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Biochemical characterization of the venom of the Bolivian endemic pit viper *Bothrops sanctaecrucis*

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ABSTRACT

Ophidic accidents are an important public health problem in South America, specifically those related to the *Bothrops* genus, due to their high incidence, complexity and severity of envenomation symptoms. The species *B. sanctaecrucis*, the only one from this genus endemic to Bolivia, is the most frequently found and involved in snakebites in the Chapare region of Cochabamba; however, its toxicological implications on human health are poorly known. Herein we conducted the first biochemical characterization of its venom. Its electrophoretic profile showed components mainly ranging from ~10 to 37 kDa, resembling other *Bothrops* venoms. The venom exhibited high activity on azocasein (47.65 U/mg) and the thrombin-specific substrate S-2238 (625.55 μmol/min/mg), and noticeably hydrolyzed gelatin and human fibrin(ogen). The venom also degraded lecithin and hyaluronic acid, but both at low levels. These *in vitro* results point out a toxic mechanism of action fundamentally at a local level, with tissue damage likely caused (although not exclusively) by SVMPs. Immunochemical reactivity was evaluated against *Bothrops* antivenoms produced in Argentina, which not only exhibited cross-reaction by Western Blotting but also neutralized the procoagulant activity of the venom. This study offers first insights into the venom components of *B. sanctaecrucis*, and provides preliminary and important information about the pathophysiological mechanisms involved in the envenomation by this species, paving the way for treatment strategies in such accidents.

1. Introduction

Although likely underestimated, the annual incidence of snakebites in Bolivia is ~8 snakebites per 100,000 inhabitants, with great disparity between districts (Chippaux and Postigo, 2014). The incidence is higher in rural areas and, considering the rise of human population in rural areas, it is predicted that the number of snakebites will increase in the coming years (Chippaux, 2017; Chippaux and Postigo, 2014). The mortality rate due to snakebites in Bolivia is the second highest in Latin America, just after Panama (Mutricy et al., 2018) and, although the genus mostly involved in snakebites in the country is unknown, considering information from its neighbor countries, the *Bothrops* genus is almost certainly among the most medically important snakes (Sant'Ana Malaque and Gutiérrez, 2015). There are more than 11 species of *Bothrops* snakes in Bolivia, and many of them are known to be of medical relevance (Albuquerque et al., 2020; Freitas-de-Sousa et al., 2024; Harvey et al., 2005; Santos-Barreto et al., 2017; Timms et al., 2019). The World Health Organization Expert Committee on Biological Standardization considers 9 *Bothrops* species as medically relevant in Bolivia: *B. atrox* and *B. mattogrossensis* classified as the highest medical importance snake species (Category 1), and *B. bilineatus*, *B. brazili*, *B. jararacussu*, *B. jonathani*, *B. moojeni*, *B. taeniatus* and *B. sanctaecrucis* of secondary medical importance (Category 2), denoting that some species of the latter category are of primary medical relevance in other countries (WHO Expert Committee on Biological Standardization, sixty-seventh report, 2017). It is important to note that the presence of *B. jararacussu* in Bolivia has been questioned by some authors (Harvey et al., 2005).

Bothrops sanctaecrucis (Hoge, 1966) is the sole endemic species of pit viper found in Bolivia (Fig. 1). It inhabits the amazon basin region of the Bolivian Andes (210–380 m above sea level), in areas with high humidity and average temperatures that range from 17 to 25 °C

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Fig. 1. *B. sanctaecrucis* specimen kept in the Santa Cruz Municipal Zoo, Bolivia. (Picture taken by Ronald Sosa, Biologist in charge of serpentarium).

(Gómez-Murillo et al., 2020; INE, 2024; Saavedra and Ureña, 2022). Specimens of this species have been found in the departments of Santa Cruz, Cochabamba, La Paz and Beni, with the majority of reports coming from the area between Santa Cruz and Cochabamba (Gómez-Murillo et al., 2020; Miranda-Calle and Aguilar-Krigin, 2011). Although no official published data exists on *B. sanctaecrucis* diet in the wild, it is highly probable that small mammals and anurous amphibians are part of its diet, given that these are common prey of other terrestrial *Bothrops* species from Bolivia (Bernarde et al., 2021; Monteiro et al., 2006, 2020; Nogueira et al., 2003).

As previously mentioned, the WHO Expert Committee on Biological Standardization classifies this species as one of secondary medical concern due to the lack of data on clinical-epidemiological profile of snakebite victims and its venom properties (WHO Expert Committee on Biological Standardization, sixty-seventh report, 2017). Although *B. sanctaecrucis* is the species most frequently found in the Chapare region, located in Cochabamba (Villca-Corani et al., 2023), and is also the responsible for most of the snakebites in this region, there is no detailed description about the clinical picture of the envenomation caused by this species (Nieto-Ariza et al., 2022).

Taking into account that the outcome of any snakebite is directly related to the venom properties of the species involved, this work aims to analyze the protein composition and biochemical properties of the venom of *B. sanctaecrucis*. Besides shedding light on the pathophysiological mechanisms following envenomation, we aimed to provide relevant information to allow medically trained personnel to assess and treat patients bitten by this species.

2. Materials and methods

2.1. *Bothrops sanctaecrucis venom*

B. sanctaecrucis (Fig. 1) is classified as a "near threatened" species according to the "Libro rojo de la fauna silvestre de Bolivia" (Ministerio de Medio Ambiente y Agua, 2009) due to its indiscriminate hunting out of fear or superstition. Thus, pooled venom from only 3 adult specimens of *B. sanctaecrucis* was used in this study. The venom was lyophilized and stored at −20 °C. When required, the venom pool was dissolved in phosphate buffered saline (PBS; pH 7.4).

2.2. *Gel electrophoresis*

The protein profile of *B. sanctaecrucis* venom was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 3% stacking and 12% resolving gels (Laemmli, 1970), and Tricine-SDS-PAGE using 4% stacking, 10% spacer and 16.5% resolving gels (Schägger, 2006). Lanes were loaded with 2.5, 5, 10 and 20 μg in both under reducing (with 2-mercaptoethanol) and non-reducing (without 2-mercaptoethanol) conditions and gels were silver stained (Blum et al., 1987). Molecular mass determination and densitometric analysis were performed using the ImageLab software, version 6.1 (BioRad).

2.3. *Proteolytic activity*

Proteolytic activity of *B. sanctaecrucis* venom was evaluated using the following substrates: azocasein, collagen, S-2238, fibrinogen and fibrin.

2.3.1. *Caseinolytic activity*

Caseinolytic activity was determined as reported previously (Wang and Huang, 2002). One unit of caseinolytic activity was defined as the amount of venom that causes an increase of 0.005 units of absorbance per min at 540 nm and the specific activity was expressed as U/mg lyophilized venom.

2.3.2. *Collagenolytic activity*

A zymographic assay (SDS-PAGE on 12% gel containing gelatin from porcine skin, 2 mg/mL) was carried out to determine collagenolytic activity (Barbaro et al., 2005).

2.3.3. *Thrombin-like activity*

Thrombin-like enzyme activity was measured by the hydrolysis of the synthetic substrate for thrombin S-2238 (D-Phe-Pip-Arg-pNA) as described by Antunes et al. (2010). Specific activity was expressed as μmol p-nitroaniline/min/mg lyophilized venom.

2.3.4. *Fibrin(ogen)olytic activity*

Specific cleavage of fibrinogen by *B. sanctaecrucis* venom was determined by SDS-PAGE using 12% polyacrylamide gels as described by (Peichoto et al., 2007). Two hundred microliters of 2 mg/mL human fibrinogen dissolved in 50 mM Tris-HCl buffer (pH 7.4) was incubated with venom (100:1 mass ratio) at 37 °C. At various time intervals, aliquots of 15 μL were withdrawn from the digestion mixture, and then denatured and reduced by boiling for 7 min with 15 μL of denaturing solution (4% SDS, 20% glycerol and 20% 2-mercaptoethanol) prior to gel electrophoresis. For fibrinolytic activity, fibrinogen aliquots (20 μL) were coagulated by adding bovine thrombin (5 U/mL final concentration, Sigma) prior to the addition of venom (40:1 ratio). At different time intervals, 20 μL of denaturing solution were added to the reaction mixture and then processed as above (Quintana et al., 2017).

2.4. *Phospholipase A_2 activity*

To determine the presence of phospholipase A_2 (PLA_2) enzymes, both direct and indirect hemolysis assays were carried out as described previously (Gutiérrez et al., 1988). Additionally, a PLA_2 zymography assay was performed to determine the corresponding active band(s) (Campos et al., 2012).

2.5. *Hyaluronidase activity*

Hyaluronidase activity was determined as described by Antunes et al. (2010). Reaction mixtures consisting of 10 μL of venom (20 mg/mL) and 40 μL of hyaluronic acid (0.5 mg/mL) were incubated at 37 °C for different time intervals (90, 180 and 360 min). After determining the time that yielded the highest level of hydrolysis, serial dilutions of venom (at concentrations starting at 40 mg/mL) were tested. Specific activity was expressed as μg of degraded hyaluronic acid/-min/mg lyophilized venom.

2.6. *Cross-reactivity with Bothrops antivenoms used in Argentina*

In order to evaluate the presence of components cross-reacting with horse antivenom against *Bothrops*, Western blot analyses were

performed. Proteins separated by SDS-PAGE (under reducing and non-reducing conditions) were transferred to 0.2 μm nitrocellulose membranes in a tank transfer system (Hoeffer mini VE, Amersham Biosciences) at 25 V for 1.5 h. Membranes were then blocked with 5% nonfat dry milk, and incubated with bothropic bivalent or tetravalent antivenom (kindly donated by the Instituto Nacional de Producción de Biológicos (INPB), ANLIS "Dr. Carlos G. Malbrán", Argentina) diluted 1:500 with 5% nonfat dry milk in PBS (0.1% Tween 20), and subsequently with 1:10,000 peroxidase-conjugated anti-horse IgG (Sigma A9292). The reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma D5637) as reported elsewhere (Antunes et al., 2010). Blots were imaged and reactive bands were analyzed by the ImageLab software, version 6.1 (BioRad).

2.7. Coagulant activity and its neutralization by Bothrops antivenoms

This was carried out by using a clotting assay described previously (Chanhome et al., 2022). Pooled normal commercial plasma (Stago, Paris, France) was incubated with different quantities of venom to determine the minimum coagulation dose (MCD), defined as the amount of venom that induces substrate coagulation in 3 min. For the neutralization evaluation, a dose of 2 MCD was pre-incubated with different dilutions of bivalent or tetravalent antivenom. The effective dose (ED) of antivenom was defined as the volume of antivenom (μL) that prolonged the clotting time to that of 3 times the control (2 DMC of venom with no antivenom). The regression analyses of plots of venom or antivenom amount (μg or μL as appropriate) against clotting time were performed

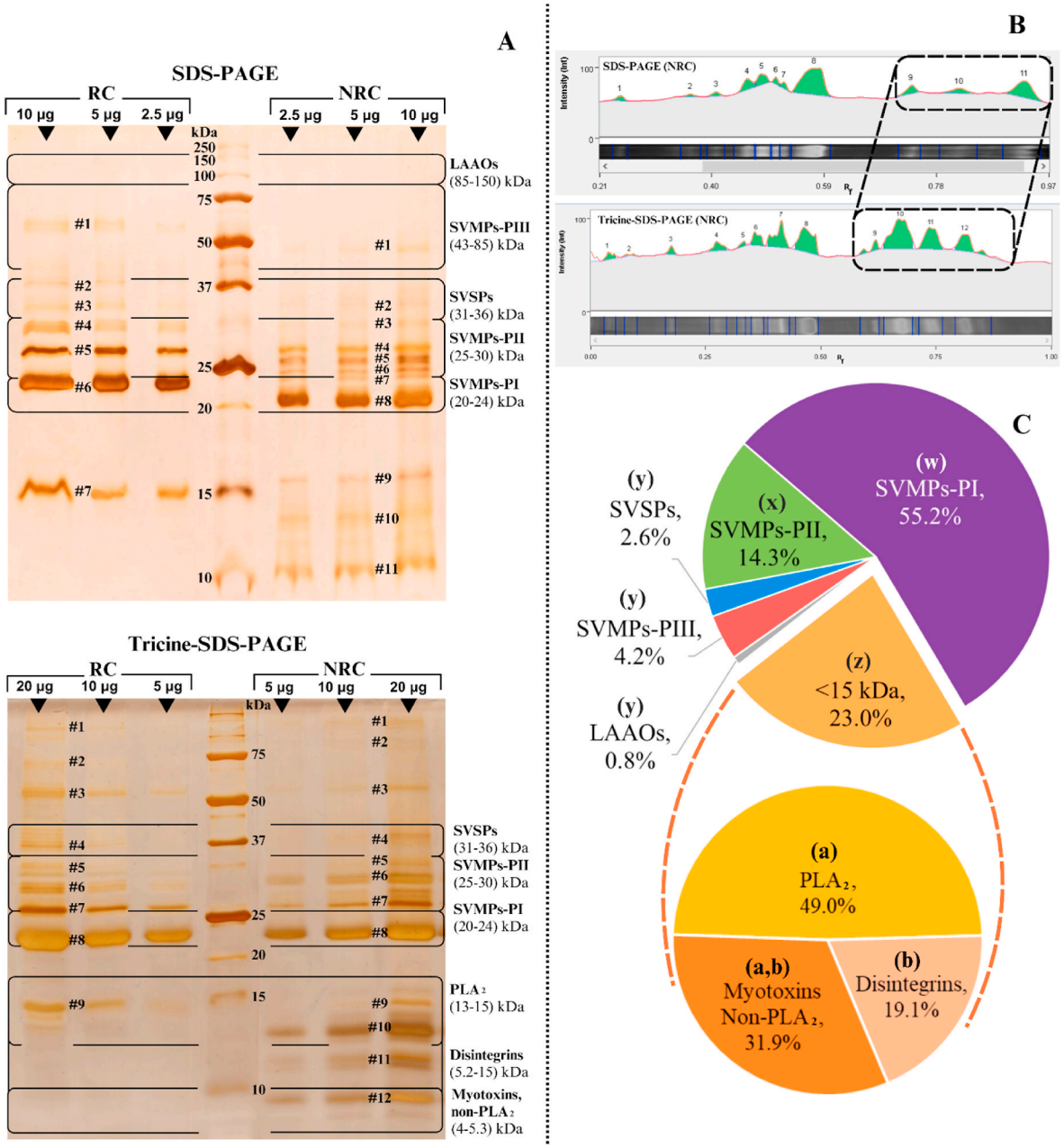

Fig. 2. Electrophoretic profile of *B. sanctaecrucis* venom. **A:** SDS PAGE (12%) and Tricine-SDS-PAGE (16.5%) under reduced (RC) and non-reduced conditions (NRC). Main protein families found in viperid venoms are noted on the right with their respective molecular mass range (Mackessy, 2009). Snake venom metalloproteinase (SVMP), snake venom serine protease (SVSP), L-amino acid oxidase (LAAO) and phospholipase A_2 (PLA_2). **B:** Densitometric analysis of the electrophoretic profile under NRC. **C:** Relative abundance pie chart of main protein families based on densitometric analysis. Values represent mean percentages (n = 3), and different letters indicate statistically significant difference ($p < 0.05$).

using the software CurveExpert version 1.40 (http://curveexpert.webhop.net/), which calculated the best fit and MCD and ED values.

2.8. Statistical analyses

Where appropriate, values were expressed as the mean ± standard deviation (SD) and statistical comparisons were done using one-way analysis of variance (ANOVA) followed by Tukey's test. All data analyses were performed using Infostat Software, with a value of $p < 0.05$ indicating statistical significance.

3. Results and discussion

3.1. Protein profile

SDS-PAGE profile of *B. sanctaecrucis* venom showed a wide distribution of bands with most of them in the molecular-mass range of ~10–37 kDa (Fig. 2-A). The band at ~20 kDa under non-reducing conditions (NRC) (#8) exhibited the highest average density (~55%; Fig. 2-B, C). A similar pattern of bands was obtained under reduced conditions (RC), though slower migrations rates are noted, which may be related with the compacted structure of the proteins due to the presence of disulfide bonds (Marangon et al., 2014). The tricine-SDS-PAGE profile showed better separation of protein bands with low-molecular-mass, specifically in the ~10–15 kDa range (Fig. 2-A).

Previously reported electrophoretic profiles of bothropic venoms show similar band patterns, such as those of *B. neuwiedi*, *B. atrox* and *B. jararaca* (Saad et al., 2012; Santoro et al., 2015), which show darker staining bands within the SVMP-PI and SVMP-PII mass range. It is important to highlight the similarities with the electrophoretic pattern of *B. mattogrossensis* (formerly known as *Bothrops neuwiedi bolivianus*; see: http://www.reptile-database.org/) (Debono et al., 2016), since the venom of this species is used for the production of *Bothrops* antivenom in Bolivia (Segura et al., 2010). However, unlike *B sanctaecrucis* venom, those venoms show also intense bands at the range corresponding to the SVMP-PIII protein family. This type of metalloproteinase is the most prevalent components of *B. atrox*, *B. jararaca, B. neuwiedi* and *B. diporus* venoms (Gay et al., 2015; Sousa et al., 2013). In contrast, SVMPs-PI predominate in *B. sanctaecrucis* venom, and although this family has shown to have fibrino(geno)lytic, pro-thrombin activating, caseinolytic and hemorrhagic activities, its toxicity is generally lower than that of SVMPs-PII and PIII (Olaoba et al., 2020).

It is important to bear in mind that only adult specimens were used to obtain venom for this study. Thus, the higher abundance of SVMPs-PI compared to that of SVMPs PII and PIII might be different for the venom of newborn and juveniles of *B. sanctaecrucis*, such as shown for other *Bothrops* species like *B. asper, B. atrox* and *B. jararaca* (Alape-Girón et al., 2008; Guércio et al., 2006; Zelanis et al., 2010). This ontogenetic variation in venom composition (mainly in SVMPs) is thought to be related to feeding habits during their growth, as members of this genus change their diet from ectothermic to endothermic prey as they age (Alape-Girón et al., 2008; Guércio et al., 2006; Zelanis et al., 2010). Additionally, although this diet change hasn't been studied in *B. sanctaecrucis*, this species does present a lighter tail coloration in juveniles (Ministerio de Medio Ambiente y Agua, 2009), which is an indicator of this shift in prey (Bernarde et al., 2021; Monteiro et al., 2020; Nogueira et al., 2003).

The serine proteinase (SVSP) content in the several aforementioned species venoms (~7–12%) (Gay et al., 2015; Sousa et al., 2013) is around three-fold higher than in *B. sanctaecrucis* venom (Sousa et al., 2013). SVSPs are primarily hemotoxic and interfere with blood coagulation, blood fibrinogen levels, blood pressure and platelet aggregation (Oliveira et al., 2022).

Herein, the authors estimated the relative abundance of different venom components of *B. sanctaecrucis* by the densitometry of their corresponding bands stained after separation by SDS-PAGE. Although this approach is valid (Uribe-Arjona et al., 2021), it is important to bear in mind the possible bias in this estimation since there are some venom proteins, as C-type lectins, that are poorly detected by conventional SDS-PAGE dyes which might result in an underestimation. Moreover, it may be difficult to distinguish components of similar native molecular mass, such as SVSPs or cysteine-rich secretory proteins (CRISPs) (Monteiro et al., 2020). Nonetheless, this study helps to provide a preliminary overview about the composition of this venom with reasonable relevance and reliability.

3.2. Proteolytic activity

Caseinolytic activity was dependent on the amount of venom used (Fig. 3), and the specific activity was 47.65 U/mg, which is much higher than that reported for the venom of *B. jararaca* (Antunes et al., 2010) but lower than that reported for the venom of *B. atrox* (Hatakeyama et al., 2020).

When venom was submitted to electrophoresis in gels containing denatured collagen (gelatin) (Fig. 4), marked gelatinolytic activity was noticed at ~28.5 kDa, which is in the mass range of SVMPs-PII. Interestingly, the venom of *B. mattogrossensis* has previously shown a major active component with electrophoretic mobility of ~25 kDa (Debono et al., 2016). Additionally, collagenolytic bands with similar migration rates have also been reported for venoms of *B. jararaca, B. jararacussu* and *B. neuwiedi*, which also presented additional bands (and even with higher activity) at molecular masses attributed to P-III metalloproteinases (Antunes et al., 2010; Bernardoni et al., 2014; Leme et al., 2009). Being collagen the main structural protein in the extracellular matrix in various connective tissues in human body, we hypothesize that the presence of active collagenolytic components in *B. sanctaecrucis* venom may contribute to bleeding by disrupting microvessels (such as generally attributed to several bothropic SVMPs; Escalante et al., 2011; Sanchez et al., 2016) at least around the bite site such as observed in a picture record of envenomation by this species (Nieto-Ariza et al., 2022). The proteolytic action of SVMPs may also contribute to local inflammation, triggered by degradation products of the extracellular matrix (Cavalcante et al., 2023). The local reaction can be further explained by the fact that SVMPs-PII and PIII have shown to co-localize with collagen, contributing to their localized activity, unlike those of the PI class which diffuse through the tissue (Olaoba et al., 2020).

To study the activity of serine proteinases, the synthetic substrate HD-Phe-Pip-Arg-pNA (S-2238) was used since, with *B. jararaca* venom, it showed higher amidolytic activity than BAPNA (Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride; commonly used to verify the SVSP activity of venoms) (Menezes et al., 2006). The activity was proportional to the amount of venom used herein (Fig. 5), obtaining a specific activity of 625.55 μmol/min/mg, which is considerably higher

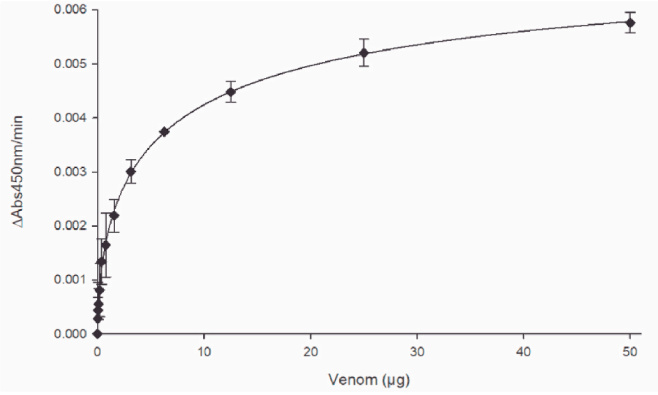

Fig. 3. Caseinolytic activity of *B. sanctaecrucis* venom. Data represent the mean ± SD of three individual experiments.

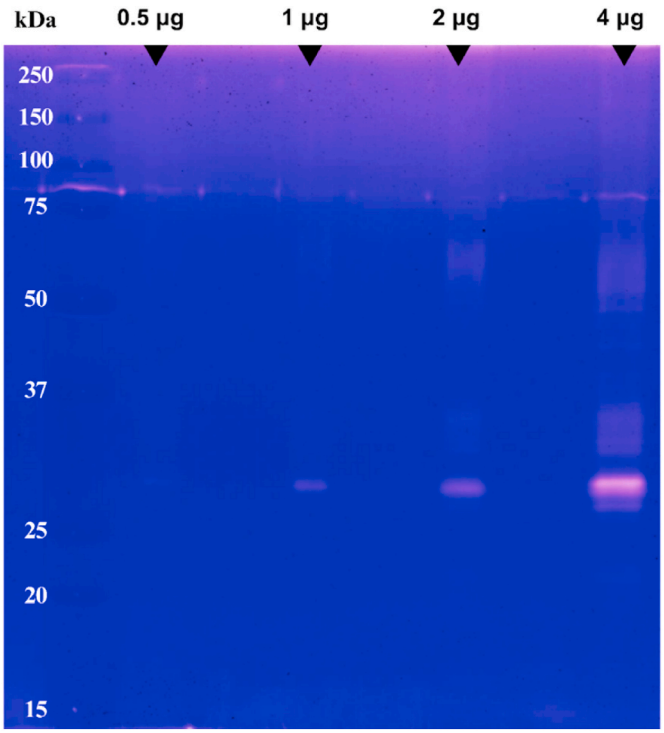

Fig. 4. Zymogram on an SDS-PAGE (12%) gel copolymerized with gelatin. Gel was stained with Coomassie Blue R-250. Note that clear zones indicate the presence of enzymes with gelatinolytic activity, which is directly dependent on the amount of *B. sanctaecrucis* venom. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

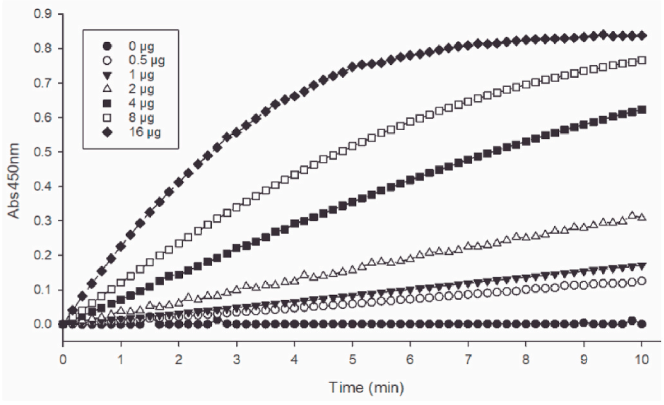

Fig. 5. Proteolytic activity against S-2238 using different amounts of *B. sanctaecrucis* venom.

than those reported for individual venoms of *B. jararaca* (Menezes et al., 2006).

The fibrinogen digestion revealed a rapid hydrolysis of the Aα-chain of fibrinogen by 5 min, and after 15 min there appeared to be some degradation of the Bβ and γ chains (Fig. 6-A), showing significant degradation of the three chains after 24 h of incubation (data not shown). Venoms from *B. atrox, B. neuwiedi* and *B. jararaca* previously exhibited a similar hydrolysis pattern (Sousa et al., 2018). It is important to comment that the formation of a loose clot was evidenced within the reaction mixture at 15 min. This is consistent with previous results showing high catalytic activity against a specific substrate for thrombin, denoting the presence of serine proteases classified as thrombin-like by having coagulant activity that is similar to plasma thrombin, but are not inhibited by heparin and do not activate coagulation factor XIII (Silva et al., 2024).

Unlike fibrinogen, fibrin has a cross-linked structure and is much less susceptible to proteolysis (Silva et al., 2024). This is in line with the fact that *B. sanctaecrucis* venom hydrolyzed only the β monomer and the γ dimer of fibrin (Fig. 6-B), whereas this degradation pattern is different from that of *Bothrops moojeni* venom, which has been shown to degrade not only the β but also the γ monomer (De Oliveira et al., 2016).

Several SVMPs and SVSPs isolated from bothropic venoms have been shown to have fibrino(geno)lytic activities (Larréché et al., 2021; Olaoba et al., 2020), provoking, on one hand, the formation of unstable fibrin clots (Silva et al., 2024) and, on the other hand, the dissolution of the fibrin clots (Amorim et al., 2018; Bernardes et al., 2008), which results in an anticoagulant effect *in vivo* (Silva et al., 2024). According to this data, we can infer that both protein families could also be responsible for such effects in *B. sanctaecrucis* venom.

3.3. Phospholipase A_2 activity

The venom exhibited hemolytic activity only in the presence of egg yolk (lecithin source). The hemolysis halo diameters were concentration-dependent (Fig. 7-A). The highest amount of *B. sanctaecrucis* venom (300 µg) induced a halo of ~12.9 mm, whereas an amount almost a hundred-fold lower of *Bothrops asper* venom (2.93 µg) provoked an indirect hemolysis halo of 20 mm (Uribe-Arjona et al., 2021). This implies that *B. sanctaecrucis* venom displays much lower level of PLA_2 enzymatic activity than *B. asper* venom, and this constitutes another characteristic shared with *B. mattogrossensis* venom (Debono et al., 2016). Phospholipase A_2 zymography showed a weak hydrolytic band between 11 and 14 kDa, with much lower activity than the positive control (Fig. 7-B). Note that very faint hydrolytic bands at ~23 and ~31 kDa are also visible, suggesting PLA_2s in dimeric forms, which have been observed in other bothropic venoms (Sousa et al., 2022). Other bothropic venoms have also been reported to have bands with PLA_2 activity within a low mass range (15 kDa or lower), such as those of *B. jararaca, B. jararacussu, B. moojeni, B. neuwiedi* and *B. alternatus* (Campos et al., 2013). It is important to highlight that PLA_2s are one of the main toxins contributing to the development of the local inflammatory response evoked by *Bothrops* venoms (Antunes et al., 2010), and they can also cause myonecrosis with the combined effect of hemorrhagic SVMPs (Gutiérrez and Ownby, 2003). Due to the low content/activity of PLA_2 in *B. sanctaecrucis* venom, envenomation by this species would not be expected to produce significant myotoxic effects, however, $PLAs_2$ may still contribute to the local inflammation observed in the case documented by Nieto-Ariza et al. (2022).

3.4. Hyaluronidase activity

Hyaluronidase activity was dependent on both incubation time and the amount of venom (Fig. 8). A low specific activity (0.443 µg/min/mg) was obtained compared to the previously reported activity for *B. jararaca* venom (Antunes et al., 2010). Taking into account that snake venom hyaluronidases are known as spreading factors of toxins (Girish and Kemparaju, 2006), we can assume that *B. sanctaecrucis* venom could have lower diffusion capacity into the tissue of the victims (Wiezel et al., 2015).

3.5. Cross-reactivity with Bothrops antivenoms used in Argentina

By Western blotting, several components of the venom of *B. sanctaecrucis* were recognized by both bivalent and tetravalent horse antivenoms (Fig. 9), with a notable difference in a band slightly lighter than 25 kDa within the range of SVMP-PII (most likely corresponding to band #5 or #6 in NRC; Fig. 2-A). By densitometry, this band represents about 60% of the venom bands recognized by the tetravalent antivenom, whereas the same band represents 28% with the bivalent antivenom.

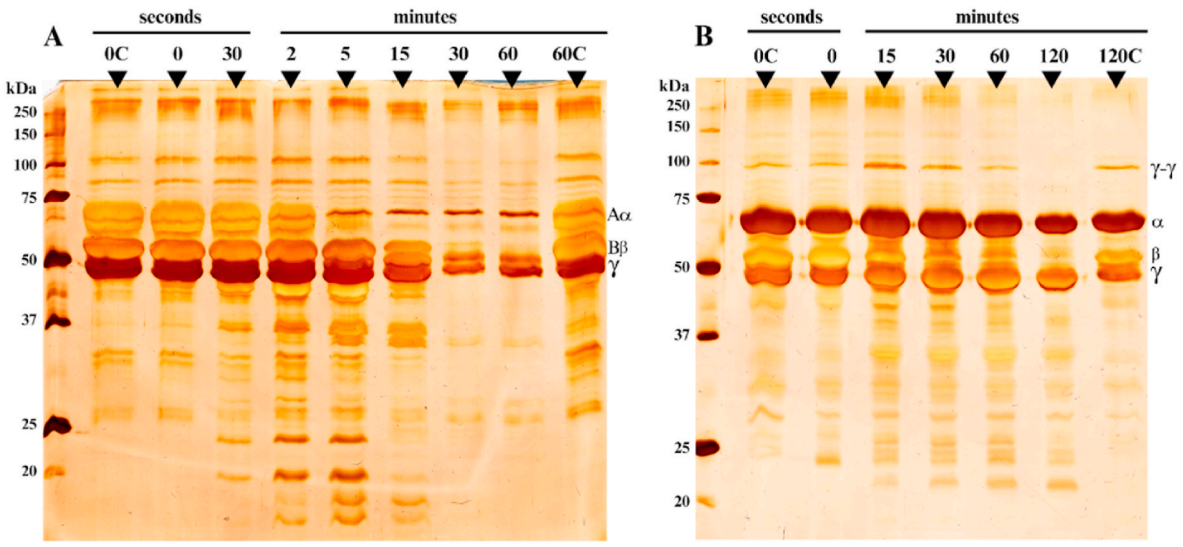

Fig. 6. Time course of the effect of *B. sanctaecrucis* venom on human fibrinogen **(A)** and fibrin **(B)**. Human fibrinogen chains are indicated (Aα - 63 kDa, Bβ - 56 kDa, and γ - 47 kDa), and the chains of human fibrin are labeled (γ-γ dimer, α-monomer, β-monomer, and γ-monomer). Controls (C) were incubated in the absence of venom.

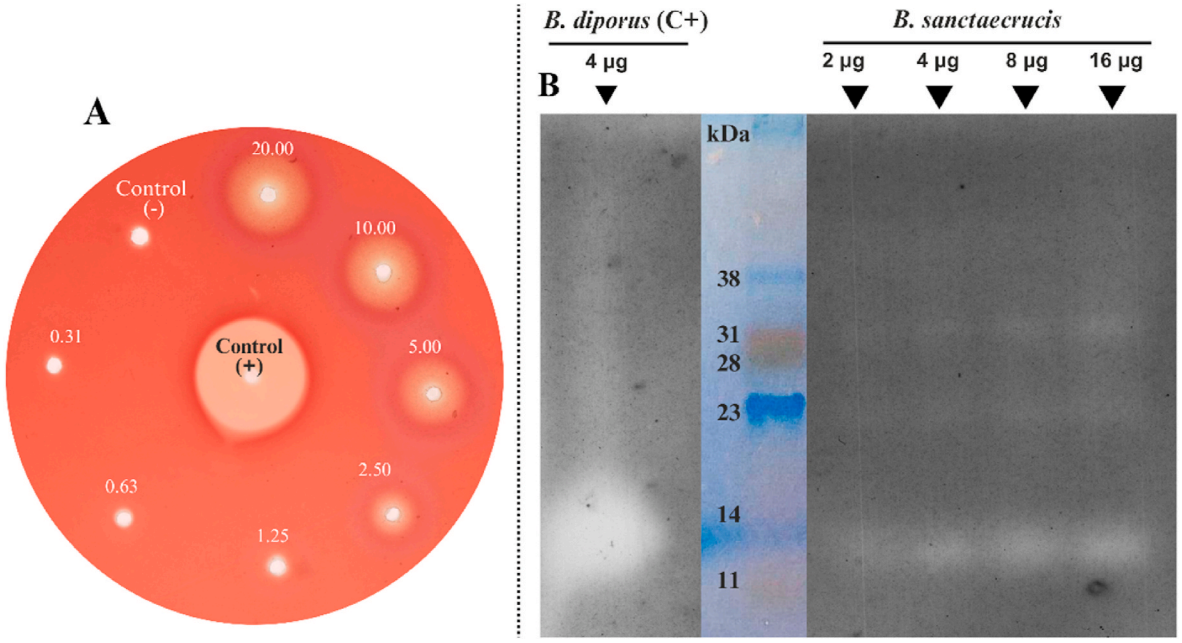

Fig. 7. PLA_2 activity of *B. sanctaecrucis* venom. **A:** Indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plate. Note the hemolytic halos produced by the venom at different concentrations (mg/mL). Triton X-100 and PBS were used as positive and negative controls respectively. **B:** PLA_2 zymogram on a 1.0% agarose gel containing 2.0% egg yolk. *B. diporus* snake venom was used as a positive control for PLA_2 activity. Note a clear area at ~12 kDa, which corresponds to the range of molecular mass of PLA_2s.

Such higher density in the former suggests that the *B. sanctaecrucis* venom proteins within the SVMP-PII mass range share a higher similarity to those found in venoms from species used in the production of the tetravalent (*B. alternatus*, *B. diporus*, *B. jararaca*, *B. jararacussu*) rather than bivalent (*B. alternatus*, *B. diporus*) antivenom.

3.6. Coagulant activity and its neutralization with Bothrops antivenoms

The venom of *B. sanctaecrucis* exhibited procoagulant activity on normal plasma (Fig. 10), with an MCD of 0.17 µg/mL (26 ng), which is substantially lower than that of *Protobothrops kelohomy* venom (Chanhome et al., 2022). The intense coagulant activity of this venom may be attributed to its thrombin-like activity (serine proteinases) already described above. However, we cannot discard the participation of procoagulant metalloproteinases since the high coagulant activity of newborn *B. jararaca* venom has been mainly attributed to metalloproteinases involved in the activation of prothrombin and factor X (Antunes et al., 2010). In adition, the detected prominent gelatinolytic band in *B. sanctaecrucis* venom (see above) presents a molecular mass similar to a prothrombin-activating metalloproteinase isolated from *B. jararaca* venom (Berger et al., 2008).

In vitro procoagulant and fibrin(ogen)olytic activities can be extrapolated to *in vivo* venom-induced consumption coagulopathy, and this may result in victims presenting unclottable blood which can

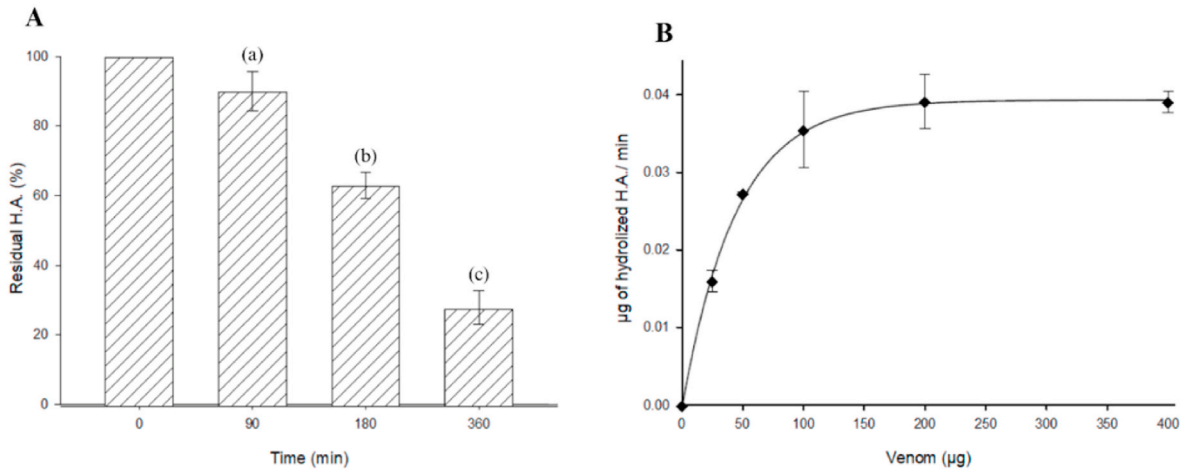

Fig. 8. Hyaluronidase activity of *B. sanctaecrucis* venom. **A:** Residual hyaluronic acid (H.A.) after different incubation times with 200 µg of *B. sanctaecrucis* venom. Different letters indicate statistically significant difference ($p < 0.05$). **B:** Hyaluronidase activity after 6 h of incubation with different amounts of *B. sanctaecrucis* venom. Data represent the mean ± SD of three individual experiments.

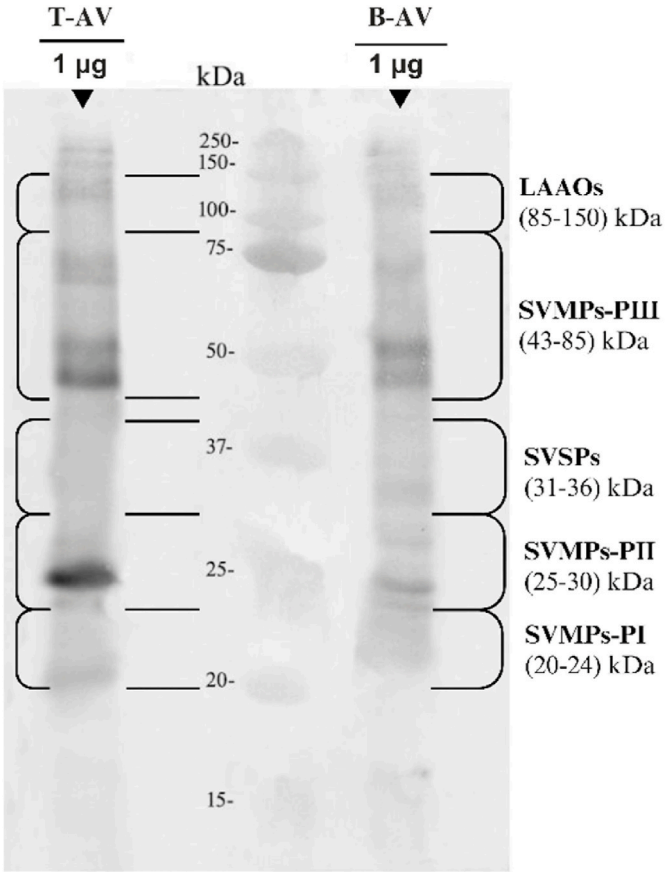

Fig. 9. Western blot analysis of *B. sanctaecrucis* venom using tetravalent (T-AV) and bivalent (B-AV) horse antivenom against *Bothrops*. Main protein families found in viperid venoms are noted on the right with their respective molecular mass range (Mackessy, 2009).

exacerbate hemorrhagic symptoms (Larréché et al., 2021). Since only human plasma was used in the present study (given the importance of knowing its toxic effects on humans), we propose that future works on *B. sanctaecrucis* venom (mainly those directed towards the ecology of this species) should include plasma from different animals given that several bothropic venoms have shown to present taxa-specific

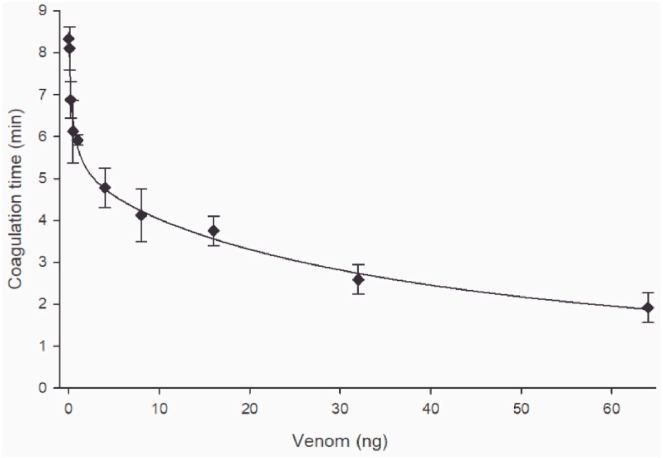

Fig. 10. Coagulant activity of *B. sanctaecrucis* venom on normal plasma. Data represent the mean ± SD of three individual experiments.

coagulopathy (Bernardoni et al., 2014; Sousa et al., 2018).

In general, the assay for neutralization of lethality has been the 'gold standard' for the approval of antivenoms in preclinical testing. However, in some viperid venoms, *in vitro* immunochemical and enzymatic tests have shown good correlation with the neutralization of lethality. A well-known example of this is the venom of *B. asper*, for which the neutralization of *in vitro* coagulant activity in normal plasma has been shown to correlate with neutralization of lethality in mice (Chacón et al., 2015). Thus, this useful approach was addressed in this study as a way to reduce the use of mice in the preclinical testing of antivenoms such as recommended in the second edition of the WHO Guidelines on antivenoms (WHO Expert Committee on Biological Standardization, sixty-seventh report, 2017).

Both Argentinian *Bothrops* antivenoms were effective to neutralize the coagulant activity of *B. sanctaecrucis* venom (Fig. 11), obtaining ED values of 15.1 nL and 30.7 nL for the tetravalent and bivalent antivenom, respectively. This means that one vial of each antivenom (10 mL) is able to neutralize 34 mg (T-AV) and 17 mg (B-AV) of *B. sanctaecrucis* venom. Similar to the Western blot results, this also suggests that the venoms used in the immunizing mixture to produce the tetravalent antivenom share a higher similarity than that of the ones used to produce the bivalent antivenom. Moreover, the ED value obtained with the tetravalent antivenom is comparable to that obtained

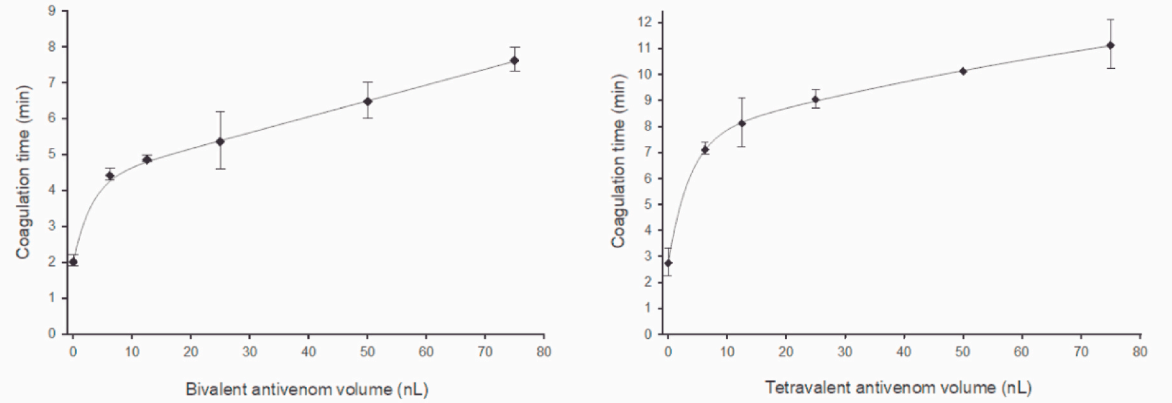

Fig. 11. Neutralization of coagulant effect of *B. sanctaecrucis* venom on normal plasma by bothropic antivenoms. The bivalent antivenom is against the venoms from *B. alternatus* and *B. diporus*, while tetravalent antivenom is against *B. alternatus*, *B. diporus, B. jararaca* and *B. jararacussu* venoms. Data represent the mean ± SD of three individual experiments.

when testing the neutralizing ability of the Bothropic/Crotalic antivenom from Instituto Nacional de Laboratorios de Salud (INLASA, Bolivia) on the coagulant activity of the venom of *B. mattogrossensis*, and, in turn, the lethality of the latter is efficiently neutralized by not only that antivenom but also the bothropic bivalent antivenom produced by the INPB-ANLIS Malbrán from Argentina (Segura et al., 2010). This implies a high extent of cross-neutralization of polyspecific viperid antivenoms manufactured in public laboratories of Argentina against the venoms of medically-relevant *Bothrops* species in Bolivia, which may constitute a relevant step forward in the efforts to develop a collaborative network between both countries.

4. Concluding remarks

By *in vitro* characterization, we revealed for the first time that *B. sanctaecrucis* venom is mainly composed of hemotoxic enzymes and/or enzymes that degrade components of connective tissue, with SVMPs being apparently the most abundant components. These *in* vitro findings support the hypothesis that local hemorrhage and inflammation are highly likely to occur upon envenomation by this species, however, further studies need to be carried out in order to fully understand the pathophysiology of this envenomation.

This venom exhibits cross-reactivity with *Bothrops* antivenoms produced by the INPB-ANLIS Malbrán from Argentina. Both antivenoms are able to neutralize its coagulant activity, one of the main ones responsible for *Bothrops* venom toxicity (Antunes et al., 2010). Although still lacking *in vivo* toxicity tests, this information is particularly useful in terms of a collaborative approach within the framework of the Latin American Network of Public Antivenom Manufacturing Laboratories (RELAPA, *Red Latinoamericana de Laboratorios Públicos Productores de Antivenenos*) (Fan et al., 2019).

Altogether, the findings of this study offer first insights into the venom components of *B. sanctaecrucis*, provide a guide for clinical approach in case of envenomation by this species, and give insight into future directions for research on its venom.

CRediT authorship contribution statement

Kevin Lobo-López: Writing – review & editing, Writing – original draft, Visualization, Resources, Investigation, Formal analysis. **Matías E. Martínez:** Visualization, Validation, Supervision, Investigation. **Micaela A. Gritti:** Visualization, Validation, Supervision, Resources, Investigation. **María E. Peichoto:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Ethical statement

I testify on behalf of all co-authors that our article "Biochemical Characterization of the Venom of the Bolivian Endemic pit viper Bothrops sanctaecrucis" meets the following criteria:

The work described has not been published previously except in the form of a preprint, an abstract, a published lecture, academic thesis or registered report. See our policy on multiple, redundant or concurrent publication.

The article is not under consideration for publication elsewhere.

The article's publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.

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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.

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Data availability

Data will be made available on request.

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