by inflammation was measured with a digital caliper (Truper) 1 h after injection. The difference between the injected feet measurements was used to quantify the edematogenic activity and the results were expressed as percentages.

2.4.3. Evaluation of damage to muscle tissue

Muscle damage caused by *B. atrox* venoms was determined by the quantification of plasma levels of a biochemical marker, following the protocol used by Almeida et al. (2020a). As before, a group of five mice per sample was injected with 50 μg of venom dissolved in 50 μl of PBS in the gastrocnemius muscle. The control group was injected in the same area with 50 μl of PBS. After 3 h, blood samples were collected from the tail of the mice in heparin capillaries that were centrifuged at 5000 rpm for 3 min. Subsequently, blood plasma was taken to measure creatine kinase activity (CK-NAC, Wiener Lab) which was monitored at 320 nm in the microplate reader and defined as U/L.

2.4.4. Survival curve

Lethality results reported by Laines (2014) and Moretto Del-Rei et al. (2019) for *B. atrox,* from Ecuador and Brazil respectively, were taking into account to evaluate the lethality of *B. atrox* venoms. For this assay, 200 μg of each venom was dissolved in 200 μl of PBS, and was administered into the intraperitoneal area of six mice per sample. After the injection, the mice were monitored every hour for the first 6 h. Subsequently, the death times were recorded after 12, 24 and 48 h, which were represented in a survival curve.

2.5. Neutralization assays

Venoms were incubated for 30 min in a solution of antivenom, which was prepared by adding 10 ml of resuspension solution and 20 ml of PBS. Depletion of hemorrhage, inflammation and lethality was evaluated using a 1:1 (mg venom/ml antivenom) proportion. Groups of five mice were used for hemorrhage and edema, and groups of six mice for lethal activity. The assays were carried out by using 10 μg, 50 μg and 200 μg of venom per mouse respectively. The results were expressed as a percentage in relation to the assays where mice were injected only with venom. Experiments were repeated with a higher amount of antivenom (1:2 venom/antivenom; 1:2.5 venom/antivenom, respectively) for the venoms with partial neutralization of hemorrhagic and lethal activities with the initial proportion. Additionally, a lethality neutralization test was performed without prior incubation to simulate a real treatment with antivenom administration after envenomation. In this last approach, groups of six mice were administered 500 μl of antivenom after 30 min of injecting 200 μg of venom (ratio 1:2.5 venom/antivenom).

2.6. Statistical analysis

An analysis of variance (ANOVA) One Way followed by the Tukey's test (for multiple comparisons) was used to verify if there were significant differences when comparing the results obtained for the six venom samples. The level of significance was established with $p \leq 0.05$. The analysis and graphs were performed in OriginLab®.

3. Results

3.1. Biochemical profile of venoms from B. atrox individuals

The biochemical profile indicated relevant differences between the analyzed venoms in the present study. SDS-PAGE of each sample (Fig. 2) showed the existence of differences in biochemical patterns. Overall, the electrophoretic profiles of Mera venoms (MR1, MR2, MR3 y MR4) have

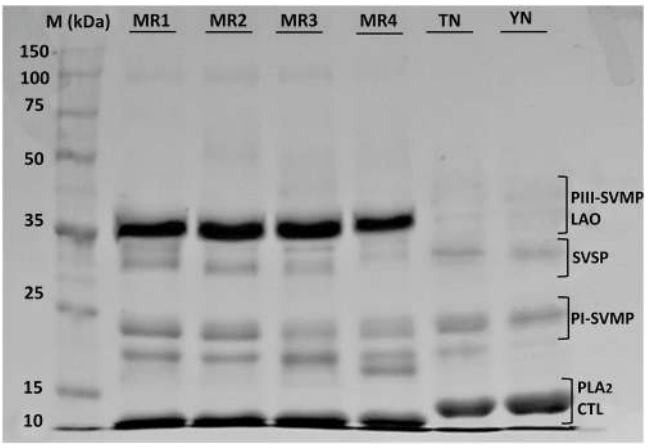

Fig. 2. Polyacrylamide electrophoresis gel (SDS-PAGE) of venoms from *B. atrox* individuals. Venom molecules were separated by one-dimension SDS-PAGE (12.5%) under reducing conditions. Molecular markers (kDa) are shown on the left side and the identification of each sample at the top. On the right side are the intervals associated with the mass range of the most abundant *Bothrops* toxins.

marked patterns in the mass ranges of 13–16 kDa (PLA_2's and CTL's: C-type lectins), 23–33 kDa (PI-SVMP: snake venom metalloproteinases), 31–38 kDa (SVSP's: snake venom serine proteases) and 46–58 kDa (PIII-SVMP's and y LAO's: L-amino oxidases). Whilst, the SDS-PAGE gels of Tena (TN) and Yasuní (YN) venoms showed faint marked bands in the PIII-SVMP and SVSP range with a predominance of low mass proteins bands.

The RP-HPLC profiles of each venom and gel analysis of their fractions corroborated the above results. In general, in MR (1, 2, 3 and 4) venoms, their chromatographic separation had similar characteristics (Fig. 3A, B, C and D), with peaks predominantly eluting from 60 min onwards and with a molecular mass of 20 kDa and 45 kDa. The data suggest that the major components in these samples would be PI-SVMP and PIII-SVMP. Additionally, their chromatograms showed a greater peak diversity with high absorbance in comparison to the remaining venoms (TN and YN). In the latter case, the absence of peaks with pronounced absorbance starting from minute 60 of the run was observed. In both venoms, peaks that could be mainly related to PI-SVMP suggest that these metalloenzymes have a high relative abundance (Fig. 3E and F). A difference between these two samples is the diversity of PLA_2 isoforms that would be greater for the TN sample (Fig. 3E insert), considering the elution times of these toxins, and the band patterns observed in fractions from 4 to 7 of the gel.

3.2. Enzymatic profile of venoms from B. atrox individuals

Enzymatic tests provided evidence of variations in the ability, of the venoms, to catalyze some hydrolytic reactions with specific chromogenic substrates (Fig. 4). For the caseinolytic activity evaluation, YN, TN and MR2 samples had the highest performance (Fig. 4A). The results reveal significant differences among MR samples in substrate degradation indicating the presence of interesting individual variations in enzyme activities. Regarding PLA_2 activity, the TN venom presented the best performance when cleaving the NOBA substrate, while the YN sample presented the lowest absorbance during the assay (Fig. 4B). On the other hand, in the BapNa substrate degradation test, three of the MR venoms (MR1, 2 and 4) showed the highest results (Fig. 4C).

3.2.1. Toxicological profile of venoms from B. atrox individuals

Hemorrhagic and myotoxic effects evaluation in animal models (CD-1 mice) showed the existence of similarities in the potency of toxicological activity among MR venoms (Fig. 5). After 1 h of sample injection

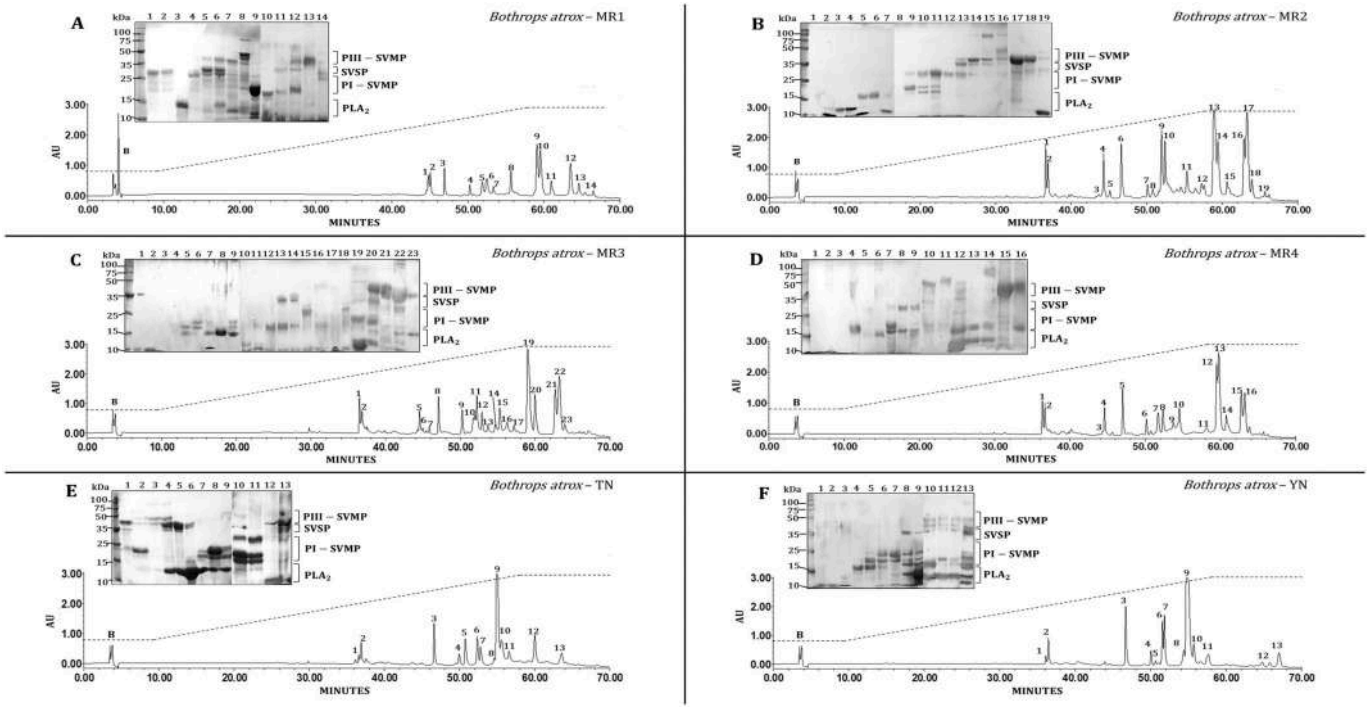

Fig. 3. Chromatographic profile of the venoms of *B. atrox* individuals monitored at 214 nm. 0.5 mg of desiccated venom was subjected to RP- HPLC on a C18 column. Inserts, SDS-PAGE under reducing conditions show the proteins contained of selected fractions. Molecular markers (kDa) are shown on the left side of the gels and sample identification in the upper right corner on each chromatogram.

in the dorsal area, the YN venom presented the most notable hemorrhagic areas, while the TN venom had the weakest effect (Fig. 5A). The local myotoxicity results contrasted with the hemorrhagic activity. The TN venom showed the highest muscle damage, observed by CK activity in the blood serum of the mice. On the contrary, the YN sample produced a minor disruption of skeletal muscle cells (Fig. 5C). In both experiments, MR venoms performed similarly, without significant statistical differences (Fig. 5A and C). On the other hand, the six samples showed close results in relation to their inflammatory capacity (Fig. 5B).

In order to distinguish the lethality of the studied venoms, a challenge was performed with 200 μg of sample inoculated in animal models (Fig. 6). The group of mice injected with the YN venom had the lowest survival rate, with about 16% at the end of the 48 h. In this same period, the lethality of TN was nil, since all the animals inoculated with this sample had overcome the trial. In fact, this same survival rate was observed for mice injected with PBS. The lethal activity for the four MR venoms was similar again, as for hemorrhagic and myotoxic effects, with a survival rate of 66% of the animal groups.

3.2.2. *Neutralization of the toxicological effects of venoms from* B. atrox *individuals*

After the toxicological assays of the Ecuadorian *B. atrox* venoms, evaluation of neutralization by the polyvalent antivenom ICP (polyvalent lyophilized -ICP) was carried out. In general, the immunoglobulins were effective against the hemorrhagic effects induced by venoms (Table 2). In four of the six samples, it was observed a complete depletion of this activity with 1 ml of antivenom per mg of venom. Furthermore, complete activity neutralization was observed in MR2 and MR4 by doubling the antivenom dose (Fig. 1 Supplementary material).

Regarding edema formation, antivenom had a low efficiency to counteract this effect, having a maximum neutralization of 28% of the total activity by incubating the venoms with immunoglobulins in a 1:1 ratio. With the same dose, the ICP immunoglobulins treatment was able to fully reduce the lethality induced by five of the venoms (Table 2). For MR4, a partial neutralization was observed. Fatalities were only observed after 24 h of the inoculation with this venom. Whilst, in lethality evaluation, the first death occurred after 4 h of injection with MR4 sample (Fig. 2 Supplementary material). When increasing the administered dose to 2.5 ml of antivenom per mg of venom, all mice inoculated with MR4 survived at the end of 48 h of the experiment (Table 1, Supplementary material).

Finally, after demonstrating that it was necessary to use 2.5 ml of antivenom per mg of venom to reach a total neutralization for one of the samples, a new challenge was carried out without prior incubation with immunoglobulins. Using this last dose, lethality was completely reduced, considering that in this last evaluation the antivenom was administered 30 min after the venom injections (Table 1, Supplementary material).

4. Discussion

Bothrops atrox is the species with the highest incidence of snakebite envenoming accidents in Amazonian locations, as shown by hospital reports described in epidemiological studies (Sant'Ana Malaque and Gutiérrez, 2016; Silva et al., 2020). It should be noted that Ecuador lacks information on the diversity of individual venom variability across its territory. Thus, in the present work we characterize the toxicological and biochemical profiles of *B. atrox* venoms, to identify possible variations between individuals and assess if these disparities can influence the antivenom capacity to neutralize the venom-induced damages.

Chromatographic and SDS-PAGE profiles of MR venoms suggest that the major components are PIII-SVMP and PI-SVMP. Nunez et al. (2009) reported this profile by analyzing pools of *B. atrox* venoms from Ecuador, Peru and Brazil. Conversely, the profiles of the TN and YN samples showed a smaller variety of isoforms compared to the MR venoms, with PI-SVMP being probably the most abundant components. Previous data has shown that metalloenzymes are the main components in the venom of this viper (Nunez et al., 2009; Monteiro et al., 2020). According to several authors these toxins, with proteolytic action, play a critical role in bothropic envenomation (Gutiérrez et al., 2016; Sousa

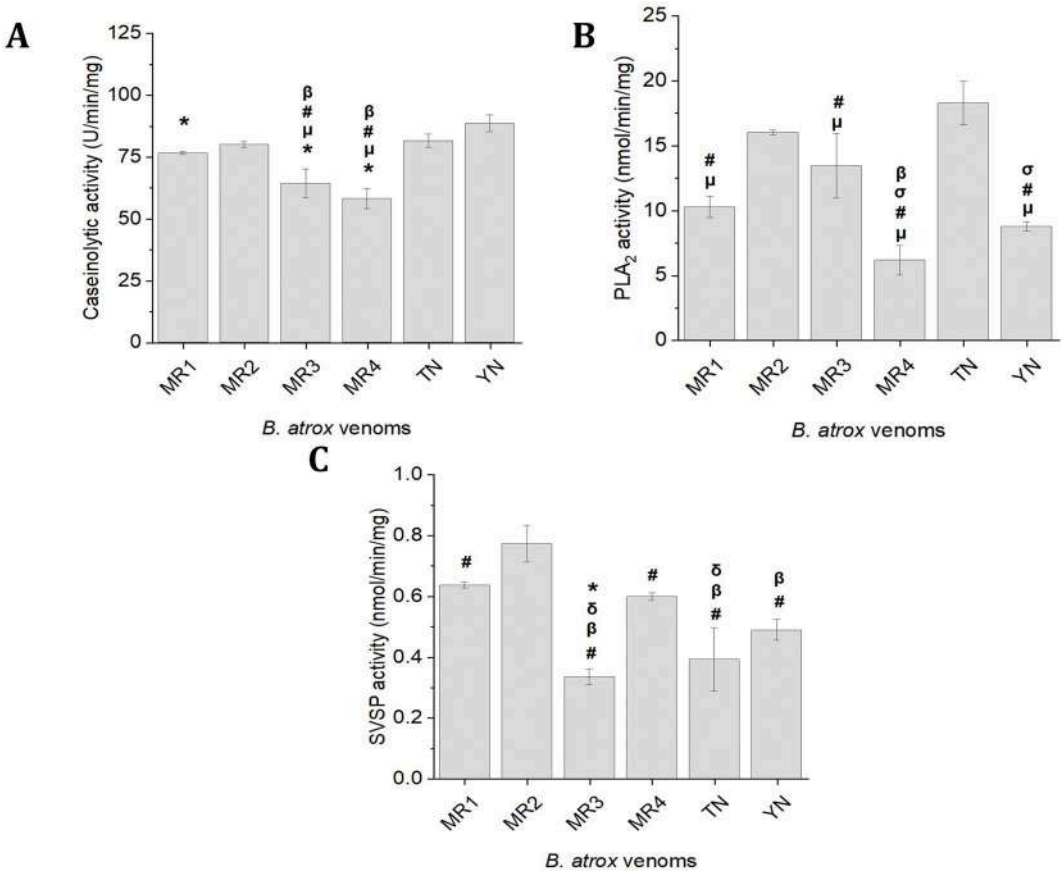

Fig. 4. Enzymatic activities of venoms of *B. atrox* individuals. Caseinolytic (A), phospholipase A_2 (B) and serine proteinase (C) activities were evaluated by spectrophotometric methods according to those described in the methodology section. Significant statistical differences were determined with a $p < 0.05$ by Tukey's test when comparing the activity between samples. Each symbol indicates that there is a significant difference with a certain sample: β (difference with MR1 activity), # (difference with MR2), σ (difference with MR3), δ (difference with MR4), μ (difference with TN), * (difference with YN).

et al., 2017; Almeida et al., 2020a; Monteiro et al., 2020).

Proteolytic activity evaluation showed slight differences with YN, TN and MR2 samples having the highest percentage of proteolysis. Caseinolytic activity has often been related to hemorrhagic effects. (Sousa et al., 2001). In general, PIII-SVMPs have been described as highly hemorrhagic enzymes due to their three domains that allow them to cleave efficiently the extracellular matrix components (Herrera et al., 2015). In comparison, single domain PI-SVMPs often cause weak damages (Escalante et al., 2006). Nonetheless, the relationship in a "linear" way of the biochemical composition, enzymatic activity and biological effects is not always possible, especially considering that azocasein is a non-specific substrate for SVMPs (Sousa et al., 2001).

In a recent study with *B. atrox* siblings' venoms, proteomic analyses showed variation in composition of SVMP toxins between female and male pools. Despite this, similar caseinolytic activity were obtained with these samples. Whilst, significant differences arise in hemorrhagic activity evaluation (Hatakeyama et al., 2020). Conversely, in a work of *Bothrops moojeni* individual venoms, the sample with the lowest expression of PIII-SVMP caused the highest proteolytic activity and the weakest hemorrhagic effect (Aguiar et al., 2019), just as observed in our study for TN venom. In agreement with this last work, similar results were observed for two pools of *Bothrops jararaca* venoms. The sample with a minor presence of SVMPs and SVSPs in its composition produced higher levels of substrate degradation (Farias et al., 2018).

A correlation between hemorrhagic and lethal activities has been suggested, being bothropic venoms with higher abundance of PIII-SVMP, like MR samples, capable to exert these damages in a notable magnitude (Nunez et al., 2009; Herrera et al., 2015). Nevertheless, Moura-da-Silva et al. (2020) identified a lack of correlation between PIII-SVMPs and local or systemic hemorrhage. Snake venoms show several structurally-related isoforms within each family, which may have different molecular targets and biological effects regardless of its structural similarity (Monteiro et al., 2020; Moura-da-Silva et al., 2020). For this reason, functional assumptions must be done carefully, even for abundant toxins like PIII-SVMPs, since non-hemorrhagic isoforms have been identified in *B. atrox* venom (Moura-da-Silva et al., 2020).

Our results evidence that YN produced the highest hemorrhagic spots and was the most lethal venom. This is an unpredictable fact considering that biochemical characterization revealed a greater abundance of PI-SVMPs than PIII-SVMPs in YN sample. This unusual result could be related to the presence of a particular and highly catalytic PI-SMVP isoform (Silva de Oliveira et al., 2017; Monteiro et al., 2020). An example is Atroxlysin-Ia a metalloenzyme from *B. atrox* venom which can hydrolyze extracellular matrix structural substrates such as fibrin, laminin and collagen IV more efficiently than Batroxrhagin (Freitas-de-Sousa et al., 2017; Almeida et al., 2020b). Batroxrhagin is the predominant PIII-SVMP isoform in *B. atrox* venom, with a potent hemorrhagic capacity (Freitas-de-Sousa et al., 2015). Furthermore, minimum necrotizing dose of Atroxlysin-Ia was lower than the observed for any other PI or PIII-SVMP (Freitas-de-Sousa et al., 2017). Considering that in YN gel a single band is observed in PI-SVMP region, this could be related with the specific isoform described above. However, this should be verified in a subsequent MS/MS analysis.

Conversely, TN sample was the weakest in hemorrhagic and lethal assays, despite having a resembling biochemical profile and similar results in the caseinolytic assay with YN. Nevertheless, TN venom had the most pronounced PLA_2 activity and myotoxic effect in our study. *Bothrops jararaca* studies have identified that venoms from female

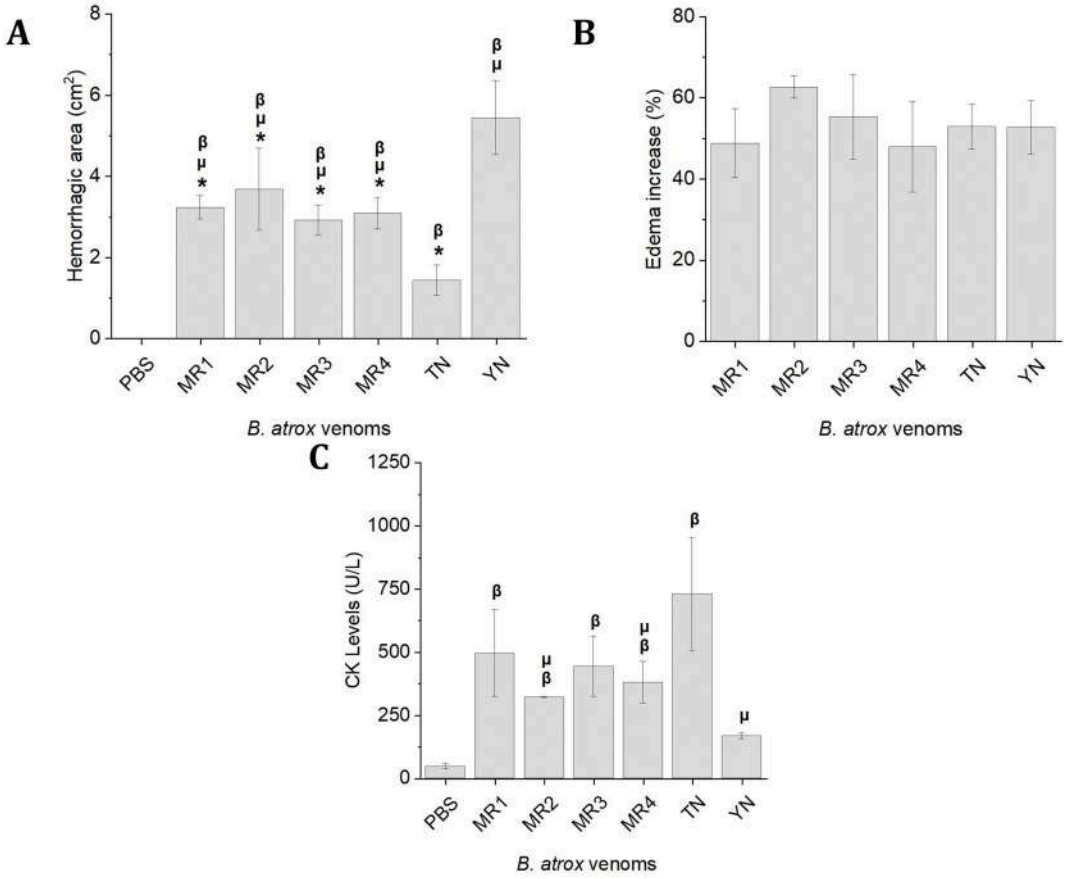

Fig. 5. Toxicological activities of venoms from *B. atrox* individuals. Hemorrhagic (A), inflammatory (B) and myotoxic (C) activities were evaluated in CD-1 biomodels (18–20 g) following protocols detailed in the methodology section. Significant statistical differences were determined with a $p < 0.05$ when comparing the activity between samples activity using Tukey's test. Each symbol indicates that there is a significant statistical difference with a certain sample: β (difference with PBS activity), μ (difference with TN), * (difference with YN).

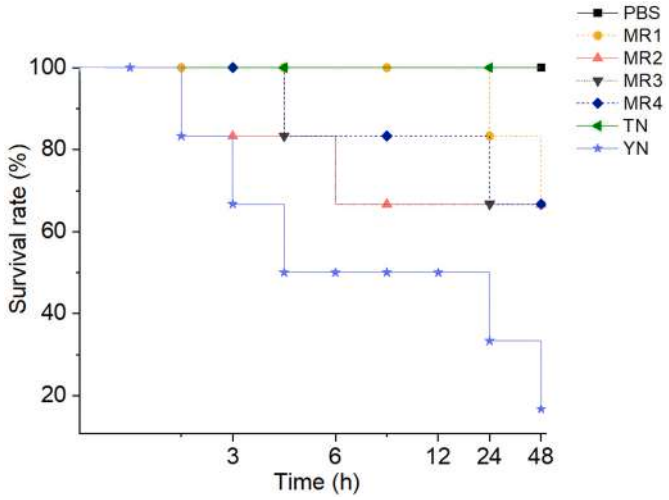

Fig. 6. Lethal activities of venoms from *B. atrox* individuals. Lethal activity for each venom was determined using male CD-1 mice (18–20 g) according to those described in the methodology section. The survival rate (%) was recorded during 48 h. The data obtained is represented in the survival curve plot.

specimens caused marked hemorrhagic and lethal consequences, like the YN and MR samples. Whilst male samples exhibit a prominent PLA_2 activity and myotoxic effect, just as the TN sample which was the only venom from a male individual (Furtado et al., 2006; da Silva et al., 2020). The functional profile of TN suggests a notable abundance of catalytic and non-catalytic PLA_2s in its composition. Our results could be related to the sexual dimorphism described for *B. atrox*, in which females are generally larger than males, requiring a higher energy demand. Female venom is probably adapted to be more lethal to capture larger and endothermic preys (Furtado et al., 2006; Hatakeyama et al., 2020; Monteiro et al., 2020).

We observed significant activity variations, especially in enzymatic assays. Individual variability in *B. atrox* adults and siblings from Brazil was identified by Amazonas et al. (2019) and (Hatakeyama et al., 2020). This could be related with the highly variable composition of isoforms in the venom (Moura-da-Silva et al., 2020). Similar fluctuation in activities of individuals was evidenced for *Bothrops moojeni* study (Aguiar et al.,

Table 2
Neutralization of *B. atrox* venoms activities with the antivenom (polyvalent lyophilized ICP), used in Ecuador. The results are shown as the percentage of neutralization, using 1 ml of antivenom per mg of venom, considering the reported data from toxicological assays as 100%. Antivenom was prepared using 10 ml of resuspension solution and 20 ml of PBS.

Venom	Hemorrhage neutralization (%)	Edema neutralization (%)	Lethality neutralization at 48 h (%)
MR1	100	16.20	100
MR2	88.53	28.17	100
MR3	100	27.13	100
MR4	88.63	18.39	0
TN	100	24.68	100
YN	100	16.43	100

2019), *Crotalus durissus* (Oliveira et al., 2019) and *Bothrops jararaca* studies (Galizio et al., 2018). However, we evidenced that changes in enzymatic results could not be reflected on *in vivo* experiments, just as it occurred with MR venoms, which perform similarly in toxicological evaluation (Gutiérrez et al., 2011; Monteiro et al., 2020).

It is also of clinical interest to determine if the above singularities can affect antivenom neutralization (Alape-Giron et al., 2008). Overall, the ICP antivenom showed an efficient response in two of the three trials, with certain exceptions. Regarding inflammatory reactions, the neutralization efficiency did not exceed 30%. Antivenoms have been documented to have low effectiveness in containing the local inflammatory process (León et al., 2000; Picolo et al., 2002). Edema imply the action of endogenous factors, which cannot be completely inhibited by antivenom administration (Rosenfeld, 1971; Araújo et al., 2007). For this reason, several compounds are being proposed as complementary therapy to mitigate local effects (Knudsen et al., 2019).

Hemorrhage and lethality neutralization of MR2 and MR4 had particular results. Antivenom did not completely reduce the hemorrhage caused by these samples, and the lethality exerted by MR4 was partially diminish using 1 ml of antivenom per mg of venom. MR venoms have a profile, which is completely immunorecognized by ICP antivenom, according to Nuñez (2009). However, MR2 and MR4 are examples of how variation between individuals from the same area could compromise antivenom efficiency. Evidence shows that the antivenoms from Butantan Institute and Clodomiro Picado Institute failed to inmmunodeplete some PI-SVMPs and PLA_2 isoforms of *B. atrox*, due to the low immunogenicity of these toxins (Calvete et al., 2011; Sousa et al., 2017; Monteiro et al., 2020).

Biochemical characterization suggests a considerable diversity of toxins in MR samples. According to Moura-da-Silva et al. (2020), efficiency of the antivenom immunoglobulins might be compromised in venoms with a high abundance of different isoforms. This fact could be the main reason behind the increment of doses in certain cases (Ministerio de Salud Pública, 2017; Monteiro et al., 2020). Furthermore, experimental studies showed a high cross-reactivity of the polyvalent lyophilized ICP antivenom efficiency against several species of viperids (Calvete et al., 2011; Gutierrez et al., 2014); however, the average neutralization dose can vary (Ferreira et al., 1992; Guercio et al., 2006; Gutierrez et al., 2010; Gutiérrez et al., 2011; Resiere et al., 2020).

Our results and data from recent works (Sousa et al., 2017; Sousa et al., 2018; Moura-da-Silva et al., 2020) evidence the functionality and structure complexity of these toxins, since the components act in a synergistic way. These issues have led to proposing alternatives to complement the neutralization of antivenoms like toxin inhibitors (Albulescu et al., 2020). These new alternatives can reduce the use of high antivenom doses and avoid the decrease of its availability for vulnerable populations (Gutiérrez, 2018), such as in the Ecuadorian Amazon. Nevertheless, our data showed that the ICP antivenom is highly efficient. Lethal activities of all venoms analyzed were completely neutralized by doubling the initial dose of immunoglobulins for the MR4 sample, and even in assays without prior incubation.

In the future it will be imperative to carry out the proteomic analysis of venoms through mass spectrometry to complement the information obtained. The data presented in this study provides suggestive and relevant evidence. However, this is an initial study and for this reason, the geographic origin was omitted as a variable. Advantageously, this work could be expanded to include venom samples taken in the Amazon provinces that were not included to discern the underlying reasons behind evidenced individual differences.

5. Conclusion

The characterization of venoms of *Bothrops atrox* individuals from the Ecuadorian Amazon evidenced the presence of relevant intraspecific variations, with a high abundance of SVMPs. Two phenotypes were also observed, mainly differentiated by the abundance of PIII-SVMPs. Furthermore, we verified differences in the biochemical and enzymatic profiles of the six samples collected. Highly intraspecific variability of isoforms reported for *Bothrops atrox* could be responsible for the increase in the doses of antivenom in order to neutralize toxicological activities in some venoms. However, ICP immunoglobulins demonstrate an efficient action against most common envenoming effects. In the future, proteomic studies should be conducted for a deeper understanding of the presented findings.

Credit author statement

RSPP participated in the experiments, analysis, investigation, writing of the original draft and project execution. DVS was involved in funding acquisition, supervision, methodology, provision of samples and editing. EMV assisted in chromatography analyses. BM helped with the visualization and conception of the study. CPB was involved in methodology. SLS assisted in drafting and structuring the manuscript and design of methodology. JRA was responsible for funding acquisition, project administration, conceived and supervised the study, assisted in formal analysis and editing. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no relevant conflict of interest.

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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.2021.01.007.

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