BioMed Research International

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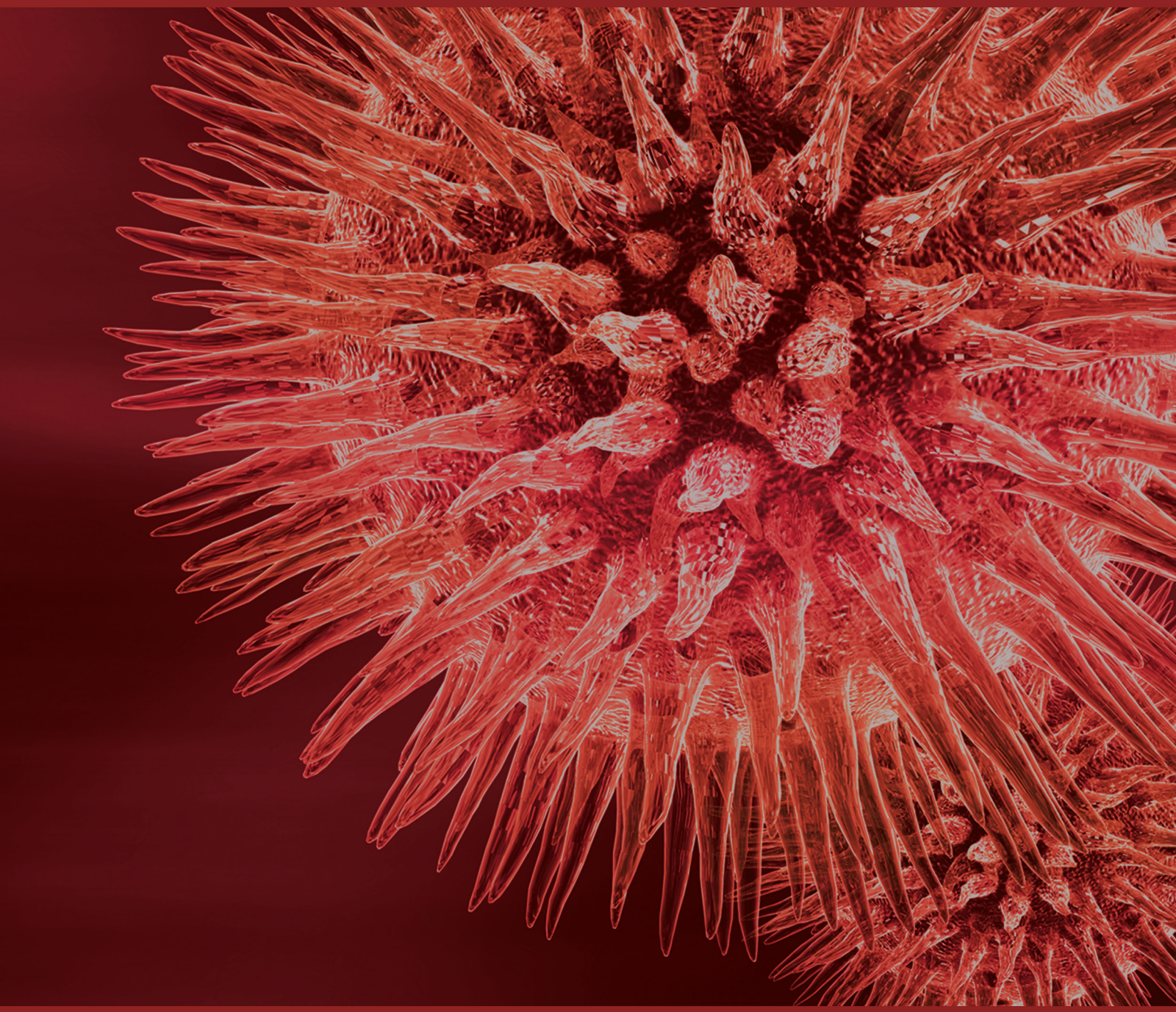
Guest Editors: S. L. Da Silva, E. G. Rowan, F. Albericio, R. G. Stábeli, L. A. Calderon, and A. M. Soares



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Contents

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 951561, 2 pages
http://dx.doi.org/10.1155/2014/951561

Editorial

Animal Toxins and Their Advantages in Biotechnology and Pharmacology

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Received 13 March 2014; Accepted 13 March 2014; Published 22 May 2014

Biodiversity provides a huge source of new chemical entities that could be useful for the development of new therapies. An example is venomous animals, which produce venoms that share common features, such as compositions characterized by a complex combination of bioactive proteins and peptides with wide structural diversity. The biological activities of these compounds are selective and specific and are currently dependent on the synergic action of several components. Thus, animal venoms are important tools for carrying out biochemical, physiological, and pathological studies, as well as for the development of new biotechnological and pharmaceutical products.

The modern approach used to characterize various compounds from animal venoms, using advanced proteomic and genomic tools, has been denominated "venomics." Modern technologies currently available in several research laboratories have allowed scientists to obtain the identification and functional-structural characterization of hundreds of toxins from snakes, scorpions, spiders, anurans, and marine invertebrates, presenting a high diversity of pharmacological activities. Venomics delineates a scientific area of high complexity that is increased by the diversity of venom proteins and the influence imposed by age, geographic location, ontogenetic variation, feeding, and individual intrinsic characteristics that produce intraspecific variations.

The study of toxins and their isoforms has allowed for a better understanding of the toxic mechanisms of envenomation. The identification/characterization of different isoforms of venom toxins, along with the search for natural and synthetic inhibitors, such as monoclonal or polyclonal antibodies, and molecules with different chemical properties, such as heparin, clotting factors, and plant extracts, has increased the possibility of using these agents as more effective therapeutic alternatives.

More recently, venomics has focused on better understanding of the clinical aspects of human envenomation, the mechanism of action of venoms and their toxins, the prospection of toxins with biotechnological/pharmaceutical potential, and the development of novel antivenom serum and alternative therapies for envenomation.

Among venom toxic components, phospholipases A_2, L-amino acid oxidases, and proteolytic enzymes, which are classified as metalloproteases and serine proteases, are able to disrupt the human haemostatic system through different mechanisms. Nonenzymatic proteins, such as disintegrins and bradykinin-potentiating peptides (BPPs), and other

components, such as carbohydrates, lipids, ions, biogenic amines, nucleotides, and free amino acids, are also components of venom.

This special issue presents three revisions and eight original papers addressing different aspects of animal venom components, their inhibitors, and possible biotechnological applications. This is relevant considering that snake envenomation is still an important public health issue in many countries, especially tropical ones.

M. B. A. Carvalho et al. present, herein, a mini-review on phospholipase A_2 inhibitors from different sources, such as Brazilian plants and their chemical synthesis. These authors report on the antivenom effect of different plant extracts used by traditional communities. These extracts have been scientifically validated through the phytochemical identification and characterization of some isolated inhibitors from these species, leading to the development of new therapeutic alternatives for envenomation. L. F. M. Izidoro et al. reviews L-amino acid oxidases isolated from snake venoms, focusing on their biochemical, biological, and enzymatic characteristics and discussing the importance of hydrogen peroxide production, antigenicity, molecular and structural characteristics, and biotechnological and pharmacological applications of LAAOs focusing on their antibiotic potential. Finally, in the third revision paper of this special issue, L. A. Calderon et al. discussed the continuous search for new bioactives in order to develop new anticancer drugs. In this review, the authors describe the potential of proteins and peptides from snake venoms, which show *in vitro* and *in vivo* cytotoxicity against cancer cells, for the development of more efficient and safe therapies.

Among the eight original papers, three of them describe the isolation and biochemical characterization of phospholipases A_2 and basic and acidic phospholipase A_2 homologs from the venoms of *Bothrops atrox* (J. L. Furtado et al., 2014), *Bothrops mattogrossensis* (A. A. de Moura et al., 2014), and *Crotalus oreganus abyssus* (W. Martins et al., 2014), which show inflammatory, leishmanicidal, and antitumoral activities, respectively.

With regard to the search for complementary alternatives for conventional serum therapies, E. C. De Oliveira et al. describe the antivenom potential of *Manilkara subsericea* extract against *Lachesis muta* venom, and C. L. S. Guimarães et al. relate the biotechnological application of polyclonal antibodies produced using alkylated myotoxic phospholipases A_2 from *Bothrops jararacussu* venom with reduced myotoxic activity.

Finally, three papers report on proteases from animal venoms, such as snakes and spiders and their biotechnological potential. F. D. Torres-Huaco et al. and K. D. Zaqueo et al. describe the biochemical characterization of two thrombin-like enzymes (SVTLE) isolated from *Lachesis muta* and *Bothrops pirajai* venoms, respectively. Both enzymes show blood-clotting activity, indicating a thrombolytic potential. In contrast, G. Gimenez et al. demonstrate the proteolytic activity of venom from the social spider *Parawixia bistriata* and its possible biotechnological application as an insecticide against *Aedes aegypti*.

Research regarding animal venoms generally focuses on the clinical features of human envenomation, the relationship between structure and function of animal toxins, bioprospection of toxins with potential for the development of therapies, diagnostic kits for human diseases, and alternative or complementary therapies for envenomation. Therefore, research involving the biologically active components of animal venoms could result in the direct or indirect use of components of venoms during the development of synthetic models in order to obtain new drugs.

Thus, understanding the mechanism of action responsible for venoms' toxic and pharmacologic effects, along with their biochemical and structural characterizations, is essential in order for them to be used as biotechnological prototypes in a range of medical and scientific areas.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 981923, 12 pages
http://dx.doi.org/10.1155/2014/981923

Research Article

Alkylation of Histidine Residues of *Bothrops jararacussu* Venom Proteins and Isolated Phospholipases A_2: A Biotechnological Tool to Improve the Production of Antibodies

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Received 14 September 2013; Accepted 2 January 2014; Published 11 May 2014

Academic Editor: Edward G. Rowan

Crude venom of *Bothrops jararacussu* and isolated phospholipases A_2 (PLA_2) of this toxin (BthTX-I and BthTX-II) were chemically modified (alkylation) by *p*-bromophenacyl bromide (BPB) in order to study antibody production capacity in function of the structure-function relationship of these substances (crude venom and PLA_2 native and alkylated). BthTX-II showed enzymatic activity, while BthTX-I did not. Alkylation reduced BthTX-II activity by 50% while this process abolished the catalytic and myotoxic activities of BthTX-I, while reducing its edema-inducing activity by about 50%. Antibody production against the native and alkylated forms of BthTX-I and -II and the cross-reactivity of antibodies to native and alkylated toxins did not show any apparent differences and these observations were reinforced by surface plasmon resonance (SPR) data. Histopathological analysis of mouse gastrocnemius muscle sections after injection of PBS, BthTX-I, BthTX-II, or both myotoxins previously incubated with neutralizing antibody showed inhibition of the toxin-induced myotoxicity. These results reveal that the chemical modification of the phospholipases A_2 (PLA_2) diminished their toxicity but did not alter their antigenicity. This observation indicates that the modified PLA_2 may provide a biotechnological tool to attenuate the toxicity of the crude venom, by improving the production of antibodies and decreasing the local toxic effects of this poisonous substance in animals used to produce antivenom.

1. Introduction

Snakebites are a public health problem in tropical countries as they can lead to death or serious sequelae. For example, in Brazil, the Viperidae family has four genera of venomous snake, the *Bothrops* genus being responsible for 87.5% of incidents reported, followed by *Crotalus* (9.2%), *Lachesis* (2.7%), and snakes that belong to the Elapidae family (0.6%) [1].

Bothrops venom is rich in phospholipases A_2 (PLA_2), a class of enzymes with catalytic activity on cell membranes of specific tissues. This observation would suggest that PLA_2 plays a crucial role in venom toxicity [2, 3]. Furthermore, snake venom PLA_2 induces several other effects such as pre- or postsynaptic neurotoxicity [3–5], cardiotoxicity [6–9], myotoxicity [10, 11], platelet aggregation inhibition or induction [12, 13], edema [14–17] and hypotension [18–20]. Thus, medical and scientific interest in PLA_2s lies in the action of these enzymes in various pathophysiological processes. In this regard, several research lines are devoted to searching for drugs or tools to inhibit or neutralize the action of PLA_2.

The development of natural or synthetic inhibitors with the capacity to neutralize the toxic effects of PLA_2s has advanced our medical knowledge of the mechanisms of action and structure-function relationships of these enzymes. In this respect, our research group focuses on the development of biotechnological tools that inhibit the PLA_2s and its toxic effects [21, 22].

Snake venom PLA_2s belong to classes I or II of this enzyme family. In this regard, the PLA_2 from venom of *Bothrops jararacussu* belongs to the latter [2–4, 22]. The enzymatic activity of the PLA_2s is characterized by the integrity of the amino acid residue His48 in the active site.

The literature describes that PLA_2 loses its catalytic activity when His48 undergoes alkylation by *p*-bromophenacyl bromide (BPB). In addition to this effect on the activity of PLA_2s, the alkylation also leads to a reduction in the toxic and pharmacological activity of this enzyme [23].

The clinical treatment for *Bothrops* snake bites basically consists of the early administration of appropriate doses of anti-Bothropic serum [24]. The production of antivenom, usually conducted with horses, involves the subcutaneous injection of increasing amounts of the corresponding venoms [25, 26]. Although these injections do not cause relevant systemic alterations in the animals, they induce significant tissue damage at the site of injection (edema, hemorrhage, and necrosis). Reduction of local tissue reaction at the site of inoculation, without impairing the immune response of the animals against venom components, is a major goal of laboratories producing antivenoms.

Here, we report on the development of a biotechnology tool based on native BjussuCV (*B. jararacussu* crude venom), BthTX-I (K49-PLA_2-like) and BthTX-II (D49-PLA_2), and also all proteins alkylated by BPB. The enzymatic and biological activities of BjussuCV, BthTX-I, and BthTX-II in their native and alkylated forms were evaluated and compared. Rabbit polyclonal antibodies against native or chemically modified BjussuCV, BthTX-I, and -II were produced, and assays were performed to assess their capacity to neutralize the phospholipasic, myotoxic, edema-inducing, and lethal activities of their corresponding antigens. Our results identify the modified toxins as potential tool to produce antivenoms and simultaneously diminish the local effects of injections on animals used to produce antivenom, without impairing their immune response.

2. Material and Methods

2.1. Venoms and Chemicals. The *B. jararacussu* snake venom pool, collected in São Paulo state ($n = 6$), was provided by L. H. A. Pedrosa (FMRP-USP). The proteins BthTX-I (K49-PLA_2-like) and BthTX-II (D49-PLA_2) were isolated as previously described [27, 28]. The venom (BjussuCV) was dried over NaOH pellets in a vacuum desiccator at room temperature immediately after collection and stored at 4°C.

Chemicals such as acrylamide, bisacrylamide, TEMED, Coomassie Brilliant Blue G-250, *p*-bromophenacyl bromide, sinapinic acid (Sigma), bromophenol Blue (Merck), mercaptoethanol (Fluka AG), CM-Sepharose (Pharmacia), pH 5.0–8.5 buffalyte (Pierce), and Freund adjuvant were of analytical grade.

2.2. Animals. Male Swiss mice (18–22 g) and rabbits (2.0–2.5 kg) were obtained from the Central Bioterium of S. Paulo University, Ribeirão Preto, SP. Animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA), and the present proposal was approved by the Animal Ethics Committee of S. Paulo University (CEUA-USP) and The Brazilian Institute of Environment (IBAMA).

2.3. Alkylation of BjussuCV, BthTX-I, and BthTX-II. The BjussuCV, BthTX-I, and BthTX-II samples (12 mg) were dissolved in 1 mL of 100 mM ammonium bicarbonate buffer, pH 7.8, containing 0.7 mM EDTA. Then, 500 μL of BPB (1.5 mg/mL ethanol) was added, and the samples were incubated at 25°C for 24 h. Excess reagent was removed by ultrafiltration through an Amicon YM3000 membrane, and the remaining protein solution was lyophilized [27, 28]. Native proteins are named BjussuCV, BthTX-I, BthTX-II, the proteins alkylated by BPB alk-BjussuCV, alk-BthTX-I, and alk-BthTX-II.

2.4. Immunization. 1.6 mg of native or alkylated BjussuCV samples, as well as 0.5 mg of native or alkylated BthTX-I and BthTX-II, was dissolved in 1 mL of phosphate-buffered saline (PBS), to which 1 mL of Freund's complete adjuvant was added. Rabbits were then injected with 0.4 mL of these mixtures at four sites on the back and thighs (s.c.). After 12 days, a second immunization at the same sites was performed using Freund's incomplete adjuvant. Finally, after 45 days, a first bleed was carried out by intracardiac puncture. Normal and immune sera were precipitated with a saturated solution of ammonium sulphate, pH 6.5, left to stand for 30 min at 4°C, and centrifuged at 3800 ×g. The precipitate obtained was washed with 40% ammonium sulfate, centrifuged, dialyzed against 20 mM sodium phosphate buffer, pH 7.5, and concentrated through an Amicon YM10,000 membrane. Antibody purification was achieved by ion exchange chromatography on a 2 × 20 cm DEAE-Sephacel column, using 0.02 M

sodium phosphate buffer, pH 7.5. Fractions of 4 mL/tube were collected by monitoring the absorbance at $\lambda = 280$ nm. Those fractions containing immunoglobulin G were pooled, concentrated through an Amicon YM10,000 membrane, lyophilized, and stored at 4°C until use [28].

2.5. Immunochemical Characterization

2.5.1. Immunodiffusion. Rabbit polyclonal antibodies to native and alkylated proteins were tested against crude venom or isolated PLA_2s (BthTX-I and BthTX-II) by gel immunodiffusion [29]. Twenty microliters of crude venom or PLA_2 solutions (1.0 mg/mL PBS) and 20 μL of antiserum were placed in wells with 1% agarose dissolved in PBS. The plates were put in a moist chamber at room temperature for 48 h for development of the precipitation lines and then washed for 24 h with 0.3 M NaCl. Experiments were performed in duplicate.

2.5.2. Enzyme Immunoassays. Microplate wells were coated with BjussuCV, BthTX-I, and BthTX-II at 0.2 μg/well by overnight incubation in 0.1 M Tris, 0.15 M NaCl, pH 9.0, buffer [30]. After five washes with solution A (50 mM Tris, 150 mM NaCl, 20 mM $MnZnCl_2$, 1 mM $MgCl_2$, and pH 7.4), the plates were air-dried and stored at 4°C. Purified rabbit antibodies against native (BjussuCV, BthTX-I, and BthTX-II) and modified proteins (alk-BjussuCV, alk-BthTX-I, and alk-BthTX-II) were diluted in solution A (containing 2% bovine serum albumin (BSA)), added to the microplate wells, and incubated at room temperature for 2 hours. After five washes, bound antibodies were detected with anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Sigma), diluted 1 : 2000 with same solution A, and incubated for 90 minutes. After this time, the microplates were washed and the color was developed with *p*-nitrophenylphosphate. Absorbance was recorded with a microplate reader (BioTek) at 410 nm. Normal rabbit serum was used as a negative control in the place of the primary antibody. Assays were done in triplicate.

2.6. Inhibition of Biological Activities. Crude venom or native PLA_2s and also crude venom or PLA_2 alkylated by BPB were selected after dose-response studies of biological activities. The inhibition caused by antibodies was evaluated by incubation with each toxin: antibody ratios (1 : 10; 1 : 50, or 1 : 100) (w/w) for 30 min at 37°C. Biological activities were expressed as percentage, where 100% corresponded to activity of BjussuCV, BthTX-I, BthTX-II, alk-BjussuCV, alk-BthTX-I, and alk-BthTX-II incubated without antiserum.

2.6.1. Myotoxic Activity. The creatine kinase (CK) assay was carried out using the CK-UV kinetic kit from Sigma. Assays were performed by injecting 50 μL (i.m.) of solutions containing 1-2 μg/μL of venom or PLA_2 (native and alkylated) dissolved in PBS, in the right gastrocnemius muscle of a group ($n = 6$) of male Swiss mice (18–22 g). The control group received only PBS. After 3 h, blood from the tail was collected in heparin-coated tubes and centrifuged for plasma separation. The amount of CK was then determined using 4 μL of plasma, which was incubated for 3 min at 37°C with 1.0 mL of the reagent, following the manufacturer's instructions. Activity was expressed in U/L, where one unit resulted from the phosphorylation of 1 μmol of creatine/min at 37°C [28, 29, 31–34].

2.6.2. Edema-Inducing Activity. Groups of six Swiss male mice (18–22 g) were injected in the subplantar region of the right paw with 50 μL of the native or modified toxins dissolved in saline solution (50 μg/50 μL). Paw edema was measured with a low pressure pachymeter (Mitutoyo, Japan) after 0.5, 1, and 3 h. Values registered at zero time point (measured before injections) were then subtracted from those obtained after injection, and the differences were reported as median% ± S.D. The minimal edematogenic dose (MED) was defined as the amount of venom or toxin (native or modified) that induced 30% edema in the paw at 0.5 h [27, 28, 34–37].

2.6.3. Lethality (LD_{50}). Solutions differing in their concentration of native and modified proteins dissolved in saline solution were injected (i.p.) in a group ($n = 6$) of Swiss male mice (18–20 g). Each injection contained 100 μL of solution. After injection, the mice were monitored for 24 h and values of lethal dose causing death of 50% of animals were calculated by Probitos method [38, 39].

2.6.4. Enzymatic Activities. The phospholipase activity of the BjussuCV, BthTX-I, BthTX-II, alk-BjussuCV, alk-BthTX-I, and alk-BthTX-II (native and modified proteins) was assayed by indirect hemolytic activity, as previously described [40–42]. The clotting time test of citrated bovine plasma was used to determine coagulant activity. The minimal coagulant dose (MCD) is the amount of venom that induces coagulation in 0.2 mL of citrated bovine plasma in 60 sec at 37°C.

2.7. Protein Interactions. Protein-protein interactions were assayed by surface plasmon resonance (SPR) with a BIAcoreT200 system [43]. Proteins BthTX-I in Fc (Flow cell, Fc = 1) and BthTX-II in (Fc = 2) were covalently immobilized on the Serie S GE-BIAcore CM-5 sensorchip; they have carboxymethylated dextran matrix, according to the manufacturer's instructions, and the flow cells Fc = 3 and Fc = 4 were the blank (negative control). The flow cells Fc = 3 and Fc = 4 were the blanks (negative control). The CM-5 chip was activated with a 1 : 1 mixture of 0.4 M EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and 0.1 M NHS (1-hydroxy-2,5-pyrrolidinedione) for 5 minutes. Proteins were dissolved in 10 mM phosphate buffer, pH 5 (BthTX-I), and acetate buffer pH 5.9 (BthTX-II) and were then injected over the activated CM-5 chip at 25°C. The matrix was treated with 1 M ethanolamine/HCl pH 8.5 to block the remaining protein activated. Sample analyses were performed at a flow rate of 30 μL/min for 1 min at 37°C. All results were analyzed using BIAevaluation software (version 1.1.1) [44]. Individual experiments were carried out three times.

2.8. Statistical Analysis. Results are presented as the mean ± S.D. obtained with the indicated number of animals. For

Table 1: Influence of BjussuCV, BthTX-I, BthTX-II, alk-BjussuCV, alk-BthTX-I, and alk-BthTX-II on LD_{50} and phospholipase A_2 (PLA_2) activity.

Sample	LD_{50} (mg/kg)	PLA_2* (U/mg)
BjussuCV	5.7 ± 0.9	71.9 ± 1.1
Alk-BjussuCV	5.8 ± 0.8	Residual
BthTX-I	8.0 ± 0.9	0.0
Alk-BthTX-I	40.0 ± 1.2	0.0
BthTX-II	7.5 ± 0.8	37.1 ± 1.2
Alk-BthTX-II	37.3 ± 1.1	0.0

*Measured through potentiometric titration [37].

statistical significance, the data were analyzed by Student's unpaired t-test, $P < 0.05$.

3. Results

B. jararacussu venom (BjussuCV) presented a chromatographic profile on CM-Sepharose column similar to that reported in previous studies [27]. BthTX-I and BthTX-II accounted for about 25% and 8% (w/w) of crude dried venom, respectively. The homogeneity of these proteins was verified by isoelectrofocusing and RP-HPLC. The electrophoretic profile in SDS-PAGE showed that alk-BthTX-I and alk-BthTX-II did not present differences when compared with the native BthTX-I and BthTX-II forms (Figure 1(a)).

Immunodiffusion and immunoelectrophoresis assays carried out with BthTX-I and BthTX-II (Figures 1(b)–1(e)) showed that a sufficient amount of antibody was produced after a 6-week period, after administration of the first dose of the antigen to the rabbits. This was verified by precipitation lines formed between native toxins (antigens) and their correspondent antibodies.

The enzyme immunoassays demonstrated that rabbit serum obtained after immunization with BjussuCV, BthTX-I, BthTX-II, alk-BjussuCV, alk-BthTX-I, and alk-BthTX-II contained antibodies that recognized the native and modified protein. The titration curves of antibodies against native and modified BthTX-I and BthTX-II revealed a moderate similarity. The lethality (LD_{50}) and phospholipasic activity of native or modified protein were significantly reduced after alkylation (Table 1).

The enzymatic activity of PLA_2 is dependent on the presence of a His residue in position 48. BthTX-I did not show enzymatic activity because this protein has a Lys in this position, thus removing the activity of enzyme. Neither BthTX-I nor alk-BthTX-I showed PLA_2 activity. In addition, as expected, alk-BjussuCV and alk-BthTX-II suppressed the PLA_2 activity of this enzyme.

We found that alk-BthTX-I and alk-BthTX-II reduce the lethal dose (LD_{50}) relative to that of BthTX-I and BthTX-II. However, the alkylation of the crude venom did not cause a reduction in mortality relative to the native form. This observation could be attributable to the presence of other toxic proteins present in crude venom, such as metalloproteases and serinoproteases (Table 1).

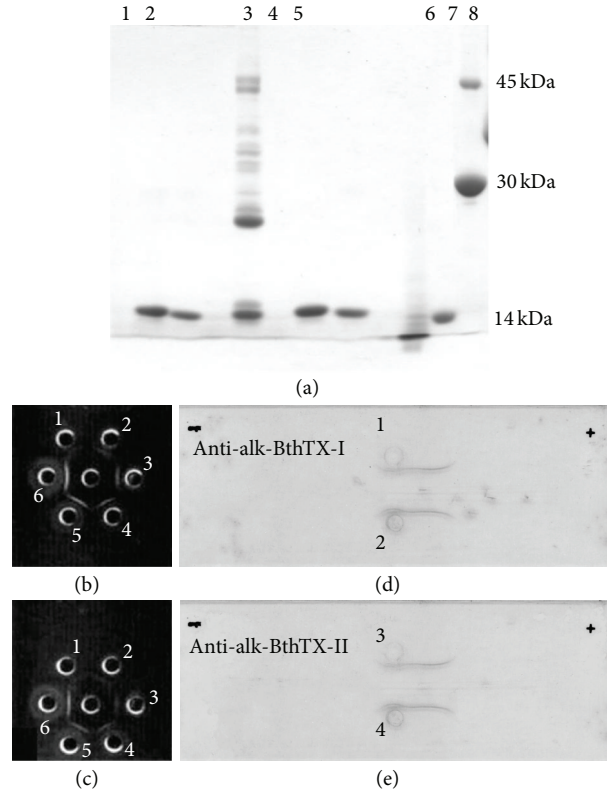


Figure 1: Electrophoretic and immunochemical analysis of the antigen-antibody reaction. (a) SDS-PAGE with reducing agents: lane 1, BthTX-I; lane 2, BthTX-II; lane 3, BjussuCV; lane 4, alk-BthTX-I; lane 5, alk-BthTX-II; lane 6, aprotinin (9 kDa); lane 7, α-lactalbumin (14 kDa); lane 8, carbonic anhydrase (30 kDa), and albumin egg (45 kDa). Antigen immunodiffusion: (b) native BthTX-I (central well) against its native anti-BthTX-I (1, 2, and 3 wells) and BPB-modified (4, 5, and 6 wells) antibodies; (c) native BthTX-II (central well) against its native anti-BthTX-II (1, 2, and 3 wells), and BPB-modified (4, 5, and 6 wells) antibodies. The precipitation lines were achieved using immunodiffusion gel in a wet chamber at 25°C for 48 h. Antigen immunoelectrophoresis: (d) anti-alk-BthTX-I (anti-BthTX-I-BPB) (central well) against its BthTX-I (1) and alk-BthTX-I (2) antigens; (e) anti-alk-BthTX-II (anti-BthTX-II-BPB) antibodies (central well) against its BthTX-II (3) and alk-BthTX-II (4) antigens. Results are representative of experiments carried out in triplicate.

Our results demonstrate that the alkylated PLA_2 has the capacity to produce antibody. To verify the efficiency of these polyclonal antibodies produced to recognize the venoms of snakes belonging to the genus *Bothrops spp.*, we purified these antibodies: anti-BjussuCV, anti-alk-BjussuCV, anti-BthTX-I, anti-BthTX-II, anti-alk-BthTX-I, and anti-alk-BthTX-II. They were then incubated (30 min at 37°C) with total venom before administration. In addition, we also extended our studies to check whether the polyclonal antibodies had the capacity to recognize the venoms of *Crotalus spp.* and *Micrurus spp.* (Figures 2(a)–2(c)). To increase the range of our results, we also analyzed the capacity of the polyclonal antibodies to recognize PLA_2s isolated from a variety of venoms. The antibodies were incubated with PLA_2 before inoculation (30 min at 37°C) (Figures 3(a) and 3(b)). All

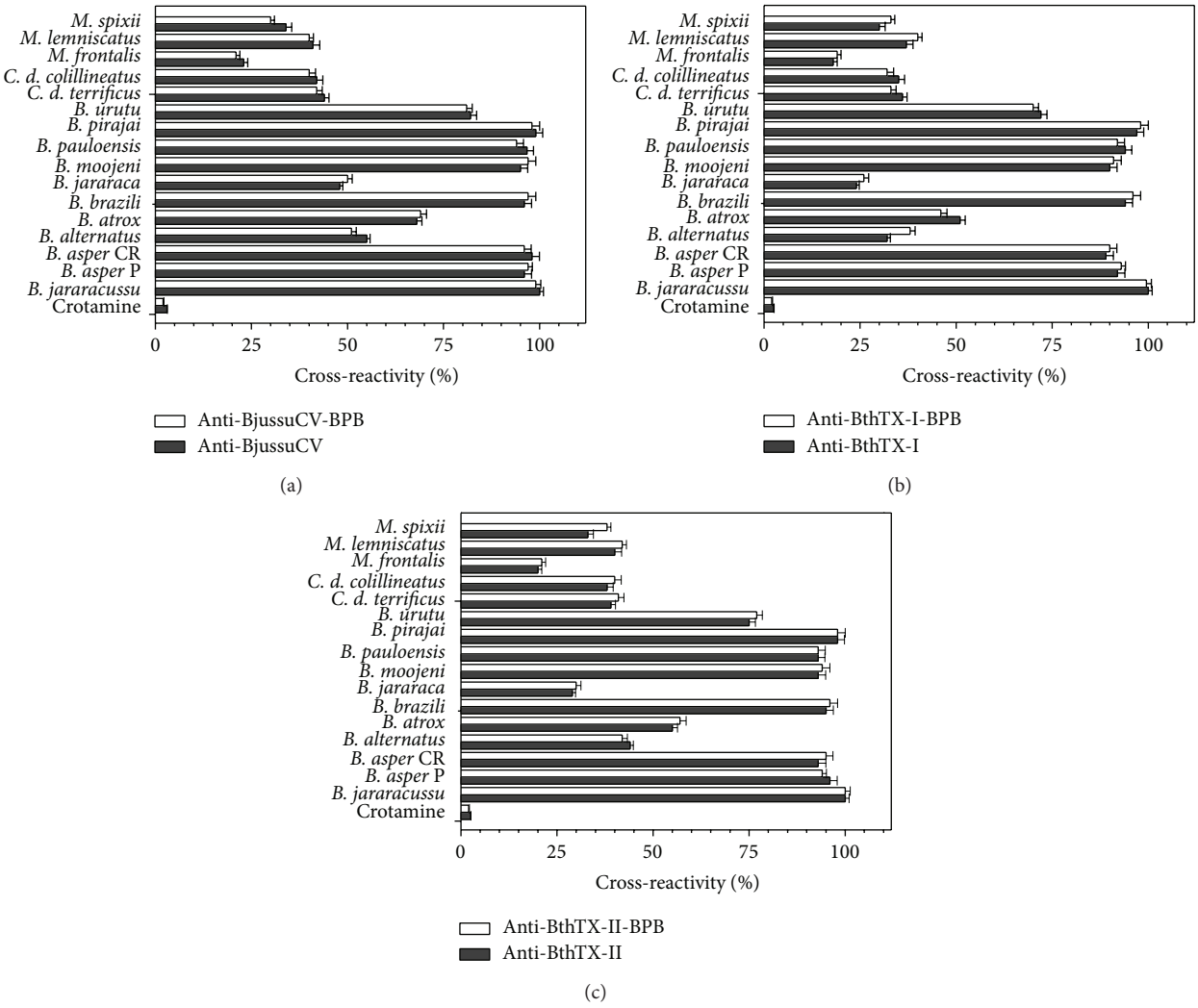


Figure 2: Cross-reactivity of antibodies produced from native or alkylated proteins from *B. jararacussu* and BthTX-I and BthTX-II (anti-BjussuCV, anti-alk-BjussuCV, anti-BthTX-I, anti-BthTX-I or anti-BthTX-II, and anti-alk-BthTX-II) against snake venoms of the *Bothrops*, *Crotalus,* and *Micrurus* genera, as shown by enzyme immunoassay. Microplate wells were coated with antigen, and antibody binding was measured, as described in Section 2.5.2. Cross-reactivity was expressed as a percentage of the absorbance signal resulting from the binding of antibodies to the homologous antigen ((a) *B. jararacussu* crude venom; (b) BthTX-I; (c) BthTX-II). Crotamine was included as an antigenically unrelated, negative control antigen. Results expressed as means ± S.D. ($n = 3$).

enzymes and myotoxins were isolated as previously reported [2–4, 6, 21, 30, 45, 46], and PLA_2 from *Apis mellifera* was purchase from the Sigma-Aldrich (PN P9279, CAS 9001-84-7, Missouri, USA).

Myotoxic activities of BjussuCV were reduced after incubation with anti-BjussuCV serum. The myotoxic activity of the isolated toxins was also reduced by the antibodies produced against native and modified toxins (anti-BthTX-I, anti-BthTX-II, anti-alk-BthTX-I, and anti-alk-BthTX-II), as well as crude venom (anti-BjussuCV and anti-alk-BjussuCV). The phospholipasic activity of BjussuCV decreased after incubation with anti-BjussuCV serum and, to a lesser extent, with anti-alk-BjussuCV serum (Table 2).

All antibodies neutralized the lethality of the venoms. No mouse died when treated with anti-BjussuCV, anti-alk-BjussuCV, anti-BthTX-I, or anti-BthTX-II, and the mortality was reduced by around 83% in response to anti-alk-BthTX-I and anti-alk-BthTX-II.

Only 15% of the edema-inducing activity was reduced in the presence of anti-BthTX-II, while anti-BthTX-I did not induce any significant reduction in this activity (only 5%), thereby indicating that the site responsible for myotoxicity (Figure 4) differs from that responsible for edema-inducing activity. Experiments addressing the phospholipasic activity showed that antibodies against the native and alkylated venom neutralized about 80% and 50% of the phospholipasic activity, respectively (Table 1).

The myotoxic effect of the crude venom or myotoxins, with or without incubation with antibodies, was estimated by the levels of CK activity in plasma 3 h after injection of the samples. Both native and alkylated antibodies reduced the myotoxic activity induced by the crude venom or isolated

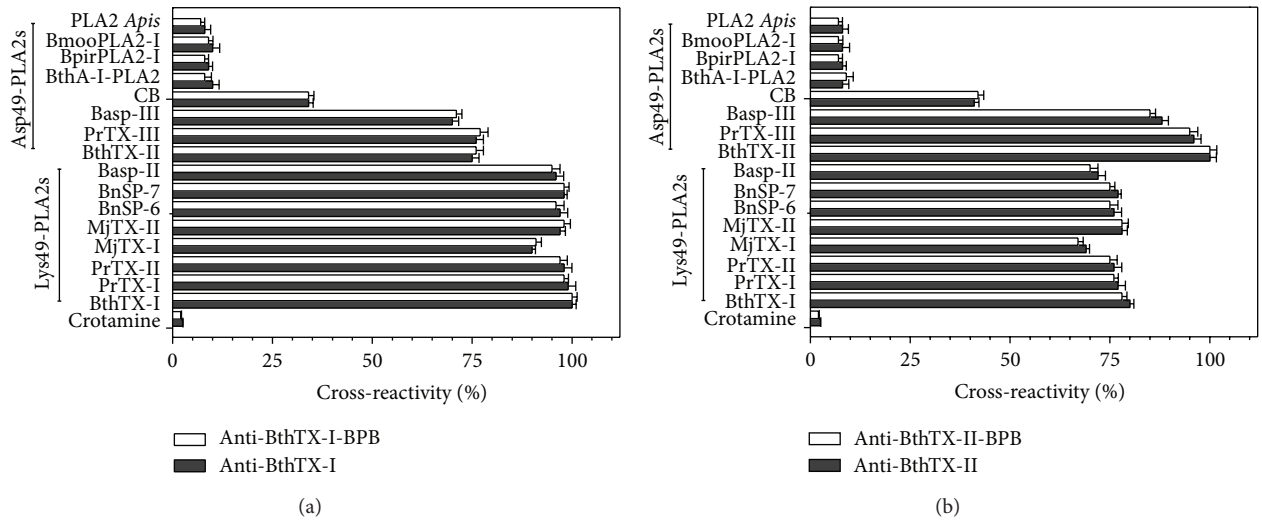


Figure 3: Cross-reactivity of antibodies produced from native or alkylated proteins BthTX-I and BthTX-II (anti-BthTX-I, anti-alk-BthTX-I, anti-BthTX-II, and anti-alk-BthTX-II) against other isolated PLA_2s, as shown by enzyme immunoassay. Lys49-PLA_2 homologs (*Bothrops jararacussu* BthTX-I, *B. pirajai* PrTX-I and II, *B. moojeni* MjTX-I and II, *B. pauloensis* BnSP-6 and 7, and *B. asper* Basp-II), basic Asp49-PLA_2s (*B. jararacussu* BthTX-II, *B. pirajai* PrTX-III, *B. asper* Basp-III, and *Crotalus d. terrificus* CB), acidic Asp49-PLA_2s (*B. jararacussu* BthA-I-PLA_2, *B. pirajai* BpirPLA$_2$-I, and *B. moojeni* BmooPLA$_2$-I), and PLA_2 from *Apis mellifera* venom [2–4, 6, 21, 30, 45, 46]. Microplate wells were coated with antigen, and antibody binding was measured, as described in Section 2.5.2. Cross-reactivity was expressed as a percentage of the absorbance signal resulting from the binding of antibodies to the homologous antigen BthTX-I or -II. Crotamine was included as an unrelated, negative control antigen. Results expressed as means ± S.D. ($n = 3$).

Table 2: Neutralization of edema, PLA_2 activity, and 2x LD_{50} induced by the respective antibodies of native and modified forms of *B. jararacussu* crude venom and isolated toxins.

Venom or isolated PLA_2	Neutralization of 2 × LD_{50}* Antibodies		Neutralization of edema Antibodies	Neutralization of PLA_2 activity Antibodies	
	Native	Alkylated	Native	Native	Alkylated
BjussuCV	100%	100%	30%	80%	50%
BthTX-I	100%	83%	5%	—	—
BthTX-II	100%	83%	15%	55%	25%

*LD_{50} assay was done after previous incubation of the venom or toxin with its respective antibody (1 : 50, w/w) for 30 min at 37°C. Results express the percentage of animal survival.

PLA_2 (Figure 4(a)). Furthermore, the inhibition of the myotoxicity induced by the toxins was clearly observed after histopathological analysis of the mouse gastrocnemius muscle sections obtained 24 h after injection of PBS (Figure 4(d)), 50 μg of PLA_2 (BthTX-I or BthTX-II) (Figure 4(b)) or PLA_2 previously incubated with the antibodies (Figure 4(c)).

SPR data showed a loss of the affinity [39] for anti-BthTX-I/BPB in comparison with the native anti-BthTX-I for interaction with BthTX-I (Figure 5(a)) but anti-BthTX-I/BPB showed high affinity for immobilized BthTX-II when compared with native anti-BthTX-I (Figure 5(b)) [44]. These results reinforce the antigenicity results revealed in the ELISA assays.

4. Discussion and Conclusion

The design of immunization experiments varies depending on the origin of the antigen and the method of inoculation. Here, we immunized Swiss mice through repeated subcutaneous injections of a fixed dose of the antigen with an interval of 15 days. At the end of 6 weeks, "proof bleeding" was done, and an immunodiffusion test was performed to check the presence of antibodies. The final bleed was then carried out. Immunoglobulin G was initially purified by precipitation of the total globulin fraction and subsequently by ion exchange chromatography on DEAE-Sephacel, leading to a high yield of purified product (Figure 1(a)).

The immunodiffusion test demonstrated that sufficient levels of antibodies were produced after the administration of 2 and 3 doses of the antigen, myotoxins, and crude venom, respectively. Our results show that the toxins, despite modification, did not lose their antigenicity (Figure 1(b)). In an analogous strategy, Soares et al. [28] described similar findings, but only with a basic Lys49-PLA_2-like protein and crude venom from *B. moojeni*.

Immunodiffusion of the toxins and rabbit plasma aliquots demonstrated the formation of precipitation lines in all cases. This observation indicates that the covalent modification of

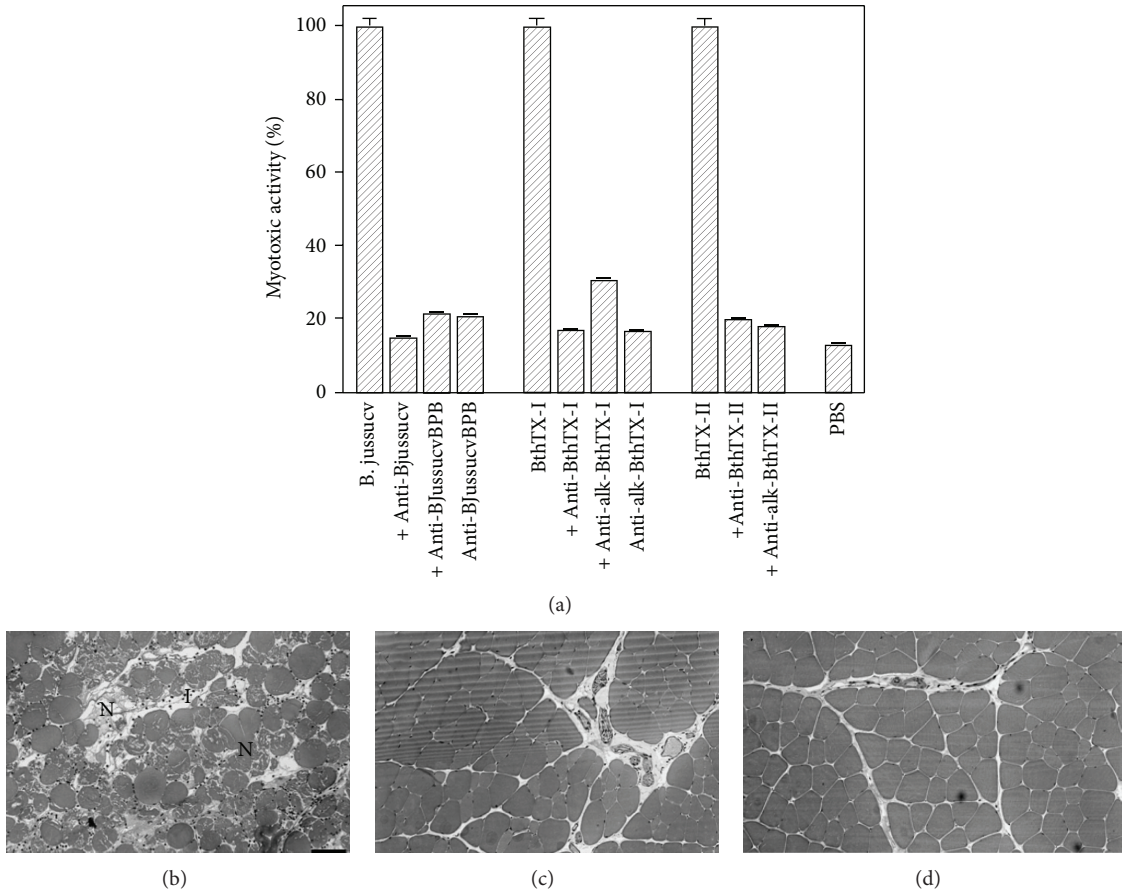


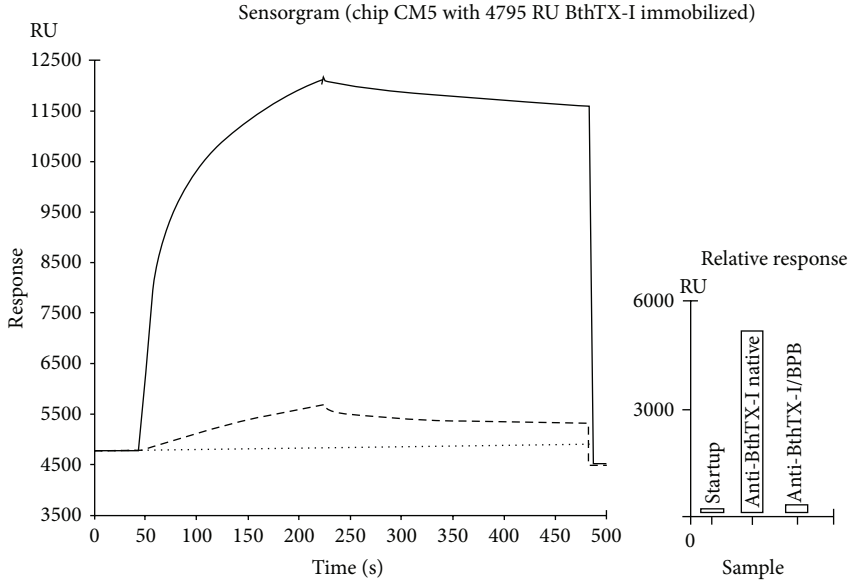
Figure 4: Inhibition of the myotoxic activity by antibodies against native and BPB-modified crude venom and myotoxins. (a) Myotoxic effect of crude venom or isolated PLA_2 alone or following incubation with antibodies (50 : 1 antibody : toxin, w/w) in mice. The values were estimated by the levels of creatine kinase (CK) activity in plasma 3 h after the injection of samples. Phosphate-buffered saline (PBS) was included as a control. Histopathological analysis induced by myotoxins. Light micrographs of sections of mouse gastrocnemius muscle 24 h after injection of 50 μg of myotoxins (BthTX-I or BthTX-II) alone (b) or incubated with antibodies (50 : 1 antibody : toxin, w/w), dissolved in 50 μL PBS, and stained with hematoxylin and eosin. (c) Control mice were injected with PBS alone; (d) normal integer fibers are seen. Note the presence of myonecrosis (N) and an abundant inflammatory infiltrate (I).

the His48 residue did not interfere with the structure of the proteins, thereby not diminishing their antigenicity. The same experiment was carried out with the antibodies against native and alkylated crude venom (Figure 1(c)). Immunoassays (ELISA) were performed to evaluate the specificity of the antibodies for their respective antigens. The antibodies recognized the BthTX-I and -II proteins (PLA_2) indistinctly. For the antibodies against BjussuCV, venoms from snakes of the Crotalidae (*B. moojeni, B. asper, B. neuwiedi, B. pirajai,* and *C. d. terrificus*) and Elapidae (*Micrurus frontalis*) families were used to test cross-reactivity. Results showed a cross-reactivity of about 100% for Crotalidae venoms, while, for Elapidae venoms, this cross-reactivity was about 70%. De Roodt et al. [49] reported that the high cross-reactivity found between venoms and various antivenins supports the use of antigens with common epitopes to induce the production of antibodies with neutralizing potential against various toxic proteins.

These results are consistent with data of the cross-reaction obtained with antivenoms produced against Crotalidae venoms, which neutralize several biological activities of other venoms belonging to snakes of the same or another family (Viperidae) [50]. This cross-reactivity occurs as a result of the high degree of similarity in the structure of homologous proteins, such as the PLA_2s of taxonomically related species [51].

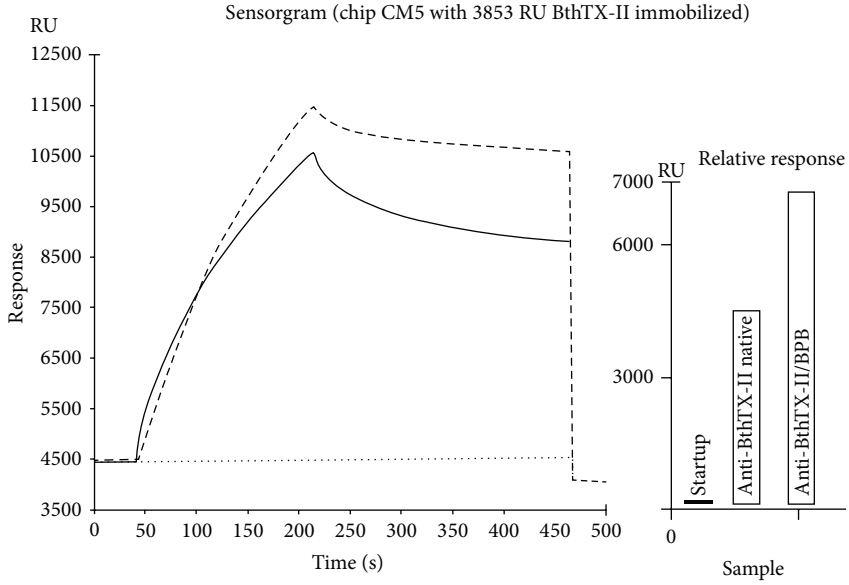
Studies show that a "family" of antigenically-related myotoxic PLA_2 occurs in many types of venom of the *Bothrops* genus (Figure 4). Conservation of at least some epitopes in several PLA_2s, which is in accordance with their high homology, both in amino acid sequences and tridimensional structure, was also discussed [2, 28, 52]. Furthermore, SPR data (Figure 5) revealed the conservation of antigenicity; if, on one hand, anti-BthTX-I/BPB loses affinity for immobilized BthTX-I, the same anti-BthTX-I/BPB gains affinity for immobilized BthTX-II. These findings were verified by an affinity curve and by sum of mass for protein immobilized affinity [53].

The lethality assay with native and alkylated BjussuCV antivenoms, capable of neutralizing $2\times LD_{50}$, resulted in 100% survival of the animals (Table 2). The antibodies recognized the antigens and completely neutralized their activity. In



Sample	RU (rel)
Anti-BthTX-I native ——	5476
Anti-BthTX-I/BPB – – –	232
Startup	0

(a)



Sample	RU (rel)
Anti-BthTX-II native ——	5676
Anti-BthTX-II/BPB – – –	6876
Startup	0

(b)

Figure 5: SPR showing protein interactions with immobilized BthTX-I (a) and BthTX-II (b) assay with native antibodies and antibodies chemically alkylated by *p*-bromophenacyl bromide (BPB). Concerning antibodies for the native and alkylated BthTX-I and BthTX-II, the assay was conducted at a flow rate of 30 μL/min for 1 min at 37°C. These samples were carried with HBS-P 1X Buffer (GE Healthcare, USA) pH 7.4.

contrast, the antibodies against alkylated BthTX-I and -II led only to partial neutralization and only 17% of the animals survived. These results suggest a low affinity between antigen and antibody and the need to increase the amount of antibody to achieve greater neutralization or even a loss of antigenic response.

For antibodies against the native and alkylated venom, inhibition assays were performed to examine the phospholipasic, edema-inducing, and myotoxic activities. Antibodies produced against the alkylated venom showed high inhibitory activity (50–100%) against PLA_2 activities, resulting in inhibition values closer to those obtained with the antibodies against the native crude venom. These results indicate that the loss of enzymatic activity of some venom toxins does not affect their immunogenicity and point to the possibility to produce identical antibodies against native and alkylated venoms or even different antibodies able to recognize the epitopes of the native and alkylated toxins.

Another experiment demonstrated that the antibodies against the native venom inhibited about 15% of edema-inducing activity. This finding corroborates the results of previous studies that reported the inefficiency of antivenom in inhibiting this activity [54, 55] since the victim's organism reacts against unknown molecules, leading to an inflammatory response. Local tissue damage (myonecrosis, dermonecrosis, and edema) is a serious consequence of the envenomation by snakes of the genus *Bothrops*. The animals used to produce anti-serum present the same damage at the sites of injections [2–5]. The pathogenesis of these alterations is complex, since these activities are induced by several toxins present in the venom [56, 57].

We performed PLA_2 activity assays only with the catalytically active isoform (BthTX-II-D49PLA_2). This activity was neutralized by about 50% by antibodies against the native toxin and by about 15% by antibodies against alkylated PLA_2. Lomonte et al. [58] demonstrated the capacity of antibodies against *B. asper* myotoxin to neutralize the phospholipase activity of myotoxin I of the same venom. This cross neutralization shows that there are conserved epitopes of relevance to the structure of these PLA_2 isoforms. We observed that neutralization of the myotoxic activity induced by the native venom, as determined by the CK levels, was about 80% for all antibodies raised against both BthTX-I and II. These results show that the basic myotoxins are key factors responsible for the toxicity of *B. jararacussu* venom [27].

Several structural studies have reported that only the His48 residue is chemically modified by BPB [23, 27, 28, 55]. This alkylation occurs in the imidazole ring (Nδ1 atom) of the His48 residue present in the active site and promotes the loss of PLA_2 enzymatic activity and/or reduction of the toxic and pharmacological effects of this molecule [23]. These findings suggest that these pharmacological effects and the catalytic activity are dependent on His48. The Lys49-PLA_2s are bounded to BPB and showed a decrease in myotoxic activity of around 40–50%, a decrease in cytotoxicity of around 85–90%, and a 15–20% decrease in capacity to induce edema. Also, Lys49-PLA_2s are bounded to BPB and significantly reduces mortality, without any significant change in the liposome-disrupting capacity, thereby also suggesting a dependence of these pharmacological effects and the "active site" of these proteins [59].

In the case of Lys49-PLA_2s, several studies have identified the C-terminal region as responsible for their cytotoxicity and other toxicological activities [60]. A specific myotoxic site formed by the Lys20, Lys115, and Arg118 residues was proposed for snakes of the *Bothrops* genus [61]. The preliminary crystal structure of BthTX-I chemically modified by BPB suggests that the binding of the inhibitor leads to significant structural modifications, especially in the C-terminal region [62].

In this structure, the phenacyl group of the BPB molecule extends along the hydrophobic channel of the protein, interacting with Tyr22, Gly23, Val31, Cys45, and Lys49 residues (Figure 6).

Recently, the structure of Lys49-PLA_2, following PrTX-I chemical modification by BPB, was resolved by X-ray crystallography [59]. This protein shares 98% similarity with BthTX-I and thus could be an interesting model for the BthTX-I-BPB structure. BPB is a small molecule that covalently binds to the His48 residue but retains the capacity to distort the Ca^{2+}-binding loop region, rearranging the C-terminus as a result of the establishment of a new interchain hydrogen bond between the Tyr119 residues [60]. This rearrangement of the C-terminus induces an alignment between the monomers, changing the quaternary conformation of the PrTX-I/BPB structure [60, 62]. These data, as previously observed Nomura et al. [32], suggest the presence of two distinct binding regions of Lys49-PLA_2s on membranes: the "active site/hydrophobic channel" and the "C-terminal/myotoxic site."

Our results indicate that the chemical modification of the myotoxins attenuate their toxic potential but do not change their antigenicity. For the native venom, the toxicity was partially reduced and the antigenicity remained unaltered when the venom was modified with BPB.

Taken together, these data demonstrate the high potential of the use of chemical modification to reduce venom toxicity [63]. These studies provide an alternative protocol for increasing the amount of venom used to vaccinate horses in order to obtain higher antibody titers in the antiophidic serum. These toxins are the main factors responsible for local damage in bothropic envenomation. Thus, it may be possible to increase the titer of antimyotoxin antibodies in antiophidic serum without major complications in the immunized animals.

Abbreviations

PLA_2:	Phospholipase A_2
BjussuCV:	*Bothrops jararacussu* crude venom
BthTX-I:	Phospholipase A_2 Lys49 from *B. jararacussu* venom
BthTX-II:	Phospholipase A_2 Asp49 from *B. jararacussu* venom
BPB:	*p*-Bromophenacyl bromide
Anti-BjussuCV:	Antibodies against *B. jararacussu* crude venom
Anti-BthTX-I:	Antibodies against *B. jararacussu* myotoxin BthTX-I

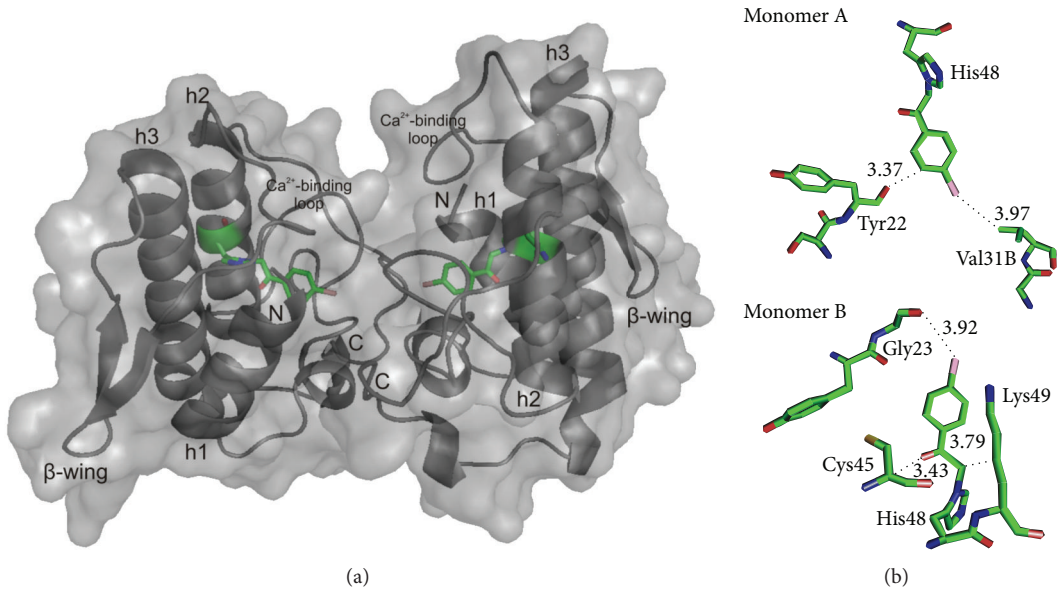


Figure 6: Preliminary crystal structure of BthTX-I chemically modified by *p*-bromophenacyl bromide (BPB). (a) Ribbon representation of BthTX-I/BPB structure with the secondary structure elements marked: the Ca^{2+}-binding loop (25 to 34), β-wing (75 to 84), and the three α-helices—"h1" (1 to 12), "h2" (40 to 58), and "h3" (89 to 110). The *p*-bromophenacyl bromide molecules are highlighted as green sticks. (b) Top view of BPB covalently bound to the His48 residue of the "active site" region of monomers A and B of the BthTX-I/BPB complex. Interactions of BPB with the protein residues are indicated. The sequences have been numbered following Renetseder and coauthors [47]. Molecules were drawn using the PyMOL program [48].

Anti-BthTX-II:	Antibodies against *B. jararacussu* myotoxin BthTX-II
Anti-BjussuCV-BPB:	Antibodies against *B. jararacussu* BPB modified crude venom
Anti-BthTX-I-BPB:	Antibodies against *B. jararacussu* myotoxin BPB modified BthTX-I
Anti-BthTX-II-BPB:	Antibodies against *B. jararacussu* myotoxin BPB modified BthTX-II.

Conflict of Interests

The authors assure and declare that there is no any conflict of interests in the research in this paper.

Acknowledgments

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Rede de Biodiversidade e Biotecnologia da Amazônia Legal (BIONORTE/CNPq/MCT), Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT-INPeTAm/CNPq/MCT), Instituto Nacional de Ciência e Tecnologia em Toxinas (INCT-Tox), Coordenação de Aperfeiçoamento de Nível Superior (CAPES, NanoBiotec), Instituto Brasileiro do Meio ambiente e dos Recursos Naturais renováveis (IBAMA), Secretary of Development of Rondonia State (SEPLAN-RO/CNPq), Institute for Research in Biomedicine (IRB) (Barcelona, Spain), and Parc Científic de Barcelona (Universidade de Barcelona, Spain).

References

[1] Health Ministry, 2005, http://portal.saude.gov.br/portal/arquivos/pdf/situacao.pdf.

[2] J. Gutiérrez and B. Lomonte, "Phospholipase A_2 myotoxins from *Bothrops* snake venoms," *Toxicon*, vol. 33, no. 11, pp. 1405–1424, 1995.

[3] A. M. Soares, M. R. M. Fontes, and J. R. Giglio, "Phospholipase A_2 myotoxins from Bothrops snake venoms: structure-function relationship," *Current Organic Chemistry*, vol. 8, no. 17, pp. 1677–1690, 2004.

[4] C. L. Ownby, H. S. Selistre De Araujo, S. P. White, and J. E. Fletcher, "Lysine 49 phospholipase A_2 proteins," *Toxicon*, vol. 37, no. 3, pp. 411–445, 1999.

[5] S. Lizano, G. Domont, and J. Perales, "Natural phospholipase A_2 myotoxin inhibitor proteins from snakes, mammals and plants," *Toxicon*, vol. 42, no. 8, pp. 963–977, 2003.

[6] Z. Mallat, G. Lambeau, and A. Tedgui, "Lipoprotein-associated and secreted phospholipases A_2 in cardiovascular disease: roles as biological effectors and biomarkers," *Circulation*, vol. 122, no. 21, pp. 2183–2200, 2010.

[7] D. De Luca, A. Minucci, P. Cogo et al., "Secretory phospholipase A_2 pathway during pediatric acute respiratory distress syndrome: A Preliminary Study," *Pediatric Critical Care Medicine*, vol. 12, no. 1, pp. e20–e24, 2011.

[8] R. Ezzeddini, M. Darabi, B. Ghasemi et al., "Circulating phospholipase-A_2 activity in obstructive sleep apnea and recurrent tonsillitis," *International Journal of Pediatric Otorhinolaryngology*, vol. 76, no. 4, pp. 471–474, 2012.

[9] S. Chalbot, H. Zetterberg, K. Blennow et al., "Blood-cerebrospinal fluid barrier permeability in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 25, no. 3, pp. 505–515, 2011.

[10] S. Marcussi, P. R. S. Santos, D. L. Menaldo et al., "Evaluation of the genotoxicity of Crotalus durissus terrificus snake venom and its isolated toxins on human lymphocytes," *Mutation Research—Genetic Toxicology and Environmental Mutagenesis*, vol. 724, no. 1-2, pp. 59–63, 2011.

[11] D. C. S. Damico, M. A. Da Cruz Höfling, M. Cintra et al., "Pharmacological study of edema and myonecrosis in mice induced by venom of the bushmaster snake (*Lachesis muta muta*) and its basic Asp49 phospholipase A_2 (LmTX-I)," *Protein Journal*, vol. 27, no. 6, pp. 384–391, 2008.

[12] S. L. Da Silva, C. A. Dias-Júnior, P. Baldasso et al., "Vascular effects and electrolyte homeostasis of the natriuretic peptide isolated from *Crotalus oreganus abyssus* (North American Grand Canyon rattlesnake) venom," *Peptides*, vol. 36, pp. 206–212, 2012.

[13] L. B. Silveira, D. P. Marchi-Salvador, N. A. Santos-Filho et al., "Isolation and expression of a hypotensive and anti-platelet acidic phospholipase A_2 from *Bothrops moojeni* snake venom," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 73, pp. 35–43, 2013.

[14] L. Romero, S. Marcussi, D. P. Marchi-Salvador et al., "Enzymatic and structural characterization of a basic phospholipase A_2 from the sea anemone Condylactis gigantea," *Biochimie*, vol. 92, no. 8, pp. 1063–1071, 2010.

[15] S. S. Teixeira, L. B. Silveira, F. M. N. Da Silva et al., "Molecular characterization of an acidic phospholipase A_2 from Bothrops pirajai snake venom: synthetic C-terminal peptide identifies its antiplatelet region," *Archives of Toxicology*, vol. 85, no. 10, pp. 1219–1233, 2011.

[16] C. Z. Oliveira, N. A. Santos-Filho, D. L. Menaldo et al., "Structural and functional characterization of a γ-type phospholipase A_2 inhibitor from *Bothrops jararacussu* Snake Plasma," *Current Topics in Medicinal Chemistry*, vol. 11, no. 20, pp. 2509–2519, 2011.

[17] J. A. F. P. Villar, F. T. D. Lima, C. L. Veber et al., "Synthesis and evaluation of nitrostyrene derivative compounds, new snake venom phospholipase A_2 inhibitors," *Toxicon*, vol. 51, no. 8, pp. 1467–1478, 2008.

[18] A. A. Farooqui, M. L. Litsky, T. Farooqui, and L. A. Horrocks, "Inhibitors of intracellular phospholipase A_2 activity: their neurochemical effects and therapeutical importance for neurological disorders," *Brain Research Bulletin*, vol. 49, no. 3, pp. 139–153, 1999.

[19] M. E. S. Martins-Santos, R. R. Resende, F. C. H. Pinto et al., "Effect of a pool of peptides isolated from crotalus durissus terrificus (South American Rattlesnake) venom on glucose levels of mice fed on a high-fat diet," *International Journal of Peptide Research and Therapeutics*, vol. 17, no. 3, pp. 225–230, 2011.

[20] S. L. Da Silva, J. R. Almeida, L. M. Resende et al., "Isolation and characterization of a natriuretic peptide from *Crotalus oreganus* abyssus (grand canyon rattlesnake) and its effects on systemic blood pressure and nitrite levels," *International Journal of Peptide Research and Therapeutics*, vol. 17, no. 3, pp. 165–173, 2011.

[21] S. Marcussi, C. D. Sant'Ana, C. Z. Oliveira et al., "Snake venom phospholipase A_2 inhibitors: medicinal chemistry and therapeutic potential," *Current Topics in Medicinal Chemistry*, vol. 7, no. 8, pp. 743–756, 2007.

[22] R. H. Schaloske and E. A. Dennis, "The phospholipase A_2 superfamily and its group numbering system," *Biochimica et Biophysica Acta*, vol. 1761, no. 11, pp. 1246–1259, 2006.

[23] A. M. Soares and J. R. Giglio, "Chemical modifications of phospholipases A_2 from snake venoms: effects on catalytic and pharmacological properties," *Toxicon*, vol. 42, no. 8, pp. 855–868, 2003.

[24] R. D. Theakston and A. S. Kamiguti, "Viper envenoming: evaluation of treatment by restoration of haemostasis and venom clearance," *Journal Venom Animal Toxins*, vol. 4, pp. 94–111, 1998.

[25] WHO, *Progress in the Characterization of Venoms and Standardization of AntiVenoms*, World Health Organization, Geneva, Switzerland, 1981.

[26] G. León, R. Estrada, F. Chaves, G. Rojas, M. Ovadia, and J. M. Gutiérrez, "Inhibition by $CaNa_2$ EDTA of local tissue damage induced by *Bothrops asper* (terciopelo) venom: application in horse immunization for antivenom production," *Toxicon*, vol. 36, no. 2, pp. 321–331, 1998.

[27] S. H. Andrião-Escarso, A. M. Soares, V. M. Rodrigues et al., "Myotoxic phospholipases A_2 in Bothrops snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*," *Biochimie*, vol. 82, no. 8, pp. 755–763, 2000.

[28] A. M. Soares, W. P. Sestito, S. Marcussi et al., "Alkylation of myotoxic phospholipases A_2 in Bothrops moojeni venom: a promising approach to an enhanced antivenom production," *International Journal of Biochemistry and Cell Biology*, vol. 36, no. 2, pp. 258–270, 2004.

[29] D. H. Campbell, J. S. Garvey, N. E. Cremer et al., *Methods in Immunology*, vol. 260 of *Edited by W. A. Denjamin*, 2nd edition, 1970.

[30] A. M. Soares, R. Guerra-Sá, C. R. Borja-Oliveira et al., "Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A_2 homologue from *Bothrops neuwiedi pauloensis* venom," *Archives of Biochemistry and Biophysics*, vol. 378, no. 2, pp. 201–209, 2000.

[31] J. A. F. P. Villar, F. T. D. Lima, C. L. Veber et al., "Synthesis and evaluation of nitrostyrene derivative compounds, new snake venom phospholipase A_2 inhibitors," *Toxicon*, vol. 51, no. 8, pp. 1467–1478, 2008.

[32] R. C. S. Nunomura, V. G. Oliveira, S. L. Da Silva, and S. M. Nunomura, "Characterization of bergenin in endopleura uchi bark and its anti-inflammatory activity," *Journal of the Brazilian Chemical Society*, vol. 20, no. 6, pp. 1060–1064, 2009.

[33] M. L. Da Silva, S. Marcussi, R. S. Fernandes et al., "Anti-snake venom activities of extracts and fractions from callus cultures of *Sapindus saponaria*," *Pharmaceutical Biology*, vol. 50, no. 3, pp. 366–375, 2012.

[34] E. S. de Alvarenga, S. A. Silva, L. C. A. Barosa et al., "Synthesis and evaluation of sesquiterpene lactone inhibitors of phospholipase A_2 from *Bothrops jararacussu*," *Toxicon*, vol. 57, no. 1, pp. 100–108, 2011.

[35] S. L. da Silva, A. K. Calgarotto, V. Maso et al., "Molecular modeling and inhibition of phospholipase A_2 by polyhydroxy phenolic compounds," *European Journal of Medicinal Chemistry*, vol. 44, no. 1, pp. 312–321, 2009.

[36] A. D. L. Souza, E. Rodrigues-Filho, A. Q. L. Souza et al., "Koninginins, phospholipase A_2 inhibitors from endophytic fungus *Trichoderma koningii*," *Toxicon*, vol. 51, no. 2, pp. 240–250, 2008.

[37] S. L. Da Silva, A. K. Calgarotto, J. S. Chaar, and S. Marangoni, "Isolation and characterization of ellagic acid derivatives isolated from *Casearia sylvestris* SW aqueous extract with anti-PLA_2 activity," *Toxicon*, vol. 52, no. 6, pp. 655–666, 2008.

[38] C. Weil, *Tables for Convenient Calculation of Median Affective Dose (DL_{50} Or ED_{50}) and Instruction in Their Use*, Mellan Institute, Pittsburgh, PC, USA, 1952.

[39] T. Nikai, N. Mori, M. Kishida et al., "Isolation and biochemical characterization of hemorrhagic toxin f from the venom of *Crotalus atrox* (Western Diamondback Rattlesnake)," *Archives of Biochemistry and Biophysics*, vol. 231, no. 2, pp. 309–319, 1984.

[40] J. M. Gutierrez, C. Avila, E. Rojas, and L. Cerdas, "An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica," *Toxicon*, vol. 26, no. 4, pp. 411–413, 1988.

[41] G. H. de Haas, N. M. Postema, W. Nieuwenhuizen, and L. L. M. van Deenen, "Purification and properties of phospholipase a from porcine pancreas," *Biochimica et Biophysica Acta*, vol. 159, no. 1, pp. 103–117, 1968.

[42] J. Alvarado and J. M. Gutiérrez, "Anticoagulant effect of myotoxic phospholipase A_2 isolated from the venom of the snake *Bothrops asper* (Viperidae)," *Revista de Biologia Tropical*, vol. 36, no. 2 B, pp. 563–565, 1988.

[43] L. Jason-Muller, M. Murphy, and J. Bruno, "Overview of Biacore Systems and their applications," *Current Protocols Protein Science*, vol. 45, pp. 1–14, 2006.

[44] R. L. Rich, D. G. Myszka, and J. Biacore, "A new platform for routine biomolecular interaction analysis," *Journal of Molecular Recognition*, vol. 14, no. 4, pp. 223–228, 2001.

[45] A. M. Soares, A. C. Mancin, A. L. Cecchini et al., "Effects of chemical modifications of crotoxin B, the phospholipase A_2 subunit of crotoxin from *Crotalus durissus* terrificus snake venom, on its enzymatic and pharmacological activities," *International Journal of Biochemistry and Cell Biology*, vol. 33, no. 9, pp. 877–888, 2001.

[46] A. M. Soares, S. H. Andrião-Escarso, R. K. Bortoleto et al., "Dissociation of enzymatic and pharmacological properties of piratoxins-I and -III, two myotoxic phospholipases A_2 from *Bothrops pirajai* snake venom," *Archives of Biochemistry and Biophysics*, vol. 387, no. 2, pp. 188–196, 2001.

[47] R. Renetseder, S. Brunie, and B. W. Dijkstra, "A comparison of the crystal structure of phospholipase A_2 from bovine pancreas and *Crotalus atrox* venom," *Journal of Biological Chemistry*, vol. 260, no. 21, pp. 11627–11634, 1985.

[48] W. L. DeLano, *The PyMOL Molecular Graphics System*, DeLano Scientific LLC, San Carlos, Calif, USA, 2002.

[49] A. R. De Roodt, J. A. Dolab, T. Fernández, L. S. Liliana Segre, and S. E. Hajos, "Cross-reactivity and heterologous neutralization of crotaline antivenoms used in Argentina," *Toxicon*, vol. 36, no. 7, pp. 1025–1038, 1998.

[50] D. Mebs and F. Kornalik, "Studies on cross reactivity of snake venom antisera," *Memorias do Instituto de Butantan*, vol. 51, pp. 127–132, 1989.

[51] R. G. Stábeli, L. M. Pimenta Magalhães, H. S. Selistre-De-Araujo, and E. B. Oliveira, "Antibodies to a fragment of the *Bothrops moojeni* L-amino acid oxidase cross-react with snake venom components unrelated to the parent protein," *Toxicon*, vol. 46, no. 3, pp. 308–317, 2005.

[52] B. Lomonte, G. Leon, Y. Angulo et al., "Neutralization of *Bothrops asper* venom by antibodies, natural products and synthetic drugs: contributions to understanding snakebite envenomings and their treatment," *Toxicon*, vol. 54, pp. 1012–1028, 2009.

[53] D. G. Myska, "Improving biosensors analysis," *Journal of Molecular Recognition*, vol. 12, pp. 279–284, 1999.

[54] B. Lomonte, J. M. Gutierrez, M. Romero, J. Nunez, A. Tarkowski, and L. A. Hanson, "An MTT-based method for the in vivo quantification of myotoxic activity of snake venoms and its neutralization by antibodies," *Journal of Immunological Methods*, vol. 161, no. 2, pp. 231–237, 1993.

[55] A. Rucavado and B. Lomonte, "Neutralization of myonecrosis, hemorrhage, and edema induced by *Bothrops asper* snake venom by homologous and heterologous pre-existing antibodies in mice," *Toxicon*, vol. 34, no. 5, pp. 567–577, 1996.

[56] J. Gutiérrez, G. Rojas, and L. Cerdas, "Ability of a polyvalent antivenom to neutralize the venom of Lachesis Muta Melanocephala, a new Costa Rican subspecies of the bushmaster," *Toxicon*, vol. 25, no. 7, pp. 713–720, 1987.

[57] D. Mebs, S. Pohlmann, and W. Von Tenspolde, "Snake venom hemorrhagins: neutralization by commercial antivenoms," *Toxicon*, vol. 26, no. 5, pp. 453–458, 1988.

[58] B. Lomonte, M. F. Furtado, M. E. Rovira et al., "South American snake venom proteins antigenically related to *Bothrops asper* myotoxins," *Brazilian Journal of Medical and Biological Research*, vol. 23, no. 5, pp. 427–435, 1990.

[59] D. P. Marchi-Salvador, C. A. H. Fernandes, L. B. Silveira, A. M. Soares, and M. R. M. Fontes, "Crystal structure of a phospholipase A_2 homolog complexed with p-bromophenacyl bromide reveals important structural changes associated with the inhibition of myotoxic activity," *Biochimica et Biophysica Acta*, vol. 1794, no. 11, pp. 1583–1590, 2009.

[60] B. Lomonte, Y. Angulo, and E. Moreno, "Synthetic peptides derived from the C-Terminal region of Lys49 phospholipase A_2 homologues from viperidae snake venoms: biomimetic activities and potential applications," *Current Pharmaceutical Design*, vol. 16, no. 28, pp. 3224–3230, 2010.

[61] J. I. dos Santos, A. M. Soares, and M. R. M. Fontes, "Comparative structural studies on Lys49-phospholipases A_2 from *Bothrops* genus reveal their myotoxic site," *Journal of Structural Biology*, vol. 167, no. 2, pp. 106–116, 2009.

[62] D. P. Marchi-Salvador, C. A. H. Fernandes, S. F. Amui, A. M. Soares, and M. R. M. Fontes, "Crystallization and preliminary X-ray diffraction analysis of a myotoxic Lys49-PLA_2 from *Bothrops jararacussu* venom complexed with p-bromophenacyl bromide," *Acta Crystallographica Section F*, vol. 62, no. 6, pp. 600–603, 2006.

[63] H. Oussedik-Oumehdi and F. Laraba-Djebari, "Irradiated Cerastes cerastes venom as a novel tool for immunotherapy," *Immunopharmacology and Immunotoxicology*, vol. 30, no. 1, pp. 37–52, 2008.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 950538, 13 pages
http://dx.doi.org/10.1155/2014/950538

Research Article

Biochemical and Functional Characterization of *Parawixia bistriata* Spider Venom with Potential Proteolytic and Larvicidal Activities

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Received 20 October 2013; Revised 19 February 2014; Accepted 20 February 2014; Published 7 May 2014

Academic Editor: Edward G. Rowan

Toxins purified from the venom of spiders have high potential to be studied pharmacologically and biochemically. These biomolecules may have biotechnological and therapeutic applications. This study aimed to evaluate the protein content of *Parawixia bistriata* venom and functionally characterize its proteins that have potential for biotechnological applications. The crude venom showed no phospholipase, hemorrhagic, or anti-Leishmania activities attesting to low genotoxicity and discrete antifungal activity for *C. albicans*. However the following activities were observed: anticoagulation, edema, myotoxicity and proteolysis on casein, azo-collagen, and fibrinogen. The chromatographic and electrophoretic profiles of the proteins revealed a predominance of acidic, neutral, and polar proteins, highlighting the presence of proteins with high molecular masses. Five fractions were collected using cation exchange chromatography, with the P4 fraction standing out as that of the highest purity. All fractions showed proteolytic activity. The crude venom and fractions P1, P2, and P3 showed larvicidal effects on *A. aegypti*. Fraction P4 showed the presence of a possible metalloprotease (60 kDa) that has high proteolytic activity on azo-collagen and was inhibited by EDTA. The results presented in this study demonstrate the presence of proteins in the venom of *P. bistriata* with potential for biotechnological applications.

1. Introduction

Animal venoms share common characteristics and typically are characterized by complex combinations of proteins and peptides with great structural diversity. Important biochemical, physiological, and pathological tools for the development of new drugs have arisen due to research on animal venoms [1, 2].

In this context, the toxins purified from the venom of spiders have a high potential for pharmacological and biochemical study and may have biomolecules with great therapeutic and biotechnological applicability [3]. However, spider

venoms are even less studied compared to marine organisms, scorpions and snakes, since components of only 174 of the 43,244 species cataloged (approximately 0.4%) have been totally or partially characterized [4, 5].

Regardless of the genus studied, it has been observed that the usual spider venoms are complex mixtures of toxins which cause numerous neurological and biochemical changes in various animals, including mammals [8]. Note also that the biological activity of spider venom is a result of its major constituents: proteins, polypeptides, neurotoxic polyamines, biogenic amines, enzymes, nucleic acids, neurotransmitters, amino acids, and inorganic salts [9–12].

Parawixia bistriata (Araneidae) is a neotropical colonial spider. The spider is found in Central America, the Amazon, and in Northeast and Southeast regions of Brazil and is the only species of the Araneidae family that presents social behavior, communal refuge, and cooperative hunting. Described as harmless to man, many adults of this species form colonies in summer. There are on average about 168 individuals per colony, although the colony size varies depending on habitat type [13, 14].

The crude venom of *P. bistriata* and its chromatographic fractions have been characterized, with some components such as beta-carbolines, polyamines, and phosphatases already having been identified. Diverse biological and pharmacological activities were described, such as insecticidal, anxiolytic, and anticonvulsant activities, reversible inhibition of A and B type monoamine-oxidases, inhibition of GABA and glycine transporters, and increased glutamate uptake [15–22].

The aim of this study included biochemically and functionally characterizing the venom of *Parawixia bistriata* through proteomics and assays measuring enzymatic, pharmacological, biological and toxic activities, contributing to the advancement of toxinology and proteomics, as well as the direct or indirect development of products of medical or scientific interest.

2. Materials and Methods

2.1. Materials. We obtained crude venom from glands of over 1800 *Parawixia bistriata* females that was collected in February 2012 in Ribeirão Preto, São Paulo. The glands were macerated with Milli-Q water in an ice bath and filtered through 0.45 micron filters. The filtrate was centrifuged (5 min at 2,500–5,000 ×g), and the supernatant was collected for lyophilization and maintained at −80°C. The study was authorized by CGEN/CNPq (010627/2011-1) and IBAMA (27131-2).

2.2. Animals. Male Swiss mice (18–20 g) were supplied by the vivarium of the Fiocruz Rondônia and received water and food ad libitum until the time of biological testing. The study was approved by the Committee of Ethics on the Use of Animals in Research (27131-1).

2.3. Functional Characterization

2.3.1. Phospholipase Activity. The phospholipase A_2 activity of the crude venom and the fractions was determined according to the protocol described by Holzer and Mackessy [23], modified for a 96-well plate. 4-Nitro-3-(octanoiloxi) benzoic acid (4N3OBA) was used as a substrate for reading the absorbance at a range of 425 nm, and the activity of phospholipase was determined in a directly proportional manner, establishing for each 0.10 AU (absorbance units) the presence of 25.8 nanomoles of chromophore (3-hydroxy-4-nitrobenzoic acid).

2.3.2. Proteolytic Activity. Protease activity was determined in the presence and absence of 1 mM EDTA by hydrolysis of the azo-collagen colorimetric substrate (Sigma), according to the manufacturer's instructions [24], adapted to a 96-well plate, and an absorbance reading at 550 nm. An increase in absorbance of 0.05 was considered to be 1 unit of enzyme activity, and the result was expressed in units of enzyme activity per milligram of protein sample (unit/mg).

The proteolytic activity on fibrinogen was verified as described by Rodrigues et al. [25]. The hydrolysis of fibrinogen was demonstrated by SDS-PAGE using 12% polyacrylamide gels. To demonstrate the variability of enzymatic activity against different varying parameters, protein hydrolysis was observed after preincubation at different pHs (2.5 to 10.0), temperatures (−10°C to 100°C), and time intervals for enzymatic action (30 min to 24 h). Likewise, the effect of inhibitors or divalent ions was tested by incubating 20 μg of venom with varying concentrations of heparin and EDTA (10, 20, and 30 mM) or ions (40 mM). The proteolytic activity on casein was tested as described by van der Walt and Joubert [26].

2.3.3. Anti-Clotting. The clotting time in the presence of different concentrations of *P. bistriata* venom was evaluated with the addition of samples of venom 10 min before the $CaCl_2$ (0.1 M) and time intervals after addition [27].

2.3.4. Hemorrhagic Activity. To check the hemorrhagic activity of *Parawixia bistriata* venom, four groups of three male Swiss mice (28–32 g) were used. The samples with about 100 micrograms of total protein content were dissolved in 50 μL of saline and intradermally inoculated in the dorsum of anesthetized mice with ethyl ether. Controls received 50 μL of saline under identical conditions. After three hours, the animals were euthanized by cervical dislocation and the skin was removed, showing the activity based on the presence of a hemorrhagic halo [28].

2.3.5. Edematogenic Activity. Groups of six male Swiss mice (18–22 g) were injected in the subplantar region, using from 10 to 200 μg/animal of *P. bistriata* crude venom diluted in 50 μL of PBS, and the negative control was injected only with PBS. After 0.5, 1, and 3 hours, edema in each paw was measured with the aid of a low-pressure spring gauge (Mitutoyo, Japan) [29]. The values taken at the beginning were subtracted and the difference was reported (mean ± standard deviation).

2.3.6. Lethality. Lethality induced by *Parawixia bistriata* venom was evaluated by intraperitoneal injections of samples

(100, 250, and 500 μg) in groups of mice of 18–22 g (sample size = 6, within 48 hours). 100 μL PBS was used as a negative control and snake venoms that are known to induce lethality in less than 5 h at these concentrations were used as positive controls [30].

2.3.7. Myotoxicity. Myotoxic activity was assayed by measuring the release of creatine kinase (CK) as recommended by Stábeli et al. [31] using a kinetic CK-UV Kit (Bioclin, Brazil). Solutions of 10 to 200 μg/animal of *P. bistriata* venom diluted in 50 μL of phosphate buffered saline (PBS) were applied intramuscularly in six male Swiss mice weighing 18–22 g. For negative controls, we used the same amount of 0.15 M phosphate buffered saline. After 3 hours, blood was collected from the tails of the mice into heparinized tubes and then centrifuged to separate the plasma. The latter was incubated for 3 min at 37°C with 1.0 mL of CK-UV and the amount of CK was determined according to the phosphorylation of a μmol of creatine/min at 25°C.

2.3.8. Genotoxicity. Genotoxicity was observed using two methods: a micronucleus test and a comet assay. The experiments were approved by the Ethics Committee of FCFRP-USP (102/2009) and the results were analyzed statistically and expressed as mean ± standard deviation (SD) (Kruskal-Wallis).

The micronucleus test in human lymphocytes *in vitro* was performed according to the technique of Moorhead et al. [32] with modifications described by Marcussi et al. [33]. *Parawixia bistriata* venom was added to the cells at concentrations of 2.5, 5, 15, and 30 μg/mL at 24 h after the start of the cultures. The antineoplastic cisplatin 6 μg/mL (PLATINIL, Chiral Chemicals of Brazil SA) was used as a positive control. The criteria used to evaluate the micronuclei were previously described by Fenech [34].

For the comet assay we used the same methodology as Singh et al. [35]. Cell suspension of 10^6 cells/mL was used to evaluate 100 nucleoides per slide in triplicate. The results were expressed in classes and arbitrary units as described by Collins et al. [7, 36], with the ranking in categories being adapted considering suitability for visual analysis and the levels of damage as described by Marcussi et al. [33].

2.3.9. Larvicidal Activity. The larvicidal activity of *P. bistriata* venom and fractions was ascertained at concentrations from 5 to 15 ppm, using 1% ethanol as a negative control and performing the tests in quadruplicate for each concentration of the sample. Lethal concentrations (LCs) were calculated from 24 to 48 hours using Probit analysis and Weibull distribution (Minitab, Minitab Inc.). The samples were solubilized in water and transferred to 50 mL plastic cups where 25 3rd-4th instar *Aedes aegypti* larvae were added in standard laboratory conditions (27°C, 70% relative humidity, and 12 hours of photoperiod).

The tests were conducted in triplicate at different times and with different groups of larvae. Dying or debilitated larvae were counted as dead, according to the criteria established by the World Health Organization and similar to that performed by Furtado et al. [37]. RM Two-Way Anova (Prism 6-GraphPad) was used to analyze the effect of venom and fractions at different times and concentrations on larval mortality.

2.3.10. Antibacterial and Antifungal Activity. To test the sensitivity of dermatophytes and yeast, we followed protocol M-38-P, described by the National Committee for Clinical and Laboratory Standards [38]. The minimal inhibitory concentration (MIC) was determined by visual reading of the development of fungal strains of *Trichophyton rubrum* (ATCC MYA-3108) and *Candida albicans* (ATCC 10231). To evaluate the sensitivity of the bacteria *Staphylococcus aureus* (ATCC 6538), *S. epidermidis* (ATCC 2228), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (USP) against crude venom, we used microdilution plates described by protocol M2-A8 of the National Committee for Clinical and Laboratory Standards [39], which was used in similar experiments described by Stábeli et al. [40].

2.4. Biochemical Characterization

2.4.1. Protein Content. The dosage of crude venom proteins was performed using the reagent Bradford [41] using a standard curve obtained with bovine serum albumin concentrations (BSA) with $R^2 \geq 0.99$. The absorbance was read using 96-well plates (Biotek) at 595 nm.

2.4.2. Chromatography. The purification of proteins was carried out using cation exchange chromatography on a CM Hytrap column and reverse phase chromatography on a C18 column. Thus, there were two chromatographic steps: first, cation exchange chromatography on a 1 mL CM FF Hytrap column followed by reverse phase HPLC on a Discovery C18 column (25 × 4.6 mm Supelco), both of which were done on an Akta purifier chromatograph-GE. In the cationic exchange chromatography, 10 mg of *Parawixia bistriata* crude venom was diluted in 1 mL of 20 mM ammonium bicarbonate and centrifuged for 5 min at 13,000 ×g and applied to the column. Elution was performed with 20 mM ammonium bicarbonate (eluent A) and 500 mM ammonium bicarbonate (eluent B) at a gradient of 0 to 100% of eluent B and a flow rate of 1 mL/minute.

Then, the fractions were applied to reverse phase chromatography, diluted in 1 mL of 0.1% trifluoroacetic acid (TF), and centrifuged for 5 min at 13,000 ×g 0.1% TFA (eluent A) and a solution of 0.1% TFA and 99.9% acetonitrile (eluent B) was used at a gradient of 0 to 100% B at a flow rate of 1 mL/minute. The peaks were monitored at 280 nm absorbance, read in Software Dataq (Dataq, Inc.) collected manually, identified, lyophilized, and stored at −20°C.

2.4.3. SDS-PAGE. SDS-PAGE was performed as described by Laemmli [42] with modifications. Approximately 2 μL of protein/peptide (200 mg) from the venom of *Parawixia bistriata* was applied to discontinuous gel with dimensions of 180 × 160 × 1.0 mm. After the protein separation, the gel was stained using Coomassie Blue G-250.

2.4.4. Two-Dimensional Electrophoresis. A sample of crude venom of *Parawixia bistriata* applied in two-dimensional electrophoresis was subjected to prior purification using *Clean-up Kit* (Sigma) according to the protocol of the manufacturer. The kit *deglycosylation enzyme mix-P6039S* (BioLabs) was used according to the manufacturer's instructions to study the deglycosylation of *Parawixia bistriata* crude venom. Two-dimensional electrophoresis was performed according to the method of O'Farrell [43] with modifications. During the focusing process, the proteins and peptides from the sample of *Parawixia bistriata* crude venom were separated based on isoelectric point in 13 cm strips of a polyacrylamide gel with pH values ranging from 3 to 10 nonlinearly.

After rehydration of the strips according to manufacturer's instructions, they underwent focusing, followed by reduction and alkylation according to the method of Vesterberg [44]. Then the strips were applied to 12.5% polyacrylamide gels for an electrophoretic run at 25 mA per gel and 100 W. The presence of proteins in the gel was revealed using colloidal Coomassie Blue G-250.

3. Results and Discussion

The biochemical, proteomic, and functional characterization of *Parawixia bistriata* venom presented in this study confirms the presence of various nonprotein toxins of low molecular weight previously described in advanced neurochemical and pharmacological research [15–22], revealing novel enzymatic and biological activities, as well as enzymatically active and inactive protein components of high molecular weight not characterized in previous studies.

Considering the predominant protein composition (670 μg protein/mg of crude venom) in the *P. bistriata* venom, we chose to investigate the probable enzymatic activities of these proteins, testing mainly for phospholipase A_2 and proteolytic activity with a number of different specific substrates. The phospholipase activity of the crude venom of *P. bistriata* was not significant against the substrate for PLA_2, demonstrating low enzymatic activity when compared to the enzymatically active phospholipases from snake venom (data not shown). However, proteolytic activity can be considered the main enzymatic activity identified in the venom of *P. bistriata* in this study, since it tested positive for several different protein substrates, which are presented below.

Proteolytic activity against fibrinogen is present in the crude venom at intensities that vary nonlinearly and are concentration-dependent. It was observed that the enzymatic activity of *P. bistriata* venom proteins oscillates when the sample (fibrinogen + crude venom) is subjected to varying parameters such as temperature, pH, and the presence of divalent ions and EDTA (Figures 1 and 2).

The proteolytic activity of the venom of *P. bistriata* on fibrinogen was concentration dependent and occurs by breaking the α and/or β chains and releasing fibrinopeptides. The enzymatic activity on fibrinogen was completely inhibited after preincubation at temperatures above 70°C (Figure 1) and when subjected to a solution with pH ≤ 2.5. After incubation in solutions of pH 3.5, 8.0, 9.0, and 10.0, there was a partial loss of fibrinogenolytic activity, similar to that observed after preincubation of the venom in the presence of Na^+ ions or EDTA at concentrations of 20 and 30 mM. The Zn^{++} ion appears to potentiate the proteolytic effect of the venom by causing total breakage of the α and β chains and partial breakage of the γ chain (black arrow in Figure 2). Heparin showed no effect on this activity (Figure 2).

Table 1: Anticoagulant activity of *Parawixia bistriata* spider venom.

Parawixia bistriata (μg)	Time of coagulation (min)
10	5:15 ± 0:23
25	7:45 ± 0:47[#]
50	10:35 ± 1:38[#]
100	22:25 ± 4:65[#]
200	Did not coagulate[**#]
250	Did not coagulate[**#]
$CaCl_2$ (0.1 M)[*]	4:00 ± 0:26

[*]Inducing coagulation control. [**]After 48 hours of observation. The anticoagulant activity of the venom was observed by adding 10–250 μg of *P. bistriata* crude venom into the plasma while monitoring clotting time. Mean values of the clotting time followed by the standard deviation expressed six individual experiments with triplicate samples. [#]Significance level ($P < 0.05$) when compared to the control.

Besides its enzymatic activities, the venom of *P. bistriata* was also investigated for its functional biological activities. The venom of *P. bistriata* was not able to coagulate plasma from mice in the observation period of up to one minute at all concentrations tested. Nevertheless, it was noted that the venom of *P. bistriata* interferes with the clotting process (Table 1), by delaying the time of clot formation with all quantities of crude venom tested. It was observed that the lowest tested amount (10 μg) was able to increase the coagulation time by approximately 30%; also observed were increased coagulation times by 86%, 160%, and 556% for doses of 25 μg, 50 μg, and 100 μg, respectively. It was also noted that there was no coagulation of the plasma after 48 hours of observation in studies in which higher amounts of venom (200 and 250 μg) were used.

Also, regarding interference from crude venom proteins in hemostasis, the venom of *P. bistriata* showed no hemorrhagic activity, since no hemorrhagic halo was observed after application of the venom in the test performed with mice (data not shown), in contrast to the hemorrhagic halo obtained in the positive control using *B. mattogrossensis* snake venom.

The venom of *P. bistriata* was also able to cause myotoxicity measured by the release of creatine kinase (CK) after intramuscular injection of venom in mice (Figure 3). Myotoxic activity of the venom of *P. bistriata* was significant starting from a dose of 25 μg/animal, showing an increase of approximately 100% CK compared to PBS, the negative control. A dose of 200 μg/animal increased the myotoxicity by 400% compared to that caused by PBS.

Although it triggers specific physiological responses, it was observed that the venom of *P. bistriata* is not lethal when injected intraperitoneally in mice at concentrations between 25 and 1,500 μg/animal.

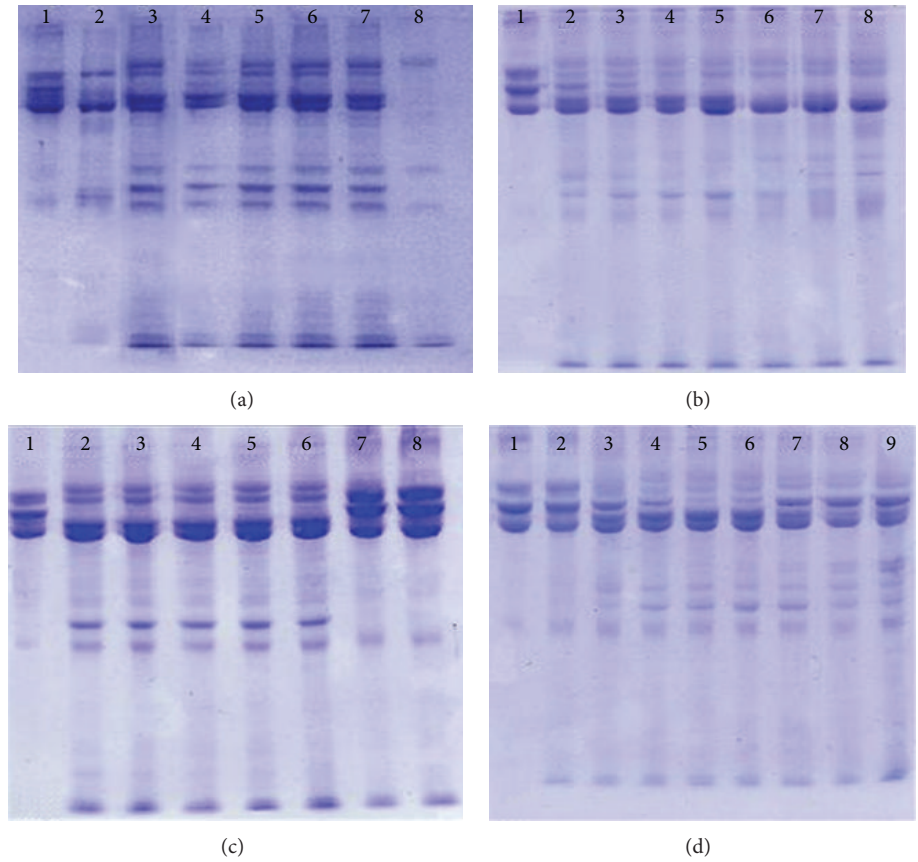

Figure 1: Proteolytic activity of *Parawixia bistriata* crude venom on Fibrogen. Fibrinogen was preincubated with the crude venom of *P. bistriata* in varying concentrations, times, temperatures, and pHs. The samples were subjected to SDS-PAGE for visualization of protein bands. Staining: Coomassie Blue.*F = Fibrogen **CV = *P. bistriata* crude venom. (a) Effect of concentration. Lanes: (a)1: *F (80 μg); (a)2: F + **CV (10 μg); (a)3: F + CV (20 μg); (a)4: F + CV (40 μg); (a)5: F + CV (60 μg); (a)6: F + CV (80 μg); (a)7: F + CV (100 μg); (a)8: CV (50 μg). (b) Effect of incubation time. Lanes: (b)1: F (80 μg); (b)2: F + 20 μg CV (30′); (b)3: F + 20 μg CV (1 h); (b)4: F + CV (2 h); (b)5: F + CV (3 h); (b)6: F + CV (6 h); (b)7: F + CV (12 h); (b)8: F + CV (24 h). (c) Effects of different temperatures. Samples: (c)1: F (80 μg); (c)2: F + CV (20 μg) (−10°C); (c)3: F + CV (4°C); (c)4: F + CV (25°C); (c)5: F + CV (37°C); (c)6: F + CV (50°C); (c)7: F + CV (70°C); (c)8: F + CV (100°C). (d) Effects of different pHs. Samples: (d)1: F (80 μg); (d)2: F + CV (20 μg) (pH 2.5); (d)3: F + CV (pH 3.5); (d)4: F + CV (pH 4.5); (d)5: F + CV (pH 5.5); (d)6: F + CV (pH 7.0); (d)7: F + CV (pH 8.0); (d)8: F + CV (pH 9.0); (d)9: F + CV (pH 10.0).

We also researched genotoxic activity in order to toxicologically characterize *P. bistriata* crude venom using two methods: the formation of micronuclei and a Comet test, which allowed for the analysis of crude venom induction of chromosomal damage or breakage of DNA strands.

The micronucleus test showed a greater percentage of mononuclear cells after treatment with the venom at all concentrations when compared to the percentage obtained for cisplatin. We also observed a lower percentage of tri or multinucleated cells compared to the same positive control, pointing out the low capacity of the venom to alter cell division (Table 2).

It also showed less micronucleus formation in binucleate cells (MN/BN) after treatment with the venom of *P. bistriata*, indicating low induction of coarse damage to chromosomes (Figure 4).

In the comet test, there was a prevalence of classes 0, 1, and 2 from Collins [7, 36], with most of the nucleoids having less than 40% damage (Figure 5).

The comet test also showed a higher significant percentage of damage in nucleoids treated with *P. bistriata* venom, at concentrations of 7.5 μg/mL and higher, compared to the positive control doxorubicin. It was found that the number of damaged nucleoids increased as the concentration of the sample increased in most of the tests (Table 3); however, global indices of damage proved to be independent of concentration or linear relationship meaning cytotoxicity should be considered.

The cytogenetic toxicological analysis of animal venoms and their isolated proteins is also of great importance for the identification and characterization of potential therapeutic agents, as well as for a better understanding of the mechanisms of action of these toxins in the human body [33]. The breakage of DNA can result in permanent damage, altering the morphology and physiological homeostasis of cells [34, 45]. Molecules from venoms capable of inducing genotoxicity may also participate in mutagenic and carcinogenic events, according to the biochemical characteristics of each individual.

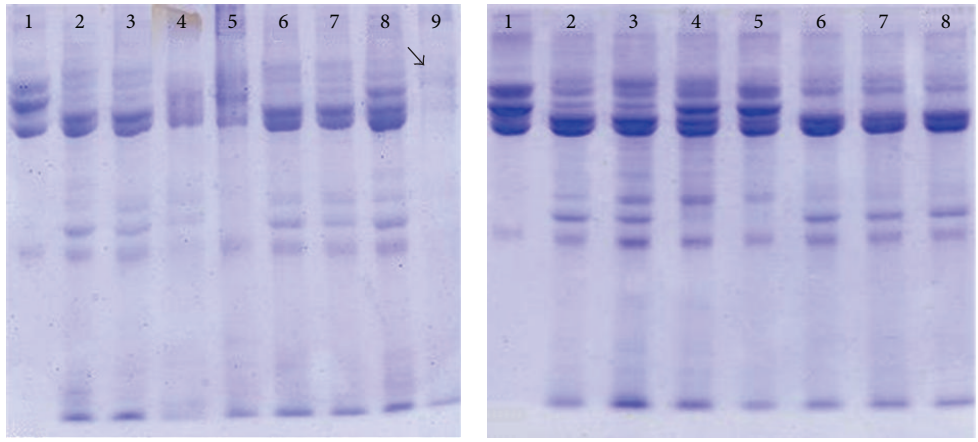

Figure 2: Fibrinogenolytic activity of *Parawixia bistriata* venom. Fibrinogen was preincubated with the crude venom of *P. bistriata* in the presence of divalent ions, EDTA and heparin. The samples were subjected to SDS-PAGE for visualization of protein bands. *F = Fibrinogen **CV = *P. bistriata* crude venom. (a) Effect of divalent ions: lanes: (a)1: *F (80 μg); (a)2: F + **CV (20 μg); (a)3: F + CV + Ca^{2+} (40 mM); (a)4: F + CV + Co^{2+} (40 mM); (a)5: F + CV + Fe^{2+} (40 mM); (a)6: F + CV + Mg^{2+} (40 mM); (a)7: F + CV + Mn^{2+} (40 mM); (a)8: F + CV + Na^{+} (40 mM); (a)9: F + CV + Zn^{2+} (40 mM). (b) Effect of EDTA and heparin. Lanes: (b)1: F (80 μg); (b)2: F + CV (20 μg); (b)3: F + CV + 10 mM EDTA; (b)4: F + CV + 20 mM EDTA; (b)5: F + CV + 30 mM EDTA; (b)6: F + CV + 10 mM heparin; (b)7: F + CV + 20 mM heparin; (b)8: F + CV + 30 mM heparin.

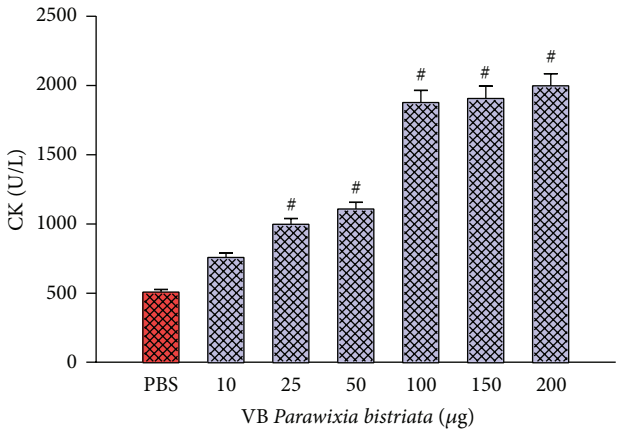

Figure 3: Myotoxic activity of *Parawixia bistriata* venom. Solutions from 10 to 200 μg/animal of *P. bistriata* venom diluted in 50 μL of PBS were applied intramuscularly in mice. The plasma was incubated for 3 min at 37°C with 1.0 mL of the reagent CK-UV. The amount of CK expressed after the phosphorylation of one μmol of creatine/min at 25°C. Negative control: 0.15 M PBS. Test conducted using a kinetic CK-UV Kit (Bioclin, Brazil). #Significantly different from the negative control ($P < 0.05$).

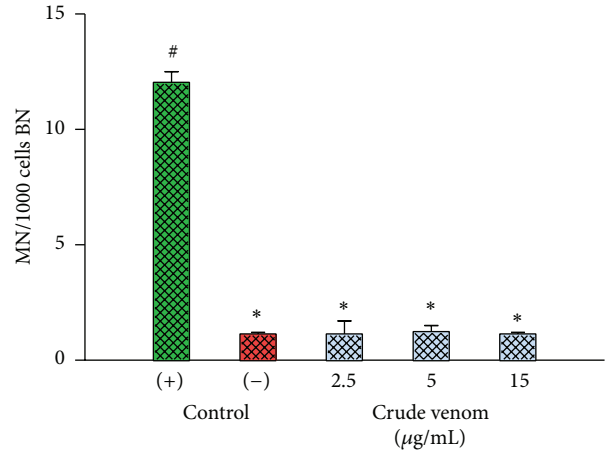

Figure 4: Distribution of micronuclei in binucleated human lymphocytes. Cells were treated with the venom of *P. bistriata* at concentrations of 2.5, 5, and 15 μg/mL. The drug cisplatin (6 μg/mL) was used as the positive control and the negative control was untreated cell cultures. The results are presented as mean ± SD ($n = 4$) of two individual experiments. *Statistically different from the positive control ($P < 0.05$). #Statistically different from the negative control ($P < 0.05$).

Some studies have associated morphological and physiological changes in victims of accidents with venomous animals, especially for late sequelae, which could be a result of the cumulative effects of the toxins, resulting in neurodegenerative diseases and chronic inflammatory diseases [46, 47].

Besides the functional biological activities of the venom of *P. bistriata,* we also investigated the action of the constituents of the crude venom on microorganisms by tracking cytotoxic activity against fungi and gram positive and negative bacteria. The test results of the minimum inhibitory concentration did not show good antimicrobial activity against gram positive and negative; however, a concentration of 1 mg/mL (MIC) of venom completely inhibited macroscopic growth of *Candida albicans*.

Most antimicrobial toxins from previously described animal venoms are peptides [48–50]. The crude venom of *Parawixia bistriata* was active against *Candida albicans*, and the identification and isolation of the toxin responsible for this effect could reveal a new potential antifungal compound.

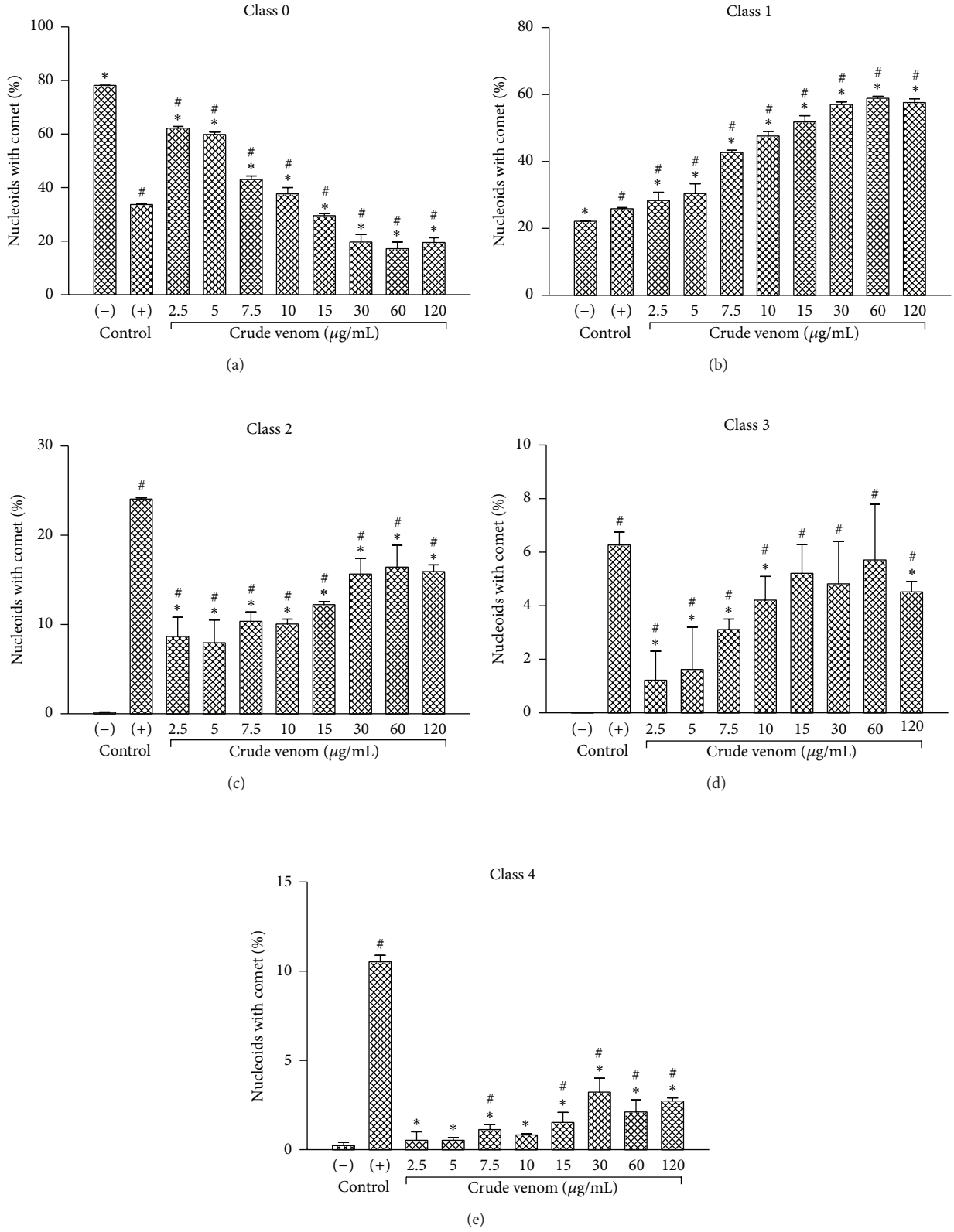

Figure 5: Frequency of nucleoids distributed in damage classes of Collins. The levels of damage to cells treated with crude venom of *Parawixia bistriata* were distributed in class 0 (no damage <5%), class 1 (low damage: 5 to 20%), class 2 (medium damage: 20 to 40%), class 3 (high damage: 40–85%), and class 4 (totally damaged >85%). Positive Control: doxorubicin (6 μg/mL). Negative control: untreated cell cultures. *Statistically different from the positive control ($P < 0.05$). #Significantly different from the negative control ($P < 0.05$).

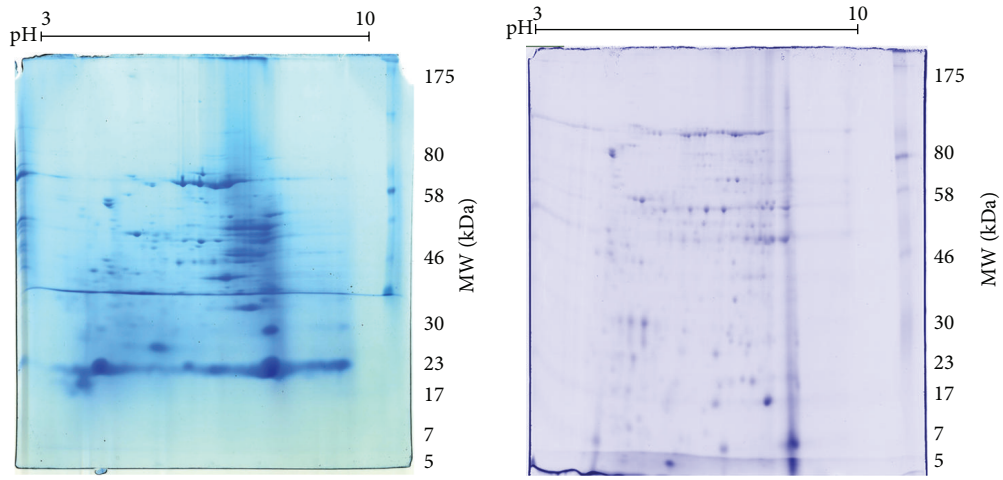

Figure 6: 2D electrophoresis of *Parawixia bistriata* venom. Samples containing 2 mg of crude venom and 2 mg of deglycosylated venom underwent focusing and were then applied to 12.5% acrylamide gels-Item 3.5.4. Methods. Staining performed by colloidal Coomassie. (a) Two-dimensional gel electrophoresis of the crude venom of *P. bistriata*. (b) Two-dimensional gel electrophoresis of deglycosylated venom of *P. bistriata*. MW = molecular weight 7–175 kDa.

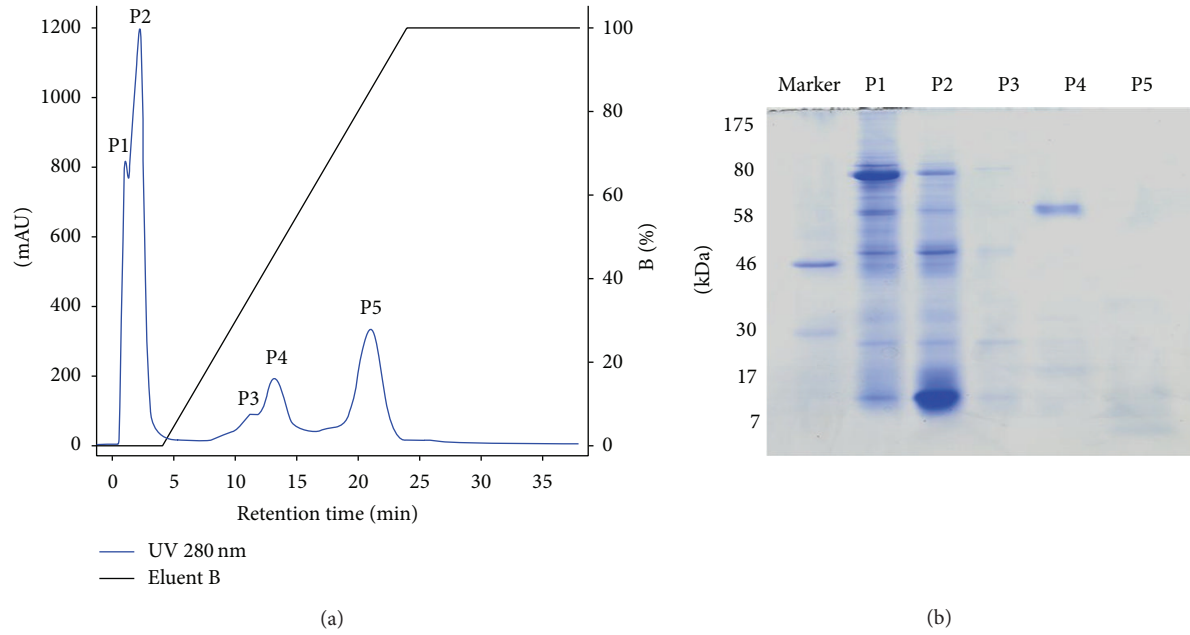

Figure 7: Chromatographic and electrophoretic profile of *Parawixia bistriata* crude venom. (a) Cation exchange chromatography of 2.7 mg of the crude venom of *Parawixia bistriata*. A 1 mL FF-CM Hytrap (7 × 25 mm) column was used at a flow rate of 0.5 mL/min. Gradient of 0 to 100% of eluent B. Eluent A: 20 mM ammonium bicarbonate, pH 8.0; eluent B: 500 mM ammonium bicarbonate, pH 8.0. Monitoring at 280 nm. (b) SDS-PAGE electrophoresis on 12.5% acrylamide gel of lyophilized fractions from cation exchange chromatography of *Parawixia bistriata* venom. Coomassie Blue staining. Molecular weight marker: 7–175 kDa.

The venom proteins of *P. bistriata* were also characterized by 2D electrophoresis, with and without deglycosylation (Figure 6).

The crude venom revealed several bands corresponding to proteins of high molecular weight, ranging mostly from acidic to neutral pH. The deglycosylated venom demonstrated a different migration profile of protein bands, especially for proteins with a low molecular weight, for gels with the same concentration of acrylamide that were run under the same experimental conditions.

Table 2: Nuclear quantification of human lymphocytes treated with *Parawixia bistriata* spider venom.

Treatments (μg/mL)	% cells/500 cells				CBPI ± S.D.
	[a]Mono	[b]Bi	[c]Tri	[d]Multi	
Control (+)					
6	48.5	38.7	7.6	5.2	1.643 ± 0.1
Control (−)					
—	50.3	39	7.3	3.4	1.604 ± 0.07
P. bistriata					
2.5	56.8	37.5	3.6	2.1	1.489 ± 0.05
5	50.4	40.2	5.4	4	1.590 ± 0.2
15	53.9	38.6	3.9	3.6	1.536 ± 0.09

[a]Mono: mononucleated, [b]Bi: binucleated, [c]Tri: trinucleated, [d]Multi: multinucleated. Positive control (+): cisplatin (6 μg/mL). Negative control (−): untreated cells in culture. CBPI: cytokinesis block proliferation index, that defines whether the cultures are multiplying normally after the addition of samples. The following formula was used according to Kirsch-Volders (1997) [6]: CBPI = 1 (mono) + 2 (bi) + 3 (tri + tetra)/500. Mean cells number followed by the standard deviation.

Table 3: Frequency of nucleoids with comet after treatment of human lymphocytes with *Parawixia bistriata* spider venom.

Treatments (μg/mL)	Damaged nucleoids (%)	A.U.
[a]Negative control		
—	22	22*
[b]Positive control		
6	39.75	134.5#
P. bistriata		
2.5	38	49#*
5	40.3	52.9#*
7.5	57.1	76.9#*
10	62.5	83.3#*
15	70.6	97.7#*
30	80.5	115.3#*
60	83	117.1#*
120	80.6	113.6#*

[a]Negative control: culture of untreated cells, [b]positive control: doxorubicin (6 μg/mL). A.U.: arbitrary units (0–400) calculated according to [7]. #Statistically different from the negative control ($P < 0.05$). *Statistically different from the positive control ($P < 0.05$).

Two-dimensional electrophoresis of *P. bistriata* venom showed various proteins of high molecular weight (mostly above 40 kDa), which could be identified as evidence of the presence of several enzymes [51] previously described in the *Loxosceles intermedia* spider [52].

Glycosylated proteins are common in animal [53, 54]. Some components of snake venom are known to possess glycosylation; however, little is known about the structure of the carbohydrates present in these proteins [55].

Through the combined analysis of chromatographic profiles from cation exchange chromatography followed by reverse phase chromatography, the predominance of polar proteins with acidic or neutral pHs was observed in the crude venom.

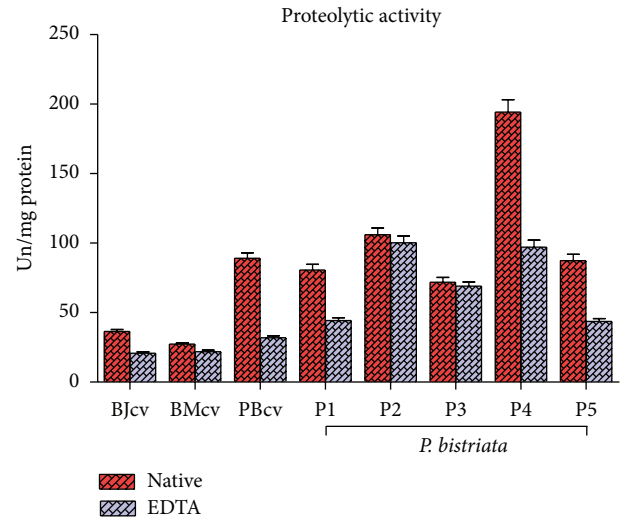

Figure 8: Proteolytic activity of *Parawixia bistriata* crude venom against the substrate azo-collagen. An increase in absorbance of 0.05 was regarded as 1 unit of enzymatic activity for each milligram of protein. The red bars represent the activity of the native protein and the light gray bars represent the activity of the proteins after incubation with 1 mM EDTA. BJcv: *B. jararacussu* crude venom; BMcv: *B. matogrossensis* crude venom. PBcv: *P. bistriata* crude venom. P1, P2, P3, P4, and P5: fractions from the venom of *P. bistriata*. Absorbance read at 550 nm.

Five fractions, named P1, P2, P3, P4, and P5, were collected during cation exchange chromatography (Figure 7(a)). The SDS-page of the fractions showed that most of the protein was concentrated in fractions P1 and P2, requiring the establishment of other purification strategies for the isolation of the proteins within these fractions to be considered in future studies. As shown in the electrophoresis, fraction P4 contained a protein of about 60 kDa with a significant degree of purity (Figure 7(b)). The electrophoresis showed protein constituents of high molecular weights in the range of 60 to 80 kDa but also showed the presence of constituents of about 14 kDa.

Considering the prior finding using SDS-page of an apparent molecular mass of approximately 60 kDa in the major protein fraction P4, we proceeded to compare possible enzymatic activities of all the fractions of *P. bistriata*, including fraction P4, with primary focus on the research of proteolytic enzyme activity in azo-collagen substrate and in the presence of the inhibitor EDTA.

Proteolytic activity was confirmed for the crude venom and all fractions (P1 to P5) for the substrate azo-collagen. The enzymatic activity of fractions P1, P4, and P5 was significantly reduced in the presence of 1 mM EDTA, suggesting that the enzymatic activity is dependent on metals (Figure 8). It was observed that the enzymatic activity of P1 was reduced by 45%, P4 reduced by about 50%, P5 reduced by about 50%, and the crude venom reduced by about 64% in the presence of 1 mM EDTA. No significant reduction in enzymatic activity was seen for fractions P2 and P3. Confirmation of proteolytic activity present in *P. bistriata* crude venom and its fractions

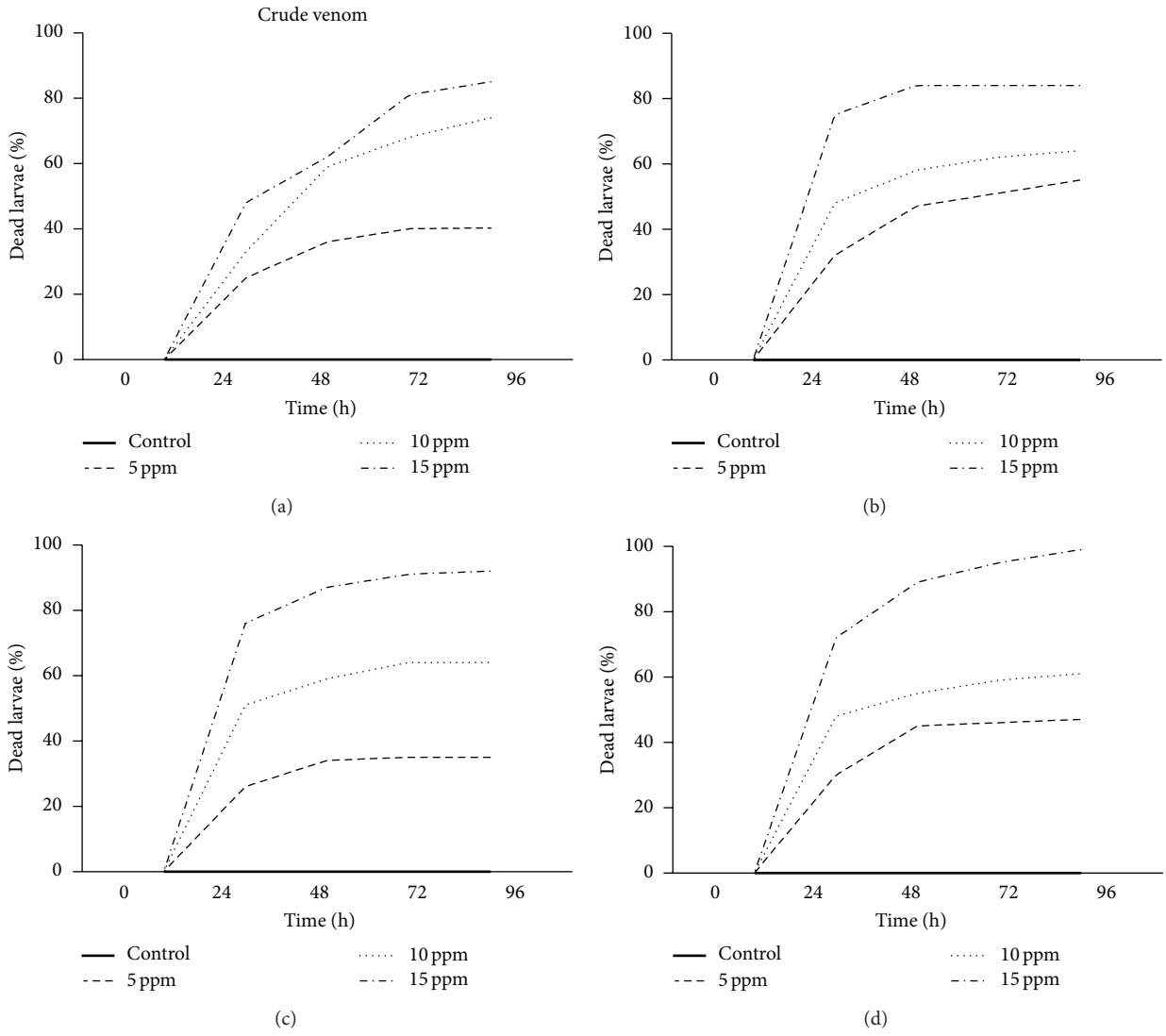

Figure 9: Larvicidal activity of the crude venom and chromatography fractions of *Parawixia bistriata* on *Aedes aegypti*. Concentrations of 5 to 15 μg/mL of the venom and its fractions were used against 3rd-4th instar *Aedes aegypti* (27°C, 70% relative humidity, and 12 h of photoperiod). Assays were performed in quadruplicate using a solution of 1% ethanol as a negative control (solid line). (a) Crude venom. (b) Fraction P1. (c) Fraction P2. (d) Fraction P3.

points to the presence of proteases, some of which are inhibited by metal chelators. The P4 fraction contains the protease with greatest proteolytic activity and the highest degree of purification measured in this study, and we suggest that it belongs to the same class of metalloproteases with molecular weight in the range of 40–60 kDa. Proteases are present in the digestive fluids of different species of spiders, just as protease inhibitors have been reported and characterized in several studies [56–59].

Proteolytic enzymes can interfere with the blood coagulation cascade and induce hemorrhaging, edema, myonecrosis, dermonecrosis, and inhibition of platelet aggregation [60–63]; however, the crude venom of *P. bistriata* showed edematogenic and anticoagulant but no hemorrhagic activity.

Among the known biological activities present in animal venoms, insecticidal/larvicidal activity has been described in venoms of many species of spiders, so we sought to investigate the larvicidal activity of the crude venom as well as of the fractions obtained from the venom of *P. bistriata*. Larvicidal activity was found in all samples (Table 4) tested (crude venom and fractions P1, P2 and P3). Fractions P4 and P5 were not tested because of the limited amount of sample available for testing.

The lethal concentrations for *A. aegypti* were similar among the crude venom and fractions, but LCs values were much lower compared to larvicidal tests against this mosquito species using plant extracts and fractions by our group [64].

The crude venom and P1, P2, and P3 fractions caused larval mortality that increased significantly with time ($F = 101.1$; $P < 0.001$, $F = 384.3$; $P < 0.001$; $F = 242.0$; $P < 0.001$ and $F = 357.7$; $P < 0.001$, resp.) and concentration ($F = 205.0$; $P < 0.001$; $F = 1044$; $P < 0.001$, $F = 1952$; $P < 0.001$

Table 4: Lethal concentrations (LC) in μg/mL of crude venom fractions from *Parawixia bistriata* for *Aedes aegypti* (Diptera: Culicidae) larvae.

Source	LC50	LC90
Crude venom	9	25
Fraction P1	6	17
Fraction P2	8	15
Fraction P3	7	16

and $F = 1188$; $P < 0.001$, resp.). Crude venom and fractions effects on larval mortality differed significantly ($F = 71.45$; $P < 0.001$) from each other after 48 hours within the concentrations tested, except between P1 and P2. At 15 μg/mL, more than 85% of larvae were dead after 96 hours in all assays (Figure 9), peaking at 99% for the fraction P3 (Figure 9(d)).

Various compounds from plants and animals can be listed as potential bioinsecticides as effective and safe alternatives to chemical insecticides [65, 66]. Venoms from spiders are a rich source of peptide insecticides, which were evolutionarily adjusted to achieve a broad range of receptors and ion channels in the nervous system of insects, possessing broad biotechnological potential [67–69].

Cesar et al. [16] described the structure of a new insecticidal compound named hydroxytrypargine isolated from the venom of *Parawixia bistriata*. The assay was performed injecting bees at a dose of 37 ng/g bee with a microsyringe, and an LD_{50} of 8 ± 2 ng/g was found for the β-carbonilic toxin and an LD_{50} of 29 ng/g for the crude venom. The present data corroborate the insecticidal activity of *P. bistriata* and also highlight the topic and/or oral effect of the crude venom and fractions of this spider species on *Aedes aegypti* with very low LC values for crude and even fractions of potential insecticides. Therefore, further characterization for the elucidation of the chemical nature of the venom components and a search for their targets will be addressed in the near future.

4. Conclusions

We observed the presence of biomolecules in *Parawixia bistriata* venom showing, individually or together, myotoxic, anticoagulant, edematogenic, mild genotoxic, insecticidal, and fungicidal activities.

Detailed analysis of bioactive proteins from the venom of *Parawixia bistriata* through more sequencing and biochemical-toxicological assays may result in better understanding of the pharmacological activity of this venom and the elucidation of its constituents, contributing to the advancement of toxinology and also to the direct or indirect development of products of medical or scientific interest.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

The authors are grateful to the Ministry of Science and Technology (MCTI), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Tecnologia do Acre (FUNTAC/FDCT), Coordenação de Aperfeiçoamento de Nível Superior (CAPES)—Projeto NanoBiotec, Rede de Biodiversidade e Biotecnologia da Amazônia Legal (BIONORTE/CNPq/MCTI), Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT-INPeTAm/CNPq/MCTI) e Instituto Nacional para Pesquisa em Toxinas (INCT-Tox), and the Secretary of Development of Rondonia State (SEPLAN-RO/PRONEX/CNPq) for financial support. They thank Dr. Rene O. Beleboni and Ana L. Facchin (Unidade de Biotecnologia, UNAERP, Ribeirão Preto-SP) for the time for the development of this work, providing the spider venom and assistance in the microbicide test, respectively. Amy Grabner provided the English editing of the paper.

References

[1] C. M. Yonamine, H. Costa, J. A. A. Silva et al., "Biodistribution studies of bee venom and spider toxin using radiotracers," *Journal of Venomous Animals and Toxins including Tropical Diseases*, vol. 11, pp. 39–50, 2005.

[2] B. Lomonte and J. Rangel, "Snake venom Lys49 myotoxins: from phospholipases A_2 to non-enzymatic membrane disruptors," *Toxicon*, vol. 60, pp. 520–530, 2012.

[3] L. D. Rash and W. C. Hodgson, "Pharmacology and biochemistry of spider venoms," *Toxicon*, vol. 40, no. 3, pp. 225–254, 2001.

[4] N. I. Platnick, *The World Spider Catalog—Version 11.5*, American Museum of Natural History, New York, NY, USA, 2012.

[5] G. F. King and M. C. Hardy, "Spider-venom peptides: structure, pharmacology, and potential for control of insect pests," *Annual Review of Entomology*, vol. 58, pp. 475–496, 2012.

[6] M. Kirsch-Volders, "Towards a validation of the micronucleus test," *Mutation Research*, vol. 392, no. 1-2, pp. 1–4, 1997.

[7] A. R. Collins, "The comet assay for DNA damage and repair: principles, applications, and limitations," *Applied Biochemistry and Biotechnology B: Molecular Biotechnology*, vol. 26, no. 3, pp. 249–261, 2004.

[8] R. D. O. Beleboni, A. B. Pizzo, A. C. K. Fontana, R. D. O. G. Carolino, J. Coutinho-Netto, and W. F. dos Santos, "Spider and wasp neurotoxins: pharmacological and biochemical aspects," *European Journal of Pharmacology*, vol. 493, no. 1–3, pp. 1–17, 2004.

[9] H. Jackson and T. N. Parks, "Spider toxins: recent applications in neurobiology," *Annual Review of Neuroscience*, vol. 12, pp. 405–414, 1989.

[10] J. Meinwald and T. Eisner, "The chemistry of phyletic dominance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 1, pp. 14–18, 1995.

[11] A. Horni, D. Weickmann, and M. Hesse, "The main products of the low molecular mass fraction in the venom of the spider *Latrodectus menavodi*," *Toxicon*, vol. 39, no. 2-3, pp. 425–428, 2000.

[12] I. R. Mellor and P. N. R. Usherwood, "Targeting ionotropic receptors with polyamine-containing toxins," *Toxicon*, vol. 43, no. 5, pp. 493–508, 2004.

[13] W. L. Levi, "Spiders of the orb-weaver genus *Parawixia* in America (Aranea: Araneidae)," *Bulletin of the Museum of Comparative Zoology*, vol. 158, pp. 1–46, 1992.

[14] M. A. R. Cairrão, A. M. Ribeiro, A. B. Pizzo et al., "Anticonvulsant and GABA uptake inhibition properties of venom fractions from the spiders *Parawixia bistriata* and *Scaptocosa raptoria*," *Pharmaceutical Biology*, vol. 40, no. 6, pp. 472–477, 2002.

[15] A. C. K. Fontana, R. Guizzo, R. de Oliveira Beleboni et al., "Purification of a neuroprotective component of *Parawixia bistriata* spider venom that enhances glutamate uptake," *British Journal of Pharmacology*, vol. 139, no. 7, pp. 1297–1309, 2003.

[16] L. M. M. Cesar, M. A. Mendes, C. F. Tormena et al., "Isolation and chemical characterization of PwTx-II: a novel alkaloid toxin from the venom of the spider *Parawixia bistriata* (Araneidae, Araneae)," *Toxicon*, vol. 46, no. 7, pp. 786–796, 2005.

[17] R. O. Beleboni, R. Guizzo, A. C. K. Fontana et al., "Neurochemical characterization of a neuroprotective compound from *Parawixia bistriata* spider venom that inhibits synaptosomal uptake of GABA and Glycine," *Molecular Pharmacology*, vol. 69, no. 6, pp. 1998–2006, 2006.

[18] J. L. Liberato, A. O. S. Cunha, M. R. Mortari et al., "Anticonvulsant and anxiolytic activity of FrPbAII, a novel GABA uptake inhibitor isolated from the venom of the social spider *Parawixia bistriata* (Araneidae: Araneae)," *Brain Research*, vol. 1124, no. 1, pp. 19–27, 2006.

[19] D. M. Saidemberg, M. A. B. Ferreira, T. N. Takahashi et al., "Monoamine oxidase inhibitory activities of indolylalkaloid toxins from the venom of the colonial spider *Parawixia bistriata*: functional characterization of PwTX-I," *Toxicon*, vol. 54, no. 6, pp. 717–724, 2009.

[20] L. M. M. Cesar-Tognoli, S. D. Salamoni, A. A. Tavares et al., "Effects of spider venom toxin PWTX-I (6-hydroxytrypargine) on the central nervous system of rats," *Toxins*, vol. 3, no. 2, pp. 142–162, 2011.

[21] H. A. Fachim, A. O. S. Cunha, A. C. Pereira et al., "Neurobiological activity of Parawixin 10, a novel anticonvulsant compound isolated from *Parawixia bistriata* spider venom (Araneidae: Araneae)," *Epilepsy and Behavior*, vol. 22, no. 2, pp. 158–164, 2011.

[22] A. C. K. Fontana, R. de Oliveira Beleboni, M. W. Wojewodzic et al., "Enhancing glutamate transport: mechanism of action of Parawixin1, a neuroprotective compound from *Parawixia bistriata* spider venom," *Molecular Pharmacology*, vol. 72, no. 5, pp. 1228–1237, 2007.

[23] M. Holzer and S. P. Mackessy, "An aqueous endpoint assay of snake venom phospholipase A_2," *Toxicon*, vol. 34, no. 10, pp. 1149–1155, 1996.

[24] N. Jiang, N. S. Tan, B. Ho, and J. L. Ding, "Azocoll protease activity assay," *Protocol Exchange*, 2007.

[25] V. M. Rodrigues, A. M. Soares, R. Guerra-Sá, V. Rodrigues, M. R. M. Fontes, and J. R. Giglio, "Structural and functional characterization of neuwiedase, a nonhemorrhagic fibrin(ogen)olytic metalloprotease from *Bothrops neuwiedi* snake venom," *Archives of Biochemistry and Biophysics*, vol. 381, no. 2, pp. 213–224, 2000.

[26] S. J. van der Walt and F. J. Joubert, "Studies on puff adder (*Bitis arietans*) venom-I. Purification and properties of protease A," *Toxicon*, vol. 9, no. 2, pp. 153–161, 1971.

[27] B. Haim, A. Rimon, J. S. Ishay, and S. Rimon, "Purification, characterization and anticoagulant activity of a proteolytic enzyme from *Vespa orientalis* venom," *Toxicon*, vol. 37, no. 5, pp. 825–829, 1999.

[28] T. Nikai, N. Mori, and M. Kishida, "Isolation and biochemical characterization of hemorrhagic toxin f from the venom of *Crotalus atrox* (Western Diamondback Rattlesnake)," *Archives of Biochemistry and Biophysics*, vol. 231, no. 2, pp. 309–319, 1984.

[29] A. M. Soares, S. H. Andrião-Escarso, R. K. Bortoleto et al., "Dissociation of enzymatic and pharmacological properties of piratoxins-I and -III, two myotoxic phospholipases A_2 from *Bothrops pirajai* snake venom," *Archives of Biochemistry and Biophysics*, vol. 387, no. 2, pp. 188–196, 2001.

[30] A. M. Soares, *Estrutura, Função e Inibição de Miotoxinas Homólogas a Fosfolipases A_2 Isoladas de Venenos de Serpentes*, Tese de Doutorado, Departamento de Bioquímica, Faculdade de Medicina de Ribeirão Preto, USP, 2000.

[31] R. G. Stábeli, S. F. Amui, C. D. Sant'Ana et al., "Bothrops moojeni myotoxin-II, a Lys49-phospholipase A_2 homologue: an example of function versatility of snake venom proteins," *Comparative Biochemistry and Physiology C: Toxicology and Pharmacology*, vol. 142, no. 3-4, pp. 371–381, 2006.

[32] P. S. Moorhead, P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford, "Chromosome preparations of leukocytes cultured from human peripheral blood," *Experimental Cell Research*, vol. 20, no. 3, pp. 613–616, 1960.

[33] S. Marcussi, P. R. S. Santos, D. L. Menaldo et al., "Evaluation of the genotoxicity of *Crotalus durissus* terrificus snake venom and its isolated toxins on human lymphocytes," *Mutation Research: Genetic Toxicology and Environmental Mutagenesis*, vol. 724, no. 1-2, pp. 59–63, 2011.

[34] M. Fenech, "Cytokinesis-block micronucleus cytome assay," *Nature Protocols*, vol. 2, no. 5, pp. 1084–1104, 2007.

[35] N. P. Singh, M. T. McCoy, R. R. Tice, and E. L. Schneider, "A simple technique for quantitation of low levels of DNA damage in individual cells," *Experimental Cell Research*, vol. 175, no. 1, pp. 184–191, 1988.

[36] A. Collins, M. Dusinska, M. Franklin et al., "Comet assay in human biomonitoring studies: reliability, validation, and applications," *Environmental and Molecular Mutagenesis*, vol. 30, pp. 139–146, 1997.

[37] R. F. Furtado, M. G. A. de Lima, A. N. Manoel, J. N. S. Bezerra, and M. G. D. V. Silva, "Larvicidal activity of essential oils against *Aedes aegypti* L. (Diptera: Culicidae)," *Neotropical Entomology*, vol. 34, no. 5, pp. 843–847, 2005.

[38] NCCLS, "Reference method for broth dilution antifungal susceptibility testing of *Filamentous fungi*: approved standard," NCCLS Document M38-A2, Clinical Laboratory Standards Institute, 2002.

[39] NCCLS, "Performance standards for antimicrobial disk susceptibility tests," NCCLS Document M2-A8, Clinical Laboratory Standards Institute, 2003.

[40] R. G. Stábeli, S. Marcussi, G. B. Carlos et al., "Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom," *Bioorganic and Medicinal Chemistry*, vol. 12, no. 11, pp. 2881–2886, 2004.

[41] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[42] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[43] P. H. O'Farrell, "High resolution two dimensional electrophoresis of proteins," *Journal of Biological Chemistry*, vol. 250, no. 10, pp. 4007–4021, 1975.

[44] O. Vesterberg, "Isoelectric focusing of proteins in polyacrylamide gels," *Biochimica et Biophysica Acta: Protein Structure*, vol. 257, no. 1, pp. 11–19, 1972.

[45] S. J. Duthie and A. R. Collins, "The influence of cell growth, detoxifying enzymes and DNA repair on hydrogen peroxide-mediated DNA damage (measured using the comet assay) in human cells," *Free Radical Biology and Medicine*, vol. 22, no. 4, pp. 717–724, 1997.

[46] J. M. Gutiérrez, B. Lomonte, G. León et al., "Trends in snakebite envenomation therapy: scientific, technological and public health considerations," *Current Pharmaceutical Design*, vol. 13, no. 28, pp. 2935–2950, 2007.

[47] L. Touqui and M. Alaoui-El-Azher, "Mammalian secreted phospholipases A_2 and their pathophysiological significance in inflammatory diseases," *Current Molecular Medicine*, vol. 1, no. 6, pp. 739–754, 2001.

[48] S.-J. Choi, R. Parent, C. Guillaume et al., "Isolation and characterization of Psalmopeotoxin I and II: two novel antimalarial peptides from the venom of the tarantula *Psalmopoeus cambridgei*," *FEBS Letters*, vol. 572, no. 1–3, pp. 109–117, 2004.

[49] V. M. Gomes, A. O. Carvalho, M. da Cunha et al., "Purification and characterization of a novel peptide with antifungal activity from *Bothrops jararaca* venom," *Toxicon*, vol. 45, no. 7, pp. 817–827, 2005.

[50] Z. Fan, L. Cao, Y. He et al., "Ctriporin, a new anti-methicillin-resistant *Staphylococcus aureus* peptide from the venom of the scorpion *Chaerilus tricostatus*," *Antimicrobial Agents and Chemotherapy*, vol. 55, no. 11, pp. 5220–5229, 2011.

[51] L. Feitosa, W. Gremski, S. S. Veiga et al., "Detection and characterization of metalloproteinases with gelatinolytic, fibronectinolytic and fibrinogenolytic activities in Brown spider (*Loxosceles intermedia*) venom," *Toxicon*, vol. 36, no. 7, pp. 1039–1051, 1998.

[52] R. B. da Silveira, A. C. M. Wille, O. M. Chaim et al., "Identification, cloning, expression and functional characterization of an astacin-like metalloprotease toxin from *Loxosceles intermedia* (brown spider) venom," *Biochemical Journal*, vol. 406, no. 2, pp. 355–363, 2007.

[53] D. C. Gowda and E. A. Davidson, "Isolation and characterization of novel mucin-like glycoproteins from cobra venom," *Journal of Biological Chemistry*, vol. 269, no. 31, pp. 20031–20039, 1994.

[54] A. V. Osipov, M. V. Astapova, V. I. Tsetlin, and Y. N. Utkin, "The first representative of glycosylated three-fingered toxins: cytotoxin from the *Naja kaouthia* cobra venom," *European Journal of Biochemistry*, vol. 271, no. 10, pp. 2018–2027, 2004.

[55] A. Zelanis, S. M. T. Serrano, and V. N. Reinhold, "N-glycome profiling of *Bothrops jararaca* newborn and adult venoms," *Journal of Proteomics*, vol. 75, no. 3, pp. 774–782, 2012.

[56] C. R. Tugmon and E. K. Tillinghast, "Protease and protease inhibitors of the spider *Argiope aurantia* (Araneae, Araneidae)," *Naturwissenschaften*, vol. 82, no. 4, pp. 195–197, 1995.

[57] M. J. Foradori, L. M. Keil, R. E. Wells, M. Diem, and E. K. Tillinghast, "An examination of the potential role of spider digestive proteases as a causative factor in spider bite necrosis," *Comparative Biochemistry and Physiology C: Toxicology and Pharmacology*, vol. 130, no. 2, pp. 209–218, 2001.

[58] M. J. Foradori, E. K. Tillinghast, J. S. Smith, M. A. Townley, and R. E. Mooney, "Astacin family metallopeptidases and serine peptidase inhibitors in spider digestive fluid," *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology*, vol. 143, no. 3, pp. 257–268, 2006.

[59] T. Soares, F. R. B. Ferreira, F. S. Gomes et al., "The first serine protease inhibitor from *Lasiodora* sp. (Araneae: Theraphosidae) hemocytes," *Process Biochemistry*, vol. 46, no. 12, pp. 2317–2321, 2011.

[60] R. A. Hutton and D. A. Warrell, "Action of snake venom components on the haemostatic system," *Blood Reviews*, vol. 7, no. 3, pp. 176–189, 1993.

[61] H. C. Castro, R. B. Zingali, M. G. Albuquerque, M. Pujol-Luz, and C. R. Rodrigues, "Snake venom thrombin-like enzymes: from reptilase to now," *Cellular and Molecular Life Sciences*, vol. 61, no. 7-8, pp. 843–856, 2004.

[62] A. S. Kamiguti, C. R. M. Hay, R. D. G. Theakston, and M. Zuzel, "Insights into the mechanism of haemorrhage caused by snake venom metalloproteinases," *Toxicon*, vol. 34, no. 6, pp. 627–642, 1996.

[63] J. M. Gutiérrez and A. Rucavado, "Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage," *Biochimie*, vol. 82, no. 9-10, pp. 841–850, 2000.

[64] F. T. T. Trindade, R. G. Stabeli, A. A. Pereira, V. A. Facundo, and A. de Almeida e Silva, "*Copaifera multijuga* ethanolic extracts, oil-resin, and its derivatives display larvicidal activity against *Anopheles darlingi* and *Aedes aegypti* (Diptera: Culicidae)," *Revista Brasileira de Farmacognosia*, vol. 23, no. 3, pp. 464–470, 2013.

[65] C. R. Carlini and M. F. Grossi-De-Sá, "Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides," *Toxicon*, vol. 40, no. 11, pp. 1515–1539, 2002.

[66] G. M. Nicholson, "Fighting the global pest problem: preface to the special Toxicon issue on insecticidal toxins and their potential for insect pest control," *Toxicon*, vol. 49, no. 4, pp. 413–422, 2007.

[67] O. M. Chaim, D. Trevisan-Silva, D. Chaves-Moreira et al., "Brown spider (Loxosceles genus) venom toxins: tools for biological purposes," *Toxins*, vol. 3, no. 3, pp. 309–344, 2011.

[68] M. J. Windley, V. Herzig, S. A. Dziemborowicz, M. C. Hardy, G. F. King, and G. M. Nicholson, "Spider-venom peptides as bioinsecticides," *Toxins*, vol. 4, no. 3, pp. 191–227, 2012.

[69] A. Rohou, J. Nield, and Y. A. Ushkaryov, "Insecticidal toxins from black widow spider venom," *Toxicon*, vol. 49, no. 4, pp. 531–549, 2007.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 196754, 19 pages
http://dx.doi.org/10.1155/2014/196754

Review Article

Snake Venom L-Amino Acid Oxidases: Trends in Pharmacology and Biochemistry

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Received 20 October 2013; Revised 13 December 2013; Accepted 16 December 2013; Published 12 March 2014

Academic Editor: Fernando Albericio

L-amino acid oxidases are enzymes found in several organisms, including venoms of snakes, where they contribute to the toxicity of ophidian envenomation. Their toxicity is primarily due to enzymatic activity, but other mechanisms have been proposed recently which require further investigation. L-amino acid oxidases exert biological and pharmacological effects, including actions on platelet aggregation and the induction of apoptosis, hemorrhage, and cytotoxicity. These proteins present a high biotechnological potential for the development of antimicrobial, antitumor, and antiprotozoan agents. This review provides an overview of the biochemical properties and pharmacological effects of snake venom L-amino acid oxidases, their structure/activity relationship, and supposed mechanisms of action described so far.

1. Composition of Snake Venoms

During the continuing evolution of snakes, according to Kardong [1], the development of more specialized glandular venom was essential in the emergence of biologically active substances capable of weakening prey to facilitate their capture. At first the discharge's main function was to lubricate the snake's food, but with the passage of time, some enzymes mixed with secretions allowing for the emergence of more elaborate and potentially toxic proteins, used in the immobilization of prey. A quantitative increase in the production of these secretions as well as a qualitative improvement of toxic proteins promoted a gain in absolute discretion in defense against predators [2]. Qualitatively, snake venoms consist of a mixture of protein with or without catalytic activity such as phospholipases A_2 (PLA_2), proteases, hyaluronidases, L-amino acid oxidases (LAAOs), acetylcholinesterases, growth factors, protein C activators, lectins, and von Willebrand factor-binding proteins; peptides mainly comprising bradykinin potentiators and disintegrins; low molecular weight organic compounds such as carbohydrates, serotonin, histamine, citrate, and nucleosides; and inorganic ions such as calcium, cobalt, magnesium, copper, iron, and potassium, as well as enzymatic inhibitors [3].

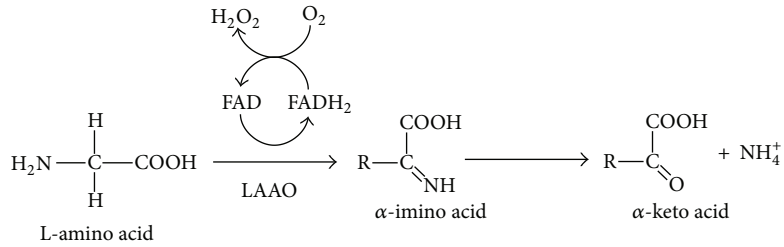


Figure 1: Mechanism of chemical reaction catalyzed by L-amino acid oxidases (LAAOs) [37].

2. L-Amino Acid Oxidases

LAAOs are widely distributed in many different species including insects, fungi, bacteria, and snakes [4] and are even found in plants where one of their catalytic products, ammonia, is used as a nitrogen source in cell metabolism [5, 6].

LAAO activity was first observed by Krebs [7] in hepatic and renal tissue homogenates. Subsequently, Blanchard et al. [8] isolated the first LAAO from a rat kidney. Regarding snake venoms, this class of molecules was only detected in 1944 by Zeller and Maritiz [9] who studied the venom of *Vipera aspis*. In 1979, Iwanaga and Suzuki [10] described the potential of LAAOs as enzymes when observing a highly specific chemical reaction with L-amino acids. Snake venom LAAOs (SV-LAAOs) are usually homodimeric with cofactors FAD (Flavin Adenine Dinucleotide) or FMN (Flavin Mononucleotide) covalently linked to their chemical structure. The yellow color of venoms rich in these enzymes is related to the presence of the pigment riboflavin present in the cofactors, a fact that facilitates its purification. Quantitatively, there are inter- and intraspecific variations in the content of this enzyme in the whole venom (Table 1), and therefore there is color variance between the venoms. In exceptional cases, one gland of the same individual may produce yellow venom and the other gland colorless venom as observed in *Crotalus viridis helleri* [11].

In snake venoms, LAAOs are found in high concentrations that vary according to each species of snakse, which may contribute to the toxicity of the venom. LAAOs exhibit catalytic specificity for long chain hydrophobic and aromatic amino acids and are active in a wide range of pHs and temperatures. Their structures, molecular masses, and isoelectric points are quite varied. They are able to induce changes in platelet function, which cause local effects on plasma clotting disorders among other things. LAAOs are capable of inducing apoptosis in various cell lines and show antimicrobial and antiparasitic activity. According to Ande et al. [36] the existence of LAAOs may be a means of protection against natural agents, parasites, and bacteria.

3. Enzymatic Activity of L-Amino Acid Oxidases

LAAOs (EC 1.4.3.2) are flavoenzymes belonging to the class of oxidoreductases that catalyze the stereospecific oxidative deamination of L-amino acids. During the reduction half-reaction, the amino acid substrate is oxidized to an imino acid, with a concomitant reduction of the FAD cofactor. The imino acid then undergoes nonenzymatic hydrolysis, yielding α-keto acid and ammonia. Another oxidation half-reaction completes the catalytic cycle, reoxidizing $FADH_2$ in the presence of molecular oxygen and thus generating hydrogen peroxide (Figure 1).

LAAOs are considered to be a class of multifunctional enzymes in view of their ability to produce hydrogen peroxide and ammonia, their participation in cell metabolism, and their possible protective effects, including their antiseptic and antimicrobial activities on different organisms. Furthermore, the correlation between the production of LAAOs and their utilization in metabolic pathways involving nitrogen, as well as the production of hydrogen peroxide, opens perspectives for new applications of these enzymes as bactericidal, antiviral, and antitumor agents, making them a promising biotechnological agent. Thus various research groups have studied LAAOs isolated from different snake species [12, 14, 15, 20, 21, 23, 24, 29, 30, 32–35, 38–48].

3.1. Importance of Hydrogen Peroxide. The hydrogen peroxide generated during the enzymatic reaction is a highly toxic oxygen reactive species that is capable of acting on nucleic acids, proteins, and plasma cell membranes [49]. This reactive oxygen species, according to Ande et al. [36], is formed extracellularly, may act directly on cell membranes by altering the permeability of the attacked area, and may also be involved in necrosis or apoptosis. The process of necrosis could be related to the direct action of hydrogen peroxide on the plasma cell membrane, since within the mechanism of apoptosis the development of morphological, biochemical, and molecular changes leads to cell death. The most common morphological changes were chromatin condensation, reduction and disintegration of nucleolus volume, and others. It also seems to be involved in the cytotoxic mechanisms of the enzyme which may ultimately represent another defense mechanism of the organism in response to the environment.

3.2. Enzymatic Kinetics of L-Amino Acid Oxidases. Kinetic studies suggest that LAAOs present preferential catalytic specificity for hydrophobic and aromatic L-amino acids (Table 1), whereas their affinity for polar and basic amino acids is low [12, 15, 16, 21, 24, 28, 30, 32, 33, 35, 44, 46, 47].

Table 1: Biochemical profile of L-amino acid oxidase isolated from snake venoms.

Toxin	Venom	MW	pI	Specific activity	Purification column	% of venom	Reference
CdcLAAO	*Crotalus durissus cumanensis*	55 kDa	8.0	L	Sephacryl S-200, RP-HPLC	ND	[12]
Cr-LAAO	*Calloselasma rhodostoma*	ND	ND	ND	ND	ND	[13]
Oh-LAAO	*Ophiophagus hannah*	64 kDa*	**ND**	M, L, H, K, I	Sephadex G-100, Q Column, HiTrap Heparin HP	**ND**	[14]
Lm-LAAO	*Lachesis muta*	60 kDa	6.28	L	Sephacryl S100, MonoQ	ND	[15]
DrLAO	*Daboia russelii*	63.6 kDa	**ND**	L	Superdex 75, Mono Q, Heparin-Sepharose	0.9%	[16]
Bl-LAAO	*Bothrops leucurus*	57 kDa	**ND**	L	Sephacryl S-200, Sephacryl S-300, DEAE Sepharose CL-6B	3.7%	[17]
ND	*Ophiophagus hannah*	65 kDa	**ND**	ND	Resource Q	ND	[18]
Akbu-LAAO	*Agkistrodon blomhoffii ussuriensis*	65 kDa	**ND**	L	DEAE Sephadex A-50 ion-exchange, Sephadex G-75 gel filtration and C4 reverse phase	5%	[19]
BmarLAAO	*Bothrops marajoensis*	72 kDa	**ND**	**ND**	Protein Pack SP 5PW HPLC, Protein Pack SP 5PW anion exchanger, Superdex 200	**ND**	[20]
LAO *B. caeruleus*	*Bungarus caeruleus*	55 kDa	**ND**	E, L, M, I, F, R	DEAE Cellulose, Sephadex G-100	25%	[21]
BF-LAAO	*Bungarus fasciatus*	55 kDa	ND	Y, D, F, E, W, H, Q, I, M, L	SP-Sepharose HP, Heparin-Sepharose FF	0.93%	[22]
***Bothrops jararaca* LAAO**	*Bothrops jararaca*	38.2 kDa*	**ND**	F, Y, L, I	MonoQ, Heparin	1.1%	[23]
Bp-LAAO	*Bothrops pauloensis*	65 kDa*	6.3	M, L, F,I	CM-Sepharose, Phenyl-Sepharose CL-4B, Benzamidine Sepharose and C18 RP-HPLC	**ND**	[24]
BatroxLAAO	*Bothrops atrox*	67 kDa	4.4	M, L, F, W, Y, I	G-75, HPLC-Shodex ES-502N 7C, Lentil Lectin	1.54%	[25]
BiLAO	*Bothrops insularis*	68 kDa	**ND**	**ND**	HPLC AP1, Superdex 75	**ND**	[26]
BjarLAAO-I	*Bothrops jararaca*	60 kDa	5.0	**ND**	Sephadex G-75, Benzamidine-Sepharose, Phenyl-Sepharose	**ND**	[27]
***N. naja oxiana* L-amino acid oxidase**	*Naja naja oxiana*	57 kDa	8.0	M, L, F, W	Sephadex G-50 SF, CM-cellulose CM52, HPS-7	0.15%	[28]
ACTX-8	*Agkistrodon acutus*	28 kDa	8.2	**ND**	DEAE Sepharose F. F., Source 30	**ND**	[29]
Akbu-LAAO	*Agkistrodon blomhoffii ussurensis*	58–60 kDa	**ND**	N, F, Y, L, I, W	Heparin-Sepharose FF, (Q-Sepharose)	1.27%	[19]
***Vipera lebetina* LAAO**	*Vipera lebetina*	66 kDa** or 60.9 kDa*	4.5	M, W, L, H, F, R, I	Sephadex G-100, HPS-7, DEAE-cellulose DE52., CM-cellulose CM52	2.5%.	[30]
Casca-LAO	*Crotalus durissus cascavella*	68 kDa	5.4	ND	Superdex 75	0.28%	[31]
BpirLAAOI	*Bothrops pirajai*	66 kDa	4.9	F, Y, W, L, M, I, V, H	Sephadex G-75, Benzamidine-Sepharose, Phenyl-Sepharose.	**ND**	[32]
***V. berus berus* LAAO**	*Vipera berus berus*	59 kDa, or 57.7** kDa	4.8	M, L, F, I, R, H	Sephadex G-100, DEAE-cellulose, phenyl-agarose	1.8%	[33]

Table 1: Continued.

Toxin	Venom	MW	pI	Specific activity	Purification column	% of venom	Reference
APIT	*Aplysia punctata*	60 kDa	4.59	K, R	Source 15Q 10/40, Superose 12 HR 10/30	**ND**	[34]
Balt-LAAO-I	*Bothrops alternatus*	66 kDa	5.37	F, Y, M, L	Sepharose-IDA, Phenyl-Sepharose, Sephadex G-100	1.0%	[35]

*Deglycosylated protein. **Estimated by MALDI-TOF. ND: not determined.

Table 2: Kinetic parameters of L-amino acid oxidase from snake venom on specific substrates.

Snake	Leu		Met		Trp		Phe		Reference
	K_m (mM)	K_{cat} (s^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_m (mM)	K_{cat} (s^{-1})	
Crotalus durissus cumanensis	9.23	1.8	ND	ND	ND	ND	ND	ND	[12]
Daboia russelii	490	2.153	373	2.193	81	4.056	142	4.130	[16]
Lachesis muta	0.97	ND	ND	ND	ND	ND	ND	ND	[15]
Bungarus fasciatus	60.69	1025.05	15.03	589.33	0.27	98.82	84.08	142.62	[55]
Naja naja oxiana	0.75	47.98	0.885	66.26	0.147	18.04	0.051	17.18	[28]
Agkistrodon blomhoffii ussurensis	0.11	48.22	0.88	24.13	0.023	6.58	0.042	48.23	[19]
Vipera berus berus	0.361	75.16	0.286	74.20	—	—	0.058	28.50	[33]
Vipera lebetina	0.40	52.0	0.65	80.3	0.17	42.65	—	—	[30]
Naja naja kaouthia	0.66	23.4	0.63	24.4	0.29	12.47	0.06	10.75	[56]
Calloselasma rhodostoma	0.63	3.30	0.24	1.65	0.08	0.88	0.05	0.72	[53]
Ophiophagus hannah	0.20	96.2	0.63	65.6	0.10	32.1	0.10	54.1	[57]

ND: not determined.

Positively charged amino acids such as L-lysine and L-arginine present unfavorable electrostatic interactions with the catalytic site of the enzyme [50].

Oxidation catalyzed by LAAOs follows Michaelis-Menten kinetics [6, 14, 15, 28, 29, 31, 33, 34, 46, 48, 49, 51–54]. The kinetic parameters K_m and K_{cat} shown in Table 2 are very useful for the study and comparison of different enzymes in relation to their substrate. Each enzyme presents optimal K_m and K_{cat} values that reflect the cellular environment, substrate concentration, and chemical characteristics of the reaction catalyzed. K_m, the Michaelis-Menten constant, often used as an indicator of the affinity of the enzyme for the substrate, is specific for each L-amino acid oxidized by LAAOs [29], whereas K_{cat} is the number of substrate molecules converted into the product per unit of time. The maximum velocity (V_{max}) reached in each enzymatic reaction is associated with the concentration of the substrate present in the medium and with K_m and is also specific for each substrate [12, 15, 16, 21, 31, 48, 49, 54].

3.2.1. Effect of pH on the Enzymatic Kinetics of L-Amino Acid Oxidases. The oxidation of L-amino acids by LAAOs happens in a wide range of pHs. This maximal specific activity of each LAAO is related to the optimum pH for each type of amino acid acting as the substrate [58]. Paik and Kim [51] extensively studied the relationship between pH and substrate reactivity for LAAOs from snake venom and found different pH curves depending on the amino acid used as the substrate. Solis et al. [59] studied the action of LAAOs isolated from the venom of *Bothrops brazili* on the substrates L-leucine, L-methionine, L-phenylalanine, and L-arginine and observed that the enzyme remained active in a wide range of pH values; however the activity was highest at a pH of 8.5. Other amino acids such as L-isoleucine, L-tryptophan, and L-lysine showed optimum pHs of 7.5, 8.0 and 9.0, respectively; indeed various LAAOs also catalyze specific oxidoreduction reactions within a broad range of medium pHs [6, 15, 21, 29, 31, 34, 44, 47, 53]. The different profiles of specificity in terms of substrate and pH are related to the acid-base behavior of the enzyme in response to the amino acid. At a certain pH, both the enzyme and the substrate are in ionic equilibrium, permitting a better fit of the substrate in the active site of the enzyme and consequent maximum oxidation.

Snake venom LAAOs can suffer two types of reversible inactivation. One factor inducing inactivation is a change in pH to values close to neutral, resulting in a spontaneous structural change of the enzyme to its inactive configuration. If the pH is lowered, the active conformation of the enzyme is restored. The steady state is reached at a pH ranging from 5.5 to 7.5, and inactivation is more extensive at more alkaline pH levels [60]. This type of inactivation can be prevented by the addition of monovalent anions, substrates, and substrate analogs and is characterized by high activation energy.

3.2.2. Effect of Metal Ions and Enzymatic Inhibitors on the Enzymatic Kinetics of L-Amino Acid Oxidases. Mackessy [61] fractionated the venom of *Crotalus ruber ruber* obtaining proteases, phosphodiesterases, and LAAOs. The activity of these enzymes, including that of the LAAOs, was inhibited in the presence of EDTA, N-ethylmaleimide, and 1,10-phenanthroline, as well as PMSF and glutathione. In the presence of enzymatic inhibitors, as mentioned above, LAAO cofactors NAD or FAD are reduced, causing inactivation of the enzyme [62].

Different bivalent ions can activate or inhibit the specific activity of some LAAOs. The LAAO of *Crotalus adamanteus* requires Mg^{2+} [51], whereas the enzymes of *Lachesis muta* and *Bothrops brazili* [59, 63] are inhibited in the presence of Zn^{2+}. Other ions such as manganese and calcium do not affect the activity of these enzymes. The inhibitory action of these ions might be related to their ability to reversibly bind to thiol groups of cysteines present in the active site of the enzyme, reducing its activity [64], so many pharmacological activities of sv-LAAOs are compromised in the presence of some specific ions.

3.2.3. Effect of Temperature on the Enzymatic Activity of L-Amino Acid Oxidases. The specific activity of some LAAOs

depends on the experimental temperature. These enzymes remain active for a variable period of time at a broad range of temperatures (0° to close to 50°C) [21, 24, 28, 30, 32–34, 44, 53, 59]. Exposure to temperatures higher than 55°C results in a gradual decrease in activity caused by disruptions in hydrophobic interactions and hydrogen bonds between the different subunits of the enzyme. Temperatures lower than 25°C are associated with increased inactivation of the enzyme [24, 28, 30, 33, 34, 59, 65]. Moreover, LAAOs are also progressively inactivated when submitted to freezing or lyophilization [15, 24, 28, 30, 33, 66]. These types of inactivation by freezing, and also by alterations in pH as cited above, induce substantial conformational changes that can be demonstrated by circular dichroism [37]. These changes involve alterations in the binding of the enzyme to substrates and lack of binding to arachidonic acid (competitive inhibitor), as well as alterations in the affinity of the flavin coenzyme for electrons. Reversible inactivation by freezing involves specific regions of the catalytic site of the enzyme, affecting the redox properties of the cofactor-substrate complex [60, 67] and decreasing catalytic activity.

4. Purification of L-Amino Acid Oxidases

The first reports of isolation of LAAOs date back to the 1950s when Singer and Kearney [65] characterized an LAAO from *Agkistrodon piscivorus* snake venom. Later Wellner and Meister [68] obtained the crystal structure of LAAO purified from *Crotalus adamanteus* venom.

Snake venom LAAOs have been purified by fast and efficient chromatographic processes, including by size exclusion, ion-exchange, hydrophobic interaction, and affinity chromatographies (Table 1). A large number of these proteins have been isolated using basically the same chromatographic strategy, that is, fractionation of the venom by size exclusion chromatography, followed by hydrophobic interaction chromatography of the fractions of interest. This step can be repeated and, finally, the highly purified protein is applied to reverse-phase HPLC. However, each research group has adapted the steps of isolation to its specific protein and laboratory conditions. Numerous LAAOs have been isolated from different species: *Trimeresurus mucrosquamatus* [52, 69], *Trimeresurus jerdonii* [70], *Agkistrodon halys pallas* [71], *Agkistrodon halys blomhoffii* [72], *Ophiophagus hannah* [57], *Lachesis muta muta* [73], *Naja naja kaouthia* [56], and *Calloselasma rhodostoma* [53, 74]. Various other LAAOs were also purified following the same steps, indicating the process efficiency (Table 1).

5. Biochemical Characterization of L-Amino Acid Oxidases

When analyzed under nondenaturing conditions, LAAOs are usually noncovalently linked homodimeric proteins with a molecular mass of approximately 110–150 kDa. Examples include the LAAO of *Agkistrodon contortrix laticinctus* [75], LAAO of *Trimeresurus mucrosquamatus* [69], Balt-LAAO-I of *Bothrops alternatus* [35], CascaLAO of *Crotalus durissus cascavella* [31], BpirLAAO-I of *Bothrops pirajai* [32], LAAO of *Vipera berus berus* [33], LAAO of *Vipera lebetina* [30], Akbu-LAAO of *Agkistrodon blomhoffii ussurensis* [19], BmooLAAO-I of *Bothrops moojeni* [66], LAAO of *Naja naja oxiana* [28], SSAP of *Sebastes schlegeli* [76], Bp-LAAO of *Bothrops pauloensis* [24], DRS-LAAO of *Daboia russelii siamensis* [47], Akbu-LAAO of *Agkistrodon blomhoffii ussurensis* [19], BmarLAAO of *Bothrops marajoensis* [20], LAO *Bungarus caeruleus* [21], LmLAAO of *Lachesis muta* [15], and DrLAO of *Daboia russelii* [16]. When these toxins are treated under denaturing conditions, the molecular mass of each monomer determined by mass spectrometry is about 50–70 kDa (Table 1).

This variation in molecular mass among different LAAOs might be related to the sites of glycosylation since these enzymes are considered to be glycoproteins [14, 24, 28, 30, 32–35, 66, 69, 77, 78]. The association of carbohydrates with the structure of LAAOs was first detected by the method described by Lowry et al. [79]. This class of enzymes is characterized by a variable percentage of sugars which vary according to snake species: 4% in *Calloselasma rhodostoma*, 2.64% in *Bothrops brazili*, 3.6% in *Bothrops jararaca*, 2 to 5% in *Crotalus adamanteus*, 15% in *Bothrops alternatus*, 13–16% in *Bothrops moojeni*, 12% in *Bothrops atrox,* and, 25% in *Bungarus caeruleus* [21, 25, 35, 53, 59, 66, 80, 81], respectively. Some carbohydrates such as fucose, mannose, galactose, N-Acetylglucosamine, and sialic acid have been identified as associated with these enzymes, accounting for approximately 5.4% (w/w) of total proteins [59, 82, 83]. These sugars are linked to the enzyme through N-glycosidic bonds and probably modulate its physicochemical properties, increasing the solubility and viscosity of the protein and maintaining the stability of electrical charges [34, 66]. Studies have demonstrated that some LAAOs do not lose their catalytic activity after deglycosylation assays using O-glycosidase and PNGase F [24, 27, 32, 35, 66, 78], a finding suggesting that the carbohydrate moiety of the enzyme only plays a structural role or protects the enzyme against proteolysis since snake venoms are rich in proteolytic enzymes [84].

Most LAAOs described so far are variably acidic, with isoelectric points above 4.4 (Table 1). In contrast, some LAAOs are slightly basic and present an isoelectric point of 8.0 or higher, including the LAAO of *Trimeresurus flavoviridis* with a pI of 8.4 [29], LAAO of *Naja naja kaouthia* with a pI of 8.1 [85], LAAO of *Agkistrodon acutus* with a pI of 8.2 [86], and LAAO of *N. naja oxiana* with a pI > 8 [28]. Isoforms of the same LAAO are often present in the same venom, which can be acidic, neutral, or basic [87]. This difference in charge density may alter the pharmacological activities of LAAOs as observed with other snake venom enzymes.

6. Antigenicity of L-Amino Acid Oxidases

In general, snake venoms are strong antigenic inductors due to their high protein content. The variability in snake venom composition raises an additional problem for the production of antivenom serum and thus provides a commercial incentive for the manufacturers of therapeutic agents against ophidian envenomation. Particularly, inter- and intraspecific

Table 3: Sequence of snake venom L-amino acid oxidase deposited in the NCBI database.

Family	Snake	bp*	gi	Reference
Viperidae	*B. n. pauloensis*	1519	195927837	[24]
Viperidae	*Bothrops jararaca*	1452	ND	[27]
Viperidae	*Viridovipera stejnegeri*	1551	33355626	[111]
Viperidae	*Bitis gabonica*	180	38000585	[22]
Viperidae	*Bothrops moojeni*	1436	398441345	[95]
Viperidae	*Bothrops jararacussu*	1491	398441343	[95]
Viperidae	*Crotalus adamanteus*	2787	3426323	[94]
Elapidae	*Bungarus fasciatus*	2815	126035652	[14]
Elapidae	*Naja atra*	1347	126035676	[14]
Elapidae	*Bungarus multicinctus*	2794	126035648	[14]
Elapidae	*Ophiophagus hannah*	2883	126035643	[14]

ND: not determined. *bp: base pairs.

variations in snake venom composition have been demonstrated to affect the neutralization capacity of antivenom sera [88].

Various studies have been carried out to develop alternative methods to improve neutralization of the toxic effects of snake venom envenomation. Our knowledge about immunological cross-reactivity of venoms has evolved from experimental evidence obtained using different approaches. The phenomenon of cross-reactivity between snake venoms is related to the observation that antiserum specifically prepared against the venom of one type of snake may react with other snake venoms [89]. Studies on the cross-reactivity of snake venoms suggest the apparent lack of a correlation between cross-reactions and phylogeny, implying that the results obtained based on antigen recognition do not completely reflect the molecular evolution of snake venoms [90, 91]. The specificity of snake venom antibodies against a fragment of *Bothrops moojeni* LAAO shows that cross-reactivity is mediated, at least in part, by antibodies that are able to recognize another functional protein [92]. This difficulty in neutralizing venoms is mainly related to the damage at the site of the bite.

7. Structural and Molecular Characteristics of L-Amino Acid Oxidases

The development of recombinant DNA techniques and nucleotide and amino acid sequencing has permitted the creation of databases that are shared by various researchers in order to identify the composition of each venom and the key activities of each protein. The N-terminal amino acid sequences of various LAAOs from the snake families, Viperidae and Elapidae, were deduced by Edman degradation, and alignment of these sequences always showed a high identity, even when toxins originating from distinct snake species were compared [31, 32, 35, 75, 83, 93, 94]. cDNA analysis using yeast or *Escherichia coli* as expression vectors showed that partial sequences of venom LAAOs from different snake species also present highly conserved regions along the primary structure of the protein, characterizing high identity between these enzymes [14, 24, 27, 34, 66, 72, 75, 95]. The number of base pairs of these sequenced toxins is highly variable, and most sequences are deposited in the NCBI database (Table 3).

Macheroux et al. [37] deduced the complete sequence of *Calloselasma rhosdostoma* LAAO from cDNA, with the sequence showing a high identity with LAAOs from *Crotalus adamanteus* and *Crotalus atrox*.

cDNA sequencing of LAAOs provides important information for the structural understanding of this class of still poorly explored enzymes. Ali et al. [83] demonstrated the presence of a highly conserved $\beta\alpha\beta$-fold domain in the N-terminal region of an LAAO from *Eristicophis macmahoni* venom, which is responsible for binding the FAD cofactor. Zhang et al. [86] identified a change to asparagine in the second amino acid residue of the N-terminal region of AHP-LAAO from *A. halys pallas* venom, which might play an important role in enzymatic activity since this region is involved in many effects induced by the enzyme.

Multiple alignment of the primary structure of a *Calloselasma rhodostoma* LAAO showed a high similarity (>84%) with other snake venom LAAOs (Table 4). Phylogenetic comparisons between FAD-dependent snake venom LAAOs and other FAD-dependent oxidases, such as monoaminoxidase (MAO), D-amino acid oxidase, and tryptophan 2-monooxygenase, reveal only distant relationships. However, all LAAOs share a highly conserved dinucleotide-binding region with MAO, D-amino acid oxidase, tryptophan 2-monooxygenase, and various other proteins that may also require FAD [37].

Sequences of LAAOs were aligned and analyzed according to the region of the world. The results of the alignment are shown in order of alignment (Figure 2). Most dissimilar regions were found in the C- and N-termini, and higher conservation between sequences was seen in the territories that were occupied more recently by humans (North America and South America). The percentage of global alignment resulted in ~60% similarity.

In the phylogenetic tree (Figure 3), four groups may be distinguished: first South America; second North America and the New World; third China, Japan, and Korea; and

Table 4: Percent of similarity between L-amino acid oxidases from snake venoms.

Sequences	GI	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Calloselasma rhodostoma	20141785	*100.00*	85.36	85.99	88.27	88.76	89.48	86.63	84.69	88.32
Bothrops moojeni	82127389	85.36	*100.00*	87.39	88.77	88.70	88.49	86.40	95.82	88.08
Crotalus adamanteus	6093636	85.99	87.39	*100.00*	87.19	87.94	87.65	85.41	87.35	87.67
Agkistrodon halys	48425312	88.27	88.77	87.19	*100.00*	91.77	99.18	90.95	90.12	90.74
Ovophis okinavensis	538260091	88.76	88.70	87.94	91.77	*100.00*	92.66	89.53	88.95	92.67
Gloydius blomhoffii	75570145	89.48	88.49	87.65	99.18	92.66	*100.00*	91.67	91.07	91.47
Trimeresurus stejnegeri	33355627	86.63	86.40	85.41	90.95	89.53	91.67	*100.00*	86.24	91.49
Bothropoides pauloensis	347602324	84.69	95.82	87.35	90.12	88.95	91.07	86.24	*100.00*	91.09
Protobothrops flavoviridis	538259837	88.32	88.08	87.67	90.74	92.67	91.47	91.49	91.09	*100.00*

fourth Australia and India. The sequence gi|327266254_Anolis_Root_Tree is an LAAO of the lizard *Anolis carolinensis* that was included for the control protocol. Phylogenetic analyses were run using the website http://www.phylogeny.fr/ [96]. Sequences were aligned using the MUSCLE program [97] according to the mer distances clustered by UGPMA. The Gblocks program [98] was used to eliminate poorly aligned positions and divergent regions. PhyML 3.0 was used for phylogenies [99] including substitution models WAG for proteins. The ALTR test (SH-like) was used to access the support values of each branch [100].

Generally, the composition of LAAOs is quantitatively similar, with many asparagine, glutamic acid, and aspartic acid residues and few methionine and tryptophan residues. The number of cysteine residues varies, indicating variations in the tertiary structure of these enzymes [35, 83, 102].

The cDNA-deduced sequence of various snake LAAOs is characterized by the presence of a highly conserved $\beta\alpha\beta$ domain in the N-terminal region that is rich in glutamic acid residues and possibly functions as a binding site for the FAD cofactor, which is fundamental for the generation of hydrogen peroxide [37, 94]. Indeed, determination of the N-terminal region of LNV-LAAO isolated from *Eristicophis macmahoni* venom by Ali et al. [103] showed similarity with other snake venom LAAOs in terms of the large number of glutamic acid residues found in this region, suggesting an important functional role of the N-terminal region of these enzymes [31, 35].

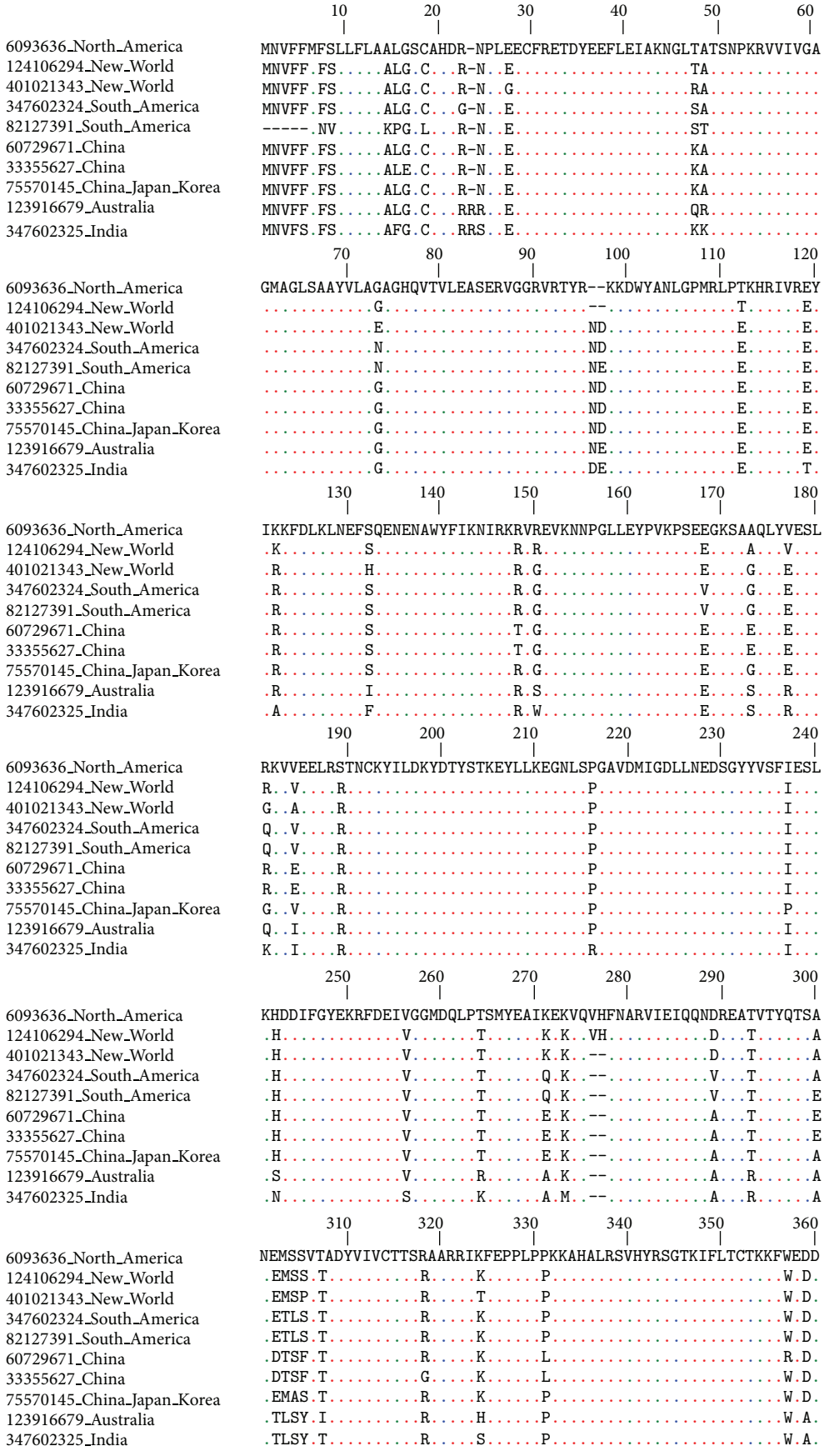
The structure of LAAO from *Calloselasma rhodostoma* was determined in the presence of the ligands citrate, aminobenzoate, and phenylalanine. This analysis showed that the protein consists of three domains: an FAD-binding domain, a substrate-binding domain, and an α-helical domain (Figure 4). The interface between the α-helical domain and the substrate-binding domain forms a 25 Å long funnel, which provides access to the active site. Three aminobenzoate molecules are visible along the funnel, a finding suggesting the trajectory of the substrate to the active site.

The innermost aminobenzoate molecule forms a hydrogen bond with the active site residues, Arg90 and Gly464, and the aromatic portion of the ligand is located in a hydrophobic region. These interactions mimic binding of natural substrates.

Analysis of the surface of the *Calloselasma rhodostoma* LAAO active site showed that the recess has a long Y-shape which allows the substrate to interact with the enzyme in such a way that one portion of the input channel interacts with O_2, and the other is where product release occurs [50]. According to these authors, the active site is dynamic and can undergo conformational changes due to the presence of two amino acid residues, His223 and Arg322. Both residues are located along the driveway to the substrate to the active center. These amino acids can take on two different conformations (A and B), and the His_{223} spends 40% of its time as conformation A and 60% as B. The His_{223} conformation A has an imidazole side chain group which is stabilized with the aid of a water molecule through hydrogen bonding with its neighbors Glu_{209} and Ser_{220}. In conformation B, the imidazole ring is attached to a neighboring phenyl group at a distance of 3.1 Å. The lack of hydrogen bonding with neighboring side chains increases the mobility of this residue. The movements of the imidazole ring of the side chain are probably related to the mechanism of deprotonation of the substrate. Minor conformational changes are observed for Arg_{322}. The two types of conformation of arginine differ in the position of carbons Cδ and Cγ depending on angles between −84° and 73° and the conformation to B.

The position of the guanidine group of the side chain is similar in both conformations, with the Nε stabilized through hydrogen bonding with the hydroxyl of Thr_{432}. The guanidine group may also interact with the side chains of amino acids Glu_{209} and Glu_{219}, making them stable. The movements observed in the side chain of His_{223} and Arg_{322} may be associated with the attachment and release of the substrate and product, respectively.

According to Moustafa et al. [50], during the binding of the substrate to the catalytic site of the enzyme, His_{223} assumes conformation A, blocking the entry of oxygen, thus allowing for the entry of the substrate. When the chemical reaction happens, His_{223} assumes conformation B again, unlocking the entry of oxygen to release the product. The movements in the side chain of Arg_{322}, which change the conformation from form A to form B, are favorable hydrophobic interactions occurring between the aliphatic amino acid side chain and the aromatic ring of the enzyme substrate. After the enzymatic reaction occurs, it returns to form B to facilitate



(a)

Figure 2: Continued.

```
                                370       380       390       400       410       420
                                 |         |         |         |         |         |
6093636_North_America           GIHGGKSTTDLPSRFIYYPNHNFTSGVGVIIAYGIGDDANFFQALDFKDCADIVINDLSL
124106294_New_World             ..............................G..............D...........
401021343_New_World             ..............................G..............D...........
347602324_South_America         ..............................G..............D...........
82127391_South_America          ..............................G..............D...........
60729671_China                  ..............................G..............D...........
33355627_China                  ..............................G..............D...........
75570145_China_Japan_Korea      ..............................G..............D...........
123916679_Australia             ..............................T..............T...........
347602325_India                 ..............................V..............T...........

                                430       440       450       460       470       480
                                 |         |         |         |         |         |
6093636_North_America           IHELPKEDIQTFCHPSMIQRWSLDKYAMGGITTFTPYQFQHFSEALTAPFKRIYFAGEYT
124106294_New_World             ............R..M.........................S.A...P.K.........
401021343_New_World             ............Y..M.........................S.A...P.K.........
347602324_South_America         ............R..M.........................S.A...P.D.........
82127391_South_America          ............R..M.........................S.A...P.D.........
60729671_China                  ............Y..M.........................S.A...H.D.........
33355627_China                  ............Y..M.........................S.A...H.D.........
75570145_China_Japan_Korea      ............Y..M.........................S.P...S.D.........
123916679_Australia             ............Y..K.........................F.I...P.G.........
347602325_India                 ............Y..L.........................I.T...P.G.........

                                490       500       510
                                 |         |         |
6093636_North_America           AQFHGWIDSTIKSGLTAARDVNRASENPSGIHLSNDNEF
124106294_New_World             .QF....D.....GLTA.R...R...NPSGIHLSNDNEF
401021343_New_World             .QF....D.....GLTA.R...R...NPSGIHLSNDNEL
347602324_South_America         .QA....D.....GLTA.R...R...----------
82127391_South_America          .QA....A.....-GPE.L...R...----------
60729671_China                  .HA....D.....GLTA.R...R...NPSGIHLSNDDEL
33355627_China                  .HA....D.....GLTA.R...R...NPSGIHLSNDNEL
75570145_China_Japan_Korea      .EA....D.....GLRA.R...R...Q----------
123916679_Australia             .SV....D.....GLTA.R...L...KPSRIQLSNDNEL
347602325_India                 .TV....D.....GLTA.R...R...KPSRIHLINDNQL
```

(b)

Figure 2: Sequence alignment of L-amino acid oxidases from snake venoms of some regions of the world. The alignment was performed using the program ClustalW [101]. Only nonconserved amino acids are showed.

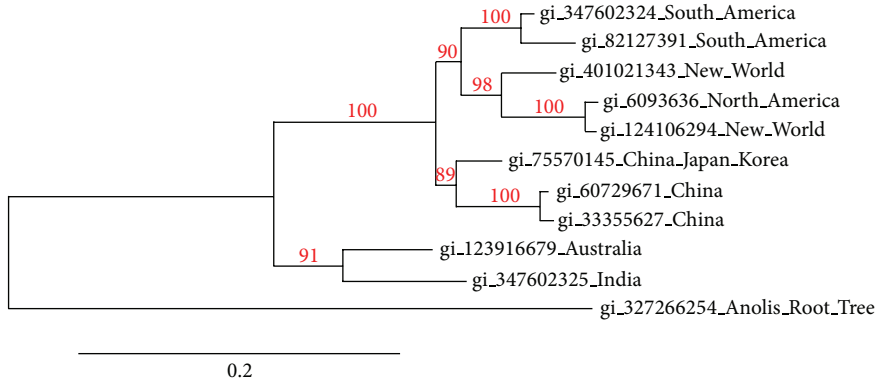


Figure 3: Phylogenetic representation of amino acid sequence alignments of L-amino acid oxidases from snake venoms of some regions of the world. Trees were obtained as described in the Methods section at http://www.phylogeny.fr/. Numbers close to the nodes represent the support value for each branch. The region of the world is shown after the accession numbers. The sequence gi|327266254_Anolis_Root_Tree is the root control of the tree.

the release of the product. The interaction of the substrate with the active site of the enzyme is mediated by the guanidine group of Arg90 and hydrogen bonding with the hydroxyl of Tyr_{372}. The amino group of the substrate forms hydrogen bonds with the carbonyl oxygen atom of the Gly_{464} residue, and then the side chains of the ligands form hydrophobic interactions with the side chains of Ile_{430} and Ile_{374} and Phe_{227}. One of the two oxygen atoms of the carboxylic group of the substrate becomes involved with a water molecule by hydrogen bonding with the flavin N-5, as well as

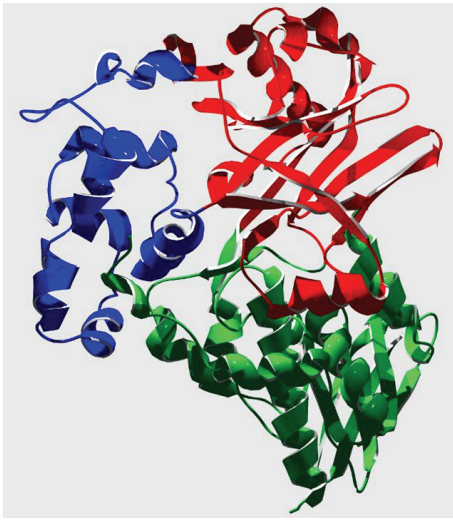


Figure 4: Structure of LAAO from *Calloselasma rhodostoma* (PDB code 2IID) with a resolution of 1.80 Å [50]. The structure was shown in ribbon representation using Swiss-PDBViewer software with renderization POV-ray [104]. In blue is the α-helical domain, in red is the substrate-binding domain, and in green is the FAD-binding domain.

the amino group of the side chain of Lys_{326}. Thus, the α carbon undergoes oxidative attack, generating the product.

Comparisons between the structure of snake venom LAAOs and mammalian D-amino acid oxidases reveal significant differences in the way the substrate arrives at the active site. In addition, a mirror-symmetrical relationship between the two substrate binding sites is observed, which facilitates enantiomer selectivity while the arrangement of atoms involved in catalysis is preserved [102].

8. Biological Effects of L-Amino Acid Oxidases

Since the 1950s when the isolation of different snake venom LAAOs from different species was initiated, studies to identify their activities and to understand their mode of action have also increased. As a result, different assays for the characterization of the toxic and pharmacological effects of these enzymes were standardized to obtain a better understanding. Until the 1990s, studies were restricted to the investigation of the structural and functional characteristics of these enzymes [4], whereas today *in vitro* and *in vivo* studies allow for a broader characterization and understanding of their effects and medical and pharmacological importance.

A range of researchers have characterized the functional properties of crude venoms from different snakes and observed that many of their toxic and pharmacological activities can be attributed to LAAOs [11, 100, 102, 105]. Comparative studies analyzing different snake venoms have demonstrated distinct effects induced by venoms that contain LAAO to a greater or lesser extent [106, 107]. Many of these effects seem to be related, at least in part, to hydrogen peroxide, a secondary product formed during the chemical reaction catalyzed by LAAOs. Du and Clemetson [4] reported a lack of evidence explaining such data, but different studies confirm that the addition of catalase in the presence of LAAOs completely suppresses the toxic effects of these enzymes [14, 20, 21, 24, 25, 27, 28, 30–34, 47–49, 66, 76–78, 95].

8.1. Local Alterations Induced by L-Amino Acid Oxidases

8.1.1. Hemorrhage. The classical route of induction of hemorrhagic processes by snake venoms involves the degradation of extracellular matrix proteins of vascular endothelium. Souza et al. [75] proposed that snake venom LAAOs trigger a process of apoptosis in vascular endothelial cells, causing rupture of the endothelium and concomitant leakage of blood to the interstice. A few LAAOs isolated from snake venoms are able to induce hemorrhage, including ACL-LAO from *Agkistrodon contortrix laticinctus* [75], Balt-LAAO-I from *Bothrops alternatus* [35], ABU-LAO from *Agkistrodon blomhoffii ussurensis* [29], and BatroxLAAO from *Bothrops atrox* [25].

8.1.2. Edema. The edematogenic activity of snake venoms is explained by an increase in vascular permeability that results in the leakage of fluid from blood vessels to the interstitial space of tissues. Some LAAOs have been described as edematogenic, including LNV-LAAO from *Eristicophis macmahoni* [83], TM-LAO from *Trimeresurus mucrosquamatus* [108], Balt-LAAO-I from *Bothrops alternatus* [35], BpirLAAO-I from *Bothrops pirajai* [32], ABU-LAO from *Agkistrodon blomhoffii ussurensis* [29], BmooLAAO-I from *Bothrops moojeni* [66], BatroxLAAO from *Bothrops atrox* [25], BF-LAAO from *Bungarus fasciatus* [55], and DrLAO from *Daboia russelii* [16].

Few studies have been conducted to determine the true mode of action of LAAOs in the induction of edema compared to other classes of snake toxins. Studies investigating different snake toxins revealed that the action of these compounds is related to the stimulation and release of inflammatory mediators such as histamine, prostaglandin, kinins, and serotonin [103]. On the other hand, the edematogenic activity of LAAOs does not seem to be mediated by the mechanisms described for other toxins since these enzymes do not lose their activity in the presence of antihistamines. Indeed, Ali et al. [103] observed that the edematogenic activity of *Ophiophagus hannah* LAAO was not inhibited in the presence of dexamethasone. However, the activity of this enzyme was completely suppressed when treated with glutathione, indicating that edema induced by LAAO is directly related to the presence of hydrogen peroxide.

8.2. Systemic Alterations Induced by L-Amino Acid Oxidases

8.2.1. Platelet Aggregation. The activity of LAAOs on platelets is still controversial, with a variable potential of these enzymes to inhibit or induce platelet aggregation. LAAOs isolated from the venoms of *Echis coloratus* [109], *A. h. blomhoffii* [72], *Vipera berus berus* [33], *Vipera lebetina* [30],

Bothrops leucurus [17], *Ophiophagus hannah* [14], *Agkistrodon blomhoffii ussurensis* [29], *Naja naja oxiana* [28], and *Daboia russelii siamensis* [47] present inhibitory activity on platelet aggregation. Du and Clemetson [4] believe that hydrogen peroxide released by enzymatic action may interfere with the interaction between platelet receptors (GPIIb/IIIa) and fibrinogen, thus impairing the mechanism of aggregation. According to Zhong et al. [47], hydrogen peroxide produced during the catalytic reaction plays a fundamental role in the inhibition of platelet function, but the exact mechanism is still unclear. On the other hand, studies have shown the ability of various LAAOs to induce platelet aggregation [21, 24, 25, 31, 32, 35, 46, 69, 70]. However, this effect was always suppressed in the presence of catalase, indomethacin, and/or aspirin. Thus, the authors suggested that hydrogen peroxide production during oxidation of the substrate is related to this activity. In addition, the possible inhibition of endogenous PLA_2 may play an important role in platelet aggregation. According to Sakurai et al. [85], the controversial results reported might be associated with differences in experimental procedures or plasma preparation.

Nathan et al. [109] demonstrated that the LAAO isolated from *O. hannah* venom induces platelet aggregation. The authors proposed that this effect may not be dependent on ADP but requires thromboxane A_2 since they observed no change in aggregation activity when the creatine phosphokinase/creatine phosphate system, which consumes ADP, was added. In contrast, platelet aggregation was inhibited when aspirin and indomethacin were added in the presence of this LAAO. Catalase and EDTA also inhibited the activity of the enzyme. The authors therefore suggested that the induction of platelet aggregation by this enzyme is intimately related to the formation of hydrogen peroxide and the subsequent synthesis of thromboxane A_2 which requires Ca^{2+}, independent of the release of ADP. According to Zhong et al. [47], the role of hydrogen peroxide in the process of inducting platelet aggregation remains uncertain since recent studies indicate that it is unlikely that hydrogen peroxide alone is responsible for the biological activities of LAAOs; that is, other mechanisms are probably triggered and cause a potent biological response.

8.2.2. Effects on Blood Coagulation. Alterations in blood coagulation induced by snake venoms have also been the target of many studies conducted in Brazil since the 1960s. This is not only due to the large amount of snake bites, but also because these venoms have become tools for the study of the complex processes involved in blood coagulation. These venoms are also used as auxiliary tools for clinical diagnosis and as therapeutic agents to provoke defibrinogenation during thrombosis treatment. Li et al. [110] isolated an LAAO from *Agkistrodon halys blomhoffii* venom, referred to as M-LAO, which presented high anticoagulant potential. This enzyme was found to inhibit the activity of factor IX, involved in the process of coagulation, in a time- and dose-dependent manner. M-LAO showed the same anticoagulant potential when membrane phospholipids were added, thus demonstrating that its inhibitory activity is directly related to the depletion of factor IX activity, destabilizing the intrinsic pathway of blood coagulation.

9. Pharmacological Effects of L-Amino Acid Oxidases

9.1. Antiviral Effect of L-Amino Acid Oxidases. The antiviral effect of LAAOs has yet to be well explored. One study reports the possible inhibition of HIV-1 replication by an LAAO isolated from *Trimeresurus stejnegeri* venom, called TSV-LAO [112]. This activity was demonstrated by a reduction in protein p24 production, which indicates HIV-1 replication and a decrease in syncytium formation. TSV-LAO was unable to block the fusion between HIV-1 and host cells, suggesting that this enzyme does not interfere with the absorption and/or binding of the virus to the host cell.

The antiviral potential of LAAOs was also studied by Sant'Ana et al. [27], who treated cells infected with DENV-3 virus strains, the etiological agent of dengue, with BjarLAAO-I isolated from *Bothrops jararaca* snake venom. The treatment's efficiency was demonstrated by a reduction of viral load in previously infected C6/36 cells exposed to the toxin when compared to controls of unexposed infected cells.

9.2. Antiparasitic Effect of L-Amino Acid Oxidases. Inhibitory activities on the growth of *Trypanosoma cruzi* and *Leishmania donovani infantum* or other species of *Leishmania* have been reported for different snake venoms [113]. This antiparasitic effect might be attributed to the activity of LAAOs [4, 15, 17, 20, 24, 27, 31, 32, 66, 78, 83, 95, 114].

Leishmaniasis is caused by parasites of the genus *Leishmania* spp. and is transmitted to the vertebrate host through the bite of mosquitoes of the genera *Lutzomyia* (Old World) and *Phlebotomus* (New World). Clinical manifestations observed in patients vary according to parasite species, with these parasites being widely distributed in tropical and subtropical regions worldwide. Leishmaniasis comprises a broad spectrum of infectious complications ranging from skin ulcerations to progressive and lethal visceral infections [115]. The first-line drugs for the treatment of this disease are pentavalent antimonials, which have serious side effects and to which the target parasites have shown clinical resistance. Thus, the study of new antiparasitic compounds from different sources, including those with antileishmanial activity, is of biotechnological and medical interest.

Chagas disease is caused by the parasite *Trypanosoma cruzi* and is transmitted by the vector *Triatoma infestans* ("barbeiro"). Epimastigotes are the potentially infectious form. The most common clinical manifestation observed during the chronic phase is cardiomyopathy, which often leads to death of the patient.

The leishmanicidal/trypanocidal effect of LAAOs purified from snake venoms is due to the oxidative stress induced by hydrogen peroxide in infected cells, resulting in proteolytic activity in treated cells. Subsequently, the mitochondrial function of these cells is compromised due to calcium influx, activating other enzymes such as nitric oxide synthetase and phospholipases. These events culminate in an increased

production of free radicals and consequent destruction of genetic material, with the cell entering apoptosis [116].

An LAAO isolated from *B. moojeni* venom presented leishmanicidal activity against promastigote forms of *Leishmania amazonensis* five times higher than that of the crude venom [66, 113]. However, this enzyme showed no activity when tested against amastigote forms, suggesting that this parasite possesses an effective protection system against free radicals and hydrogen peroxide. Pessatti et al. [117] discussed the possibility that while the promastigote is totally deficient in catalase and glutathione peroxidase, the amastigote accumulates high concentrations of the antioxidant enzymes catalase and superoxide dismutase, which were susceptible to the action of the secondary metabolite formed during the oxidation of L-amino acids, justifying effective leishmanicidal activity of this class of toxins.

Murray [118] showed that amastigote forms of *Leishmania donovani* contain three times more catalase and 14 times more glutathione peroxidase than promastigote forms and are therefore four times more resistant to the enzymatic production of hydrogen peroxide, which raised several studies.

Various researchers have attributed the antiprotozoan potential of LAAOs to hydrogen peroxide since the effect disappeared when the enzyme was incubated with catalase [24, 27, 31, 32, 66, 78, 95]. A similar explanation was provided for the trypanocidal effect of LAAOs [27, 66, 95]. svLAAOs may be involved in several other pharmacological activities.

9.3. Bactericidal Effect of L-Amino Acid Oxidases. Since Skarnes [119] observed, for the first time, the bactericidal potential of an LAAO isolated from *Crotalus adamanteus* venom, many other snake venom LAAOs have been shown to be effective against bacteria, irrespective of the genus from which they were isolated: *Trimeresurus jerdonii* [70], *Trimeresurus mucrosquamatus* [69], *Bothrops alternatus* [35], *A. halys* [86], *Crotalus durissus cascavella* [31], *Vipera lebetina* [30], *Bothrops pirajai* [32], *Bothrops moojeni* [66], *Naja naja oxiana* [28], *Bothrops pauloensis* [24], *Bothrops jararaca* [78], *Daboia russelii siamensis* [47], *Agkistrodon blomhoffii ussurensis* [19], *Bothrops marajoensis* [20], *Ophiophagus hannah* [120], *Calloselasma rhodostoma* and *Ophiophagus hannah* [18], and *Crotalus durissus cumanensis* [12].

The true bactericidal mode of action of LAAOs is still not completely understood but seems to be related to the oxidized form of the cofactor of the enzyme (FAD or FMN). This cofactor interacts with L-amino acids that can then act on nucleic acids, proteins, and the plasma membrane. Thus, when in contact with the bacterial membrane hydrogen peroxide can provoke lipoperoxidation [13, 31, 49], DNA fragmentation [29, 32, 75], and consequent cell death. The probable mechanisms induced by LAAOs on proteins involve the enzymatic oxidation of L-amino acids [36].

According to Zhang et al. [86] inhibitory activity of AHP-LAAO isolated from *A. h. pallas* venom on the growth of *Bacillus subtilis* (Gram-positive) and *E. coli* (Gram-negative) was observed. When treated with L-vinylglycine, a substrate that reversibly inhibits the oxidative activity of LAAO [121], the enzyme completely lost its bactericidal activity. The same was observed in the presence of catalase. The authors suggested that the bactericidal activity of AHP-LAAO is related to its oxidative potential. Since L-vinylglycine is a substrate that interacts with the catalytic site of LAAOs and not with the glycan moiety, the loss of bactericidal activity in the presence of this substrate indicates that the presence of carbohydrates is not fundamental for this activity.

The LAAO isolated from *Crotalus durissus cascavella* venom by Toyama et al. [31], called CascaLAO, presented bactericidal activity against *Xanthomonas axonopodis pv. passiflorae* (Gram-negative) and *Streptococcus mutans* (Gram-positive). This activity was demonstrated using transmission electron microscopy by rupturing the plasma membrane of microorganisms and observing consequent leakage of cytoplasmic content and cell death. An immunohistochemical study also demonstrated that achacin, an LAAO purified from *Achatina fulica* [26], binds to the plasma membrane of *Staphylococcus aureus* and *E. coli* during the growth phase, causing death of the bacteria by the mechanisms mentioned above.

According to several investigators [31, 76, 86, 122], the most likely mode of action involved in the bactericidal activity of LAAOs is that hydrogen peroxide causes oxidative stress in the target cell, triggering disorganization of the plasma membrane and cytoplasm and consequent cell death.

9.4. Cytotoxic and Apoptotic Effects of L-Amino Acid Oxidases. The mechanisms of cytotoxicity induced by LAAOs involve two processes: necrosis and apoptosis. Necrosis is related to the direct action of the enzyme or its catabolic products on the plasma membrane of the cell, promoting its degeneration [29, 36, 123]. In contrast, apoptosis is initiated through various pathways; one of them is the generation of reactive oxygen species and free radicals [124]. One common pathway of cell death involves the products of tumor suppressor genes, including p53, which induce apoptosis. Protein p53 participates in growth inhibition and DNA repair or apoptosis after DNA damage induced by cytotoxic agents [125]. Due to the central role of p53 in many human cancers, including gliomas, its regulation and expression might be a potential target in glial cell cancer therapy [126].

The induction of apoptosis in tumor cells is one of the most important mechanisms of anticancer agents. Apoptotic events coincide with morphological, biochemical, and molecular alterations that lead to cell death. The most frequent morphological alterations include chromatin condensation, disintegration of the nucleolus, and a reduction in cell volume. Biochemical alterations culminate in the production of oxidant enzymes, and molecular changes are associated with the fragmentation of DNA, as shown in Table 5.

According to Ponnudurai et al. [53], this biological effect may result from a secondary action of hydrogen peroxide produced during the oxidation reaction of the substrate. This theory is supported by the finding that the addition of catalase or GSH (glutathione) to LAAOs coincides with the loss of activity of these enzymes. Increasing evidence supporting this hypothesis includes the finding that hydrogen peroxide is a mediator of apoptosis that directly acts on oxidative cell metabolism [4].

Table 5: Cytotoxic and apoptotic effects of L-amino acid oxidases isolated from snake venom.

Venom	Toxin	Cell line (DNA)	Concentration and treatment time	Reference
Lachesis muta	LmLAAO	LL-24, AGS, MCF-7, and HUTU	1.17–75 μg/mL for 24 h	[15]
Bothrops leucurus	Bl-LAAO	MKN-45 and RKO	0.1–20 μg/mL for 24 h	[17]
Bungarus fasciatus	BF-LAAO	A549	0.03–3.0 μg/mL for 12 h	[55]
Bothrops atrox	LAAO	PC12, B16F10, HL-60, and Jurkat	5–50 μg/mL for 4 h	[45]
Bothrops moojeni	BmooLAAO-I	HL-60	8–16 μg/mL for 12 h	[66]
Agkistrodon acutus	ACTX-8	HeLa	20 μg/mL for 12–48 h	[29]
Bothrops pirajai	BpirLAAO-I	Fago M13mp18	1–20 μg/mL for 24 h	[32]
Vipera berus berus	*V. berus berus* LAAO	HeLa and K562	2.5–10 μg/mL for 7–24 h	[33]
Trimeresurus flavoviridis	OHAP-1	RBR17T and C6	2.5 and 5 μg/mL for 24 h	[48]
Eristicophis macmahoni	LNV-LAO	MM6	25–100 μg/mL for 18 h	[83]
Agkistrodon contortrix laticinctus	ACL LAO	HL-60	2.5–100 μg/mL for 16 h	[75]

Mosmann [127] observed that hydrogen peroxide induces the upregulation of Fas in human endothelial cells and that the activation of tyrosine kinase might be involved in the hydrogen peroxide-induced expression of Fas. Fas is a type I membrane protein belonging to the tumor necrosis factor and nerve growth factor receptor family and mediates a death signal. Thus, Fas-mediated apoptosis in human endothelial cells may contribute to the mechanism of hydrogen peroxide-induced endothelial cell injury.

Several of the LAAOs isolated have been considered cytotoxic, including APIT to Jurkat T cells [34], *Vipera berus berus* LAAO to HeLa and K562 cells [33], BpirLAAO-I to S180 cells and macrophages [32], ABU-LAO to human monocytes and T cells [29], BmooLAAO-I to Ehrlich ascites tumor cells [66], BatroxLAAO to HL-60, Jurkat, B16F10, and PC12 cells [25], ACTX-8 to HeLa cells [29], BjarLAAO-I to Ehrlich ascites tumour [27], BF-LAAO to A549 cells [55], BpLAAO to SKBR3 breast carcinoma and Jurkat leukemia cells [24], BmarLAAO to macrophages [20], Bl-LAAO to LL-24, RKO, HUTU, and MKN-45 cells [17], and LmLAAO to AGS cells-gastric adenocarcinoma and MCF-7 cells-breast adenocarcinoma [15].

DiPietrantonio et al. [128] detected an increased activity of caspase 3 in HL-60 cells exposed to hydrogen peroxide. Caspases are proteases of the cysteine family, which are common signaling molecules of apoptosis. Zhang et al. [129] demonstrated that TSV-LAO isolated from *T. stejnegeri* venom presents cytotoxicity in a human leukemia T cell line (C8166) by inducing chromatin condensation and nuclear morphological changes, which are typical phenomena of apoptosis.

Parallel to the treatment of svLAAOs cells infected with parasites, some authors do a first screening with different concentrations of the toxin on normal cells of the same lineage. The dose range that results in cell viability analyzed by MTT above 80% was adopted as criteria for further experiments. Under these conditions, the cells remain viable, but when they are infected with parasites and treated with different doses of the toxin in the range of previously found good viability, the intracellular multiplication of the parasite is inhibited [32].

10. Pharmacological Applications of L-Amino Acid Oxidases

According to Kitani et al. [130], SV-LAAOs have a large potential as cytotoxic drugs, based on cell viability obtained by MTT, according to the protocol described by Mosmann [127]. Previously published data have demonstrated cytotoxic activity to be an important characteristic of these enzymes, which started to receive attention for the development of new antimicrobial agents.

Sun et al. [19] demonstrated a protective effect of rat milk LAAOs on mammary glands. This effect is due to a probable antiseptic action of the enzyme when incorporated into milk. Inflammation and infections in the mammary glands are reduced by the presence of the antimicrobial agent, when continually secreted with milk.

According to Nuutinen and Timonen [6], the basidiomycetes *Laccaria bicolor* and *Hebeloma* spp., which live in symbiosis with tree roots of temperate and boreal forests, express the LAAO enzyme, whose function is to oxidize classical amino acids, generating nitrogen that undergoes mineralization to ammonia (NH^{4+}). Later, the nitrogen from this ammonia is utilized in the construction of amino acids by the host plant, besides ensuring the recycling of nitrogen derived from amino acids.

Achacin, an LAAO secreted in the mucus of the giant African snail (*Achatina fulica*), seems to protect this snail against aggressors, with the mucus forming a protective barrier against bacteria and fungi. Another LAAO, called SSAP, which is synthesized by the skin of the rockfish (*Sebastes schlegeli*) in the form of mucus, also exerts protective effects similar to those promoted by achacin [128, 130]. This antimicrobial activity is probably related to the mechanism of action of the protein, which exerts a bactericidal effect by producing hydrogen peroxide and can be used as a natural repellent.

There are few leishmanicidal agents for the current leishmaniasis clinical therapy; in addition, visceral leishmaniasis may affect the liver and spleen and can become potentially lethal. Again, hydrogen peroxide was found to play a key role in the cytotoxic effect of the enzyme. However, partial

retention of enzymatic activity after the addition of catalase showed the existence of other unknown mechanisms involved in the leishmanicidal and bactericidal effects of LAAO.

There are few effective drugs for the treatment of leishmaniasis, and in the visceral form this disease can affect the liver and spleen and can become potentially lethal; thus, LAAOs could be used as a potentiator of leishmanicidal action. However, its role as a toxin prevents everyday use, due to its nonspecific action. One way to use these toxins as possible therapeutic drugs is to alleviate them without losing the potential toxic effects, permitting their use in therapies or as models for the development of new drugs.

In 1997, the Korean group of Ahn et al. [108] published a study on the *Ophiophagus hannah* LAAO in which its cytotoxicity to tumors cells was evaluated using radioactively labeled thymidine uptake assays. Cytotoxicity was observed in stomach cancer, murine melanoma (B16F10), fibrosarcoma, colorectal cancer, and Chinese hamster ovary cell lines. Markland [131] suggested that this enzyme probably prevents the adhesion of tumor cells and metastases in the host by inhibiting platelet aggregation, in addition to promoting the attack of natural phagocytic cells of the immune system.

Another activity of svLAAOs is their antifung effect. According to Costa Torres et al. [20], a toxin called Bmar-LAAO isolated from the snake venom of *Bothrops marajoensis* is able to inhibit the growth of *Candida albicans*. Recently Cheng et al. [132] published a study showing that LAAOs induce apoptosis by DNA fragmentation of *Botrytis cinerea*.

11. Concluding Remarks

SvLAAOs, potentially toxic proteins, are present in different genera and families of snakes and are responsible for several biological activities. They catalyze a redox reaction of different groups of amino acids, generating hydrogen peroxide as a catabolic product. This reactive oxygen species so far seems to be the molecule responsible for the pharmacological effects of this class of enzymes.

Different LAAOs have been found to be valuable molecules with possible future applications to the treatment of many diseases and as models for the development of antiviral, antitumor, antiparasitic, and antimicrobial drugs. However, the development of therapeutic agents based on the structure of widely characterized molecules previously isolated from snake venoms is gaining popularity in the search for future drugs.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

The authors are grateful to the Ministry of Science and Technology (MCT), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Tecnologia do Acre (FUNTAC/FDCT), Coordenação de Aperfeiçoamento de Nível Superior (CAPES) Projeto NanoBiotec, Rede de Biodiversidade e Biotecnologia da Amazônia Legal (BIONORTE/CNPq/MCT), Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT-INPeTAm/CNPq/MCT) e Instituto Nacional para Pesquisa em Toxinas (INCT-Tox), and the Secretary of Development of Rondonia State (SEPLAN/PRONEX/CNPq) for financial support.

References

[1] K. V. Kardong, "The evolution of the venom apparatus in snakes from Colubrids and Elapids," *Memórias do Instituto Butantan*, vol. 46, pp. 105–118, 1982.

[2] C. Gans, "Reptilian venom: some evolutionary considerations," in *Biology of the Reptilia*, C. Gans and K. A. Gans, Eds., vol. 8, pp. 1–42, Academic Press, London, UK, 1978.

[3] O. H. P. Ramos and H. S. Selistre-De-Araujo, "Snake venom metalloproteases—structure and function of catalytic and disintegrin domains," *Comparative Biochemistry and Physiology C*, vol. 142, no. 3-4, pp. 328–346, 2006.

[4] X.-Y. Du and K. J. Clemetson, "Snake venom L-amino acid oxidases," *Toxicon*, vol. 40, no. 6, pp. 659–665, 2002.

[5] T. Nishizawa, C. C. Aldrich, and D. H. Sherman, "Molecular analysis of the rebeccamycin L-amino acid oxidase from *Lechevalieria aerocolonigenes* ATCC 39243," *Journal of Bacteriology*, vol. 187, no. 6, pp. 2084–2092, 2005.

[6] J. T. Nuutinen and S. Timonen, "Identification of nitrogen mineralization enzymes, l-amino acid oxidases, from the ectomycorrhizal fungi *Hebeloma* spp. and *Laccaria bicolor*," *Mycological Research*, vol. 112, no. 12, pp. 1453–1464, 2008.

[7] H. A. Krebs, *The Enzymes*, vol. 217, Delmar Publishers, New York, NY, USA, 1st edition, 1933.

[8] M. Blanchard, D. Green, V. Nocito et al., "L-Amino acid oxidase of animal tissue," *The Journal of Biological Chemistry*, vol. 155, pp. 421–440, 1944.

[9] A. Zeller and A. Maritiz, "Uber eine neue L-aminosaure Oxidase," *Helvetica Chimica Acta*, vol. 27, pp. 1888–1902, 1944.

[10] S. Iwanaga and T. Suzuki, "Enzymes in snake venoms," in *Handbook of Experimental Pharmacology. (Snake Venoms)*, C. Y. Lee, Ed., vol. 52, pp. 75–84, Springer, Berlin, Germany, 1979.

[11] E. K. Johnson, K. V. Kardong, and C. L. Ownby, "Observations on white and yellow venoms from an individual southern Pacific rattlesnake (*Crotalus viridis helleri*)," *Toxicon*, vol. 25, no. 11, pp. 1169–1180, 1987.

[12] L. J. Vargas, J. C. Quintana, J. A. Pereañez et al., "Cloning and characterization of an antibacterial L-amino acid oxidase from *Crotalus durissus cumanensis* venom," *Toxicon*, vol. 64, pp. 1–11, 2013.

[13] T. Ehara, S. Kitajima, N. Kanzawa, T. Tamiya, and T. Tsuchiya, "Antimicrobial action of achacin is mediated by L-amino acid oxidase activity," *The FEBS Letters*, vol. 531, no. 3, pp. 509–512, 2002.

[14] Y. Jin, W.-H. Lee, L. Zeng, and Y. Zhang, "Molecular characterization of l-amino acid oxidase from *king cobra* venom," *Toxicon*, vol. 50, no. 4, pp. 479–489, 2007.

[15] C. Bregge-Silva, M. C. Nonato, S. Albuquerque et al., "Isolation and biochemical, functional and structural characterization of

a novel L-amino acid oxidase from *Lachesis muta* snake venom," *Toxicon*, vol. 60, pp. 1263–1276, 2012.

[16] H.-S. Chen, Y.-M. Wang, W.-T. Huang, K.-F. Huang, and I.-H. Tsai, "Cloning, characterization and mutagenesis of Russell's viper venom l-amino acid oxidase: insights into its catalytic mechanism," *Biochimie*, vol. 94, no. 2, pp. 335–344, 2012.

[17] G. B. Naumann, L. F. Silva, L. Silva et al., "Cytotoxicity and inhibition of platelet aggregation caused by an l-amino acid oxidase from *Bothrops leucurus* venom," *Biochimica et Biophysica Acta*, vol. 1810, no. 7, pp. 683–694, 2011.

[18] S. Kunalan, J. Vejayan, P. Navaratnam et al., "Purification and determination of antibacterial constituent, L-amino acid oxidase from *Calloselasma rhodostoma* and *Ophiophagus Hannah*," *Abstracts Toxins/Toxicon*, vol. 60, pp. 95–248, 2012.

[19] M.-Z. Sun, C. Guo, Y. Tian, D. Chen, F. T. Greenaway, and S. Liu, "Biochemical, functional and structural characterization of Akbu-LAAO: a novel snake venom l-amino acid oxidase from *Agkistrodon blomhoffii ussurensis*," *Biochimie*, vol. 92, no. 4, pp. 343–349, 2010.

[20] A. F. Costa Torres, R. T. Dantas, M. H. Toyama et al., "Antibacterial and antiparasitic effects of *Bothrops marajoensis* venom and its fractions: phospholipase A_2 and l-amino acid oxidase," *Toxicon*, vol. 55, no. 4, pp. 795–804, 2010.

[21] S. S. More, K. M. Kiran, S. M. Veena, and J. R. Gadag, "Purification of an l-amino acid oxidase from *Bungarus caeruleus* (Indian krait) venom," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 16, no. 1, pp. 60–75, 2010.

[22] I. M. B. Francischetti, V. My-Pham, J. Harrison, M. K. Garfield, and J. M. C. Ribeiro, "*Bitis gabonica* (Gaboon viper) snake venom gland: toward a catalog for the full-length transcripts (cDNA) and proteins," *Gene*, vol. 337, pp. 55–69, 2004.

[23] P. Ciscotto, R. A. Machado de Avila, E. A. F. Coelho et al., "Antigenic, microbicidal and antiparasitic properties of an l-amino acid oxidase isolated from *Bothrops jararaca* snake venom," *Toxicon*, vol. 53, no. 3, pp. 330–341, 2009.

[24] R. S. Rodrigues, J. F. da Silva, J. Boldrini França et al., "Structural and functional properties of Bp-LAAO, a new l-amino acid oxidase isolated from *Bothrops pauloensis* snake venom," *Biochimie*, vol. 91, no. 4, pp. 490–501, 2009.

[25] R. M. Alves, G. A. Antonucci, H. H. Paiva et al., "Evidence of caspase-mediated apoptosis induced by l-amino acid oxidase isolated from *Bothrops atrox* snake venom," *Comparative Biochemistry and Physiology*, vol. 151, no. 4, pp. 542–550, 2008.

[26] M. D. M. Braga, A. M. C. Martins, D. N. Amora et al., "Purification and biological effects of l-amino acid oxidase isolated from *Bothrops insularis* venom," *Toxicon*, vol. 51, no. 2, pp. 199–207, 2008.

[27] C. D. Sant'Ana, D. L. Menaldo, T. R. Costa et al., "Antiviral and antiparasite properties of an l-amino acid oxidase from the Snake *Bothrops jararaca*: cloning and identification of a complete cDNA sequence," *Biochemical Pharmacology*, vol. 76, no. 2, pp. 279–288, 2008.

[28] M. Samel, K. Tõnismägi, G. Rönnholm et al., "l-Amino acid oxidase from Naja naja oxiana venom," *Comparative Biochemistry and Physiology B*, vol. 149, no. 4, pp. 572–580, 2008.

[29] L. Zhang and L.-J. Wei, "ACTX-8, a cytotoxic l-amino acid oxidase isolated from *Agkistrodon acutus* snake venom, induces apoptosis in Hela cervical cancer cells," *Life Sciences*, vol. 80, no. 13, pp. 1189–1197, 2007.

[30] K. Tõnismägi, M. Samel, K. Trummal et al., "l-Amino acid oxidase from *Vipera lebetina* venom: isolation, characterization, effects on platelets and bacteria," *Toxicon*, vol. 48, no. 2, pp. 227–237, 2006.

[31] M. H. Toyama, D. D. O. Toyama, L. F. D. Passero et al., "Isolation of a new L-amino acid oxidase from *Crotalus durissus cascavella* venom," *Toxicon*, vol. 47, no. 1, pp. 47–57, 2006.

[32] L. F. M. Izidoro, M. C. Ribeiro, G. R. L. Souza et al., "Biochemical and functional characterization of an l-amino acid oxidase isolated from *Bothrops pirajai* snake venom," *Bioorganic and Medicinal Chemistry*, vol. 14, no. 20, pp. 7034–7043, 2006.

[33] M. Samel, H. Vija, G. Rönnholm, J. Siigur, N. Kalkkinen, and E. Siigur, "Isolation and characterization of an apoptotic and platelet aggregation inhibiting l-amino acid oxidase from *Vipera berus berus* (common viper) venom," *Biochimica et Biophysica Acta*, vol. 1764, no. 4, pp. 707–714, 2006.

[34] D. Butzke, R. Hurwitz, B. Thiede, S. Goedert, and T. Rudel, "Cloning and biochemical characterization of APIT, a new L-amino acid oxidase from *Aplysia punctata*," *Toxicon*, vol. 46, no. 5, pp. 479–489, 2005.

[35] R. G. Stábeli, S. Marcussi, G. B. Carlos et al., "Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom," *Bioorganic and Medicinal Chemistry*, vol. 12, no. 11, pp. 2881–2886, 2004.

[36] S. R. Ande, H. Fussi, H. Knauer et al., "Induction of apoptosis in yeast by L-amino acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*," *Yeast*, vol. 25, no. 5, pp. 349–357, 2008.

[37] P. Macheroux, O. Seth, C. Bollschweiler et al., "L-amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*: comparative sequence analysis and charaterization of active and inactive forms of the enzyme," *European Journal of Biochemistry*, vol. 268, no. 6, pp. 1679–1686, 2001.

[38] S. S. Chen, J. Hudspeth Walgate, and J. A. Duerre, "Oxidative deamination of sulfur amino acids by bacterial and snake venom l-amino acid oxidase," *Archives of Biochemistry and Biophysics*, vol. 146, no. 1, pp. 54–63, 1971.

[39] T. Ubuka and K. Yao, "Oxidative deamination of L-cystine by L-amino acid oxidase from snake venom: formation of S-(2-oxo-2carboxyethylthiol) cysteine and S-(carboxymethylthiol) cystein," *Biochemical and Biophysical Research Communications*, vol. 55, no. 4, pp. 1305–1310, 1973.

[40] E. A. Zeller, "Snake venom action: are enzymes involved in it?" *Experientia*, vol. 33, no. 2, pp. 143–150, 1977.

[41] T. Ubuka, Y. Ishimoto, and K. Kasahara, "Determination of 3-mercaptopyruvate-cysteine disulfide, a product of oxidative deamination of l-cystine by l-amino acid oxidase," *Analytical Biochemistry*, vol. 67, no. 1, pp. 66–73, 1975.

[42] H.-S. Gewitz, J. Piefke, K. Langowska, and B. Vennesland, "The formation of hydrogen cyanide from histidine in the presence of amino acid oxidase and peroxidase," *Biochemistry and Biophisical*, vol. 611, no. 1, pp. 11–26, 1980.

[43] L.-K. Sun, Y. Yoshii, A. Hyodo et al., "Apoptotic effect in the glioma cells induced by specific protein extracted from Okinawa Habu (*Trimeresurus flavoviridis*) venom in relation to oxidative stress," *Toxicology in Vitro*, vol. 17, no. 2, pp. 169–177, 2003.

[44] K. Kasai, T. Ishikawa, T. Komata et al., "Novel l-amino acid oxidase with antibacterial activity against methicillin-resistant *Staphylococcus aureus* isolated from epidermal mucus of the flounder *Platichthys stellatus*," *The FEBS Journal*, vol. 277, no. 2, pp. 453–465, 2010.

[45] R. M. Alves, G. A. Antonucci, H. H. Paiva et al., "Evidence of caspase-mediated apoptosis induced by l-amino acid oxidase

isolated from Bothrops atrox snake venom," *Comparative Biochemistry and Physiology*, vol. 151, no. 4, pp. 542–550, 2008.

[46] X.-L. Wei, J.-F. Wei, T. Li et al., "Purification, characterization and potent lung lesion activity of an l-amino acid oxidase from *Agkistrodon blomhoffii* ussurensis snake venom," *Toxicon*, vol. 50, no. 8, pp. 1126–1139, 2007.

[47] S.-R. Zhong, Y. Jin, J.-B. Wu et al., "Purification and characterization of a new l-amino acid oxidase from *Daboia russellii siamensis* venom," *Toxicon*, vol. 54, no. 6, pp. 763–771, 2009.

[48] L.-K. Sun, Y. Yoshii, A. Hyodo et al., "Apoptotic effect in the glioma cells induced by specific protein extracted from Okinawa Habu (*Trimeresurus flavoviridis*) venom in relation to oxidative stress," *Toxicology in Vitro*, vol. 17, no. 2, pp. 169–177, 2003.

[49] Z. Findrik, B. Geueke, W. Hummel, and D. Vasić-Rački, "Modelling of L-DOPA enzymatic oxidation catalyzed by L-amino acid oxidases from *Crotalus adamanteus* and *Rhodococcus opacus*," *Biochemical Engineering Journal*, vol. 27, no. 3, pp. 275–286, 2006.

[50] I. M. Moustafa, S. Foster, A. Y. Lyubimov, and A. Vrielink, "Crystal structure of LAAO from *Calloselasma rhodostoma* with an l-phenylalanine substrate: insights into structure and mechanism," *Journal of Molecular Biology*, vol. 364, no. 5, pp. 991–1002, 2006.

[51] W. K. Paik and S. Kim, "pH-substrate relationship of l-amino acid oxidases from snake venom and rat kidney," *Biochimica and Biophysica Acta*, vol. 96, no. 1, pp. 66–74, 1965.

[52] M. Ueda, C.-C. Chang, and M. Ohno, "Purification and characteriaztion of L-amino acid oxidase from the venom of *Trimeresurus mucrosquamatus* (Taiwan habu snake)," *Toxicon*, vol. 26, no. 8, pp. 695–706, 1988.

[53] G. Ponnudurai, M. C. M. Chung, and N.-H. Tan, "Purification and properties of the L-amino acid oxidase from Malayan pit viper (*Calloselasma rhodostoma*) venom," *Archives of Biochemistry and Biophysics*, vol. 313, no. 2, pp. 373–378, 1994.

[54] Z. Findrik and D. Vasić-Rački, "Mathematical modelling of amino acid resolution catalyzed by l-amino acid oxidases from *Crotalus adamanteus* and *Crotalus atrox*," *Process Biochemistry*, vol. 43, no. 11, pp. 1186–1194, 2008.

[55] J.-F. Wei, H.-W. Yang, X.-L. Wei, L.-Y. Qiao, W.-Y. Wang, and S.-H. He, "Purification, characterization and biological activities of the l-amino acid oxidase from *Bungarus fasciatus* snake venom," *Toxicon*, vol. 54, no. 3, pp. 262–271, 2009.

[56] N.-H. Tan and S. Swaminathan, "Purification and properties of the L-amino acid oxidase from monocellate cobra (*Naja naja kaouthia*) venom," *International Journal of Biochemistry*, vol. 24, no. 6, pp. 967–973, 1992.

[57] N. H. Tan and M. N. Saifuddin, "Isolation and characterization of an unusual form of L-amino acid oxidase from King cobra (*Ophiophagus hannah*) venom," *Biochemistry International*, vol. 19, no. 4, pp. 937–944, 1989.

[58] D. S. Page and R. L. Van Etten, "L-amino acid oxidase. II. Deuterium isotope effects and the actino mechanism for the reduction of L-amino acid oxidase by L-leucine," *Biochemistry and Biophisical*, vol. 227, no. 1, pp. 16–31, 1971.

[59] C. Solis, E. Escobar, A. Yarleque et al., "Purificación y caracterización de la L-aminoácido oxidasa del veneno de la serpiente *Bothrops brazili* 'Jergón shushupe'," *Revista Peruana de Biologia*, vol. 6, pp. 75–84, 1999.

[60] C. J. Coles, D. E. Edmondson, and T. P. Singer, "Reversible inactivation of L-amino acid oxidase. Properties of the three conformational forms," *The Journal of Biological Chemistry*, vol. 252, no. 22, pp. 8035–8039, 1977.

[61] S. P. Mackessy, "Fractionation of red diamond rattlesnake (*Crotalus ruber ruber*) venom: protease, phosphodiesterase, L-amino acid oxidase activities and effects of metal ions and inhibitors on protease activity," *Toxicon*, vol. 23, no. 2, pp. 337–340, 1985.

[62] B. Mannervick, V. Boggaram, I. Carlberg et al., *The Catalytic Mechanism of Mechanism of Glutathione Reductasa-Flavin and Flavoproteins*, Scientific Societies Press, Tokyo, Japan, 1st edition, 1980.

[63] Y. Cisneros, *Características bioquímicas de una proteína antibacteriana aislada del veneno de Lachesis Muta "Shushupe" [Tesis para optar al Titulo profesional de Biólogo]*, UNMSM, Lima, Peru, 1996.

[64] M. Bender and L. Brubacher, *Catálisis y acción enzimática*, Reverte, Barcelona, Spain, 1st edition, 1977.

[65] T. P. Singer and E. B. Kearney, "The L-amino acid oxidases of snake venoms. II Isolation and Characterization of homogeneous L-amino acid oxidase," *Archives Biochemistry and Biophysics*, vol. 29, no. 1, pp. 190–209, 1950.

[66] R. G. Stábeli, C. D. Sant'Ana, P. H. Ribeiro et al., "Cytotoxic L-amino acid oxidase from *Bothrops moojeni*: biochemical and functional characterization," *International Journal of Biological Macromolecules*, vol. 41, pp. 132–140, 2007.

[67] S. Soltysik, C. M. Byron, G. H. Einarsdottir, and M. T. Stankovich, "The effects of reversible freezing inactivation and inhibitor binding on redox properties of l-amino-acid oxidase," *Biochimica et Biophysica Acta*, vol. 911, no. 2, pp. 201–208, 1987.

[68] D. Wellner and A. Meister, "Crystalline L-amino acid oxidase of *Crotalus adamanteus*," *The Journal of Biological Chemistry*, vol. 235, pp. 2013–2018, 1960.

[69] J.-F. Wei, Q. Wei, Q.-M. Lu et al., "Purification, characterization and biological activity of an L-amino acid oxidase from *Trimeresurus mucrosquamatus* venom," *Acta Biochimica et Biophysica Sinica*, vol. 35, no. 3, pp. 219–224, 2003.

[70] Q.-M. Lu, Q. Wei, Y. Jin, J.-F. Wei, W.-Y. Wang, and Y.-L. Xiong, "L-amino acid oxidase from *Trimeresurus jerdonii* snake venom: purification, characterization, platelet aggregation-inducing and antibacterial effects," *Journal of Natural Toxins*, vol. 11, no. 4, pp. 345–352, 2002.

[71] J. W. Liu, M. Q. Chai, X. Y. Du et al., "Purification and characterization of L-amino acid oxidase from *Agkistrodon halys pallas* venom," *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao*, vol. 34, pp. 305–310, 2002.

[72] H. Takatsuka, Y. Sakurai, A. Yoshioka et al., "Molecular characterization of L-amino acid oxidase from *Agkistrodon halys blomhoffii* with special reference to platelet aggregation," *Biochimica et Biophysica Acta*, vol. 1544, no. 1-2, pp. 267–277, 2001.

[73] E. O. Sánchez and A. Magalhães, "Purification and partial characterization of an L-amino acid oxidase from bushmaster snake (Surucucu Pico de Jaca) *Lachesis muta muta* venom," *Brazilian Journal of Medical and Biological Research*, vol. 24, no. 3, pp. 249–260, 1991.

[74] A. Geyer, T. B. Fitzpatrick, P. D. Pawelek et al., "Structure and characterization of the glycan moiety of L-amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*," *European Journal of Biochemistry*, vol. 268, no. 14, pp. 4044–4053, 2001.

[75] D. H. F. Souza, L. M. Eugenio, J. E. Fletcher et al., "Isolation and structural characterization of a cytotoxic L-amino acid

oxidase from *Agkistrodon contortrix* laticinctus snake venom: preliminary crystallographic data," *Archives of Biochemistry and Biophysics*, vol. 368, no. 2, pp. 285–290, 1999.

[76] Y. Kitani, N. Kikuchi, G. Zhang et al., "Antibacterial action of L-amino acid oxidase from the skin mucus of rockfish *Sebastes schlegelii*," *Comparative Biochemistry and Physiology B*, vol. 149, no. 2, pp. 394–400, 2008.

[77] R. Iijima, J. Kisugi, and M. Yamazaki, "L-Amino acid oxidase activity of an antineoplastic factor of a marine mollusk and its relationship to cytotoxicity," *Developmental and Comparative Immunology*, vol. 27, no. 6-7, pp. 505–512, 2003.

[78] P. Ciscotto, R. A. Machado de Avila, E. A. F. Coelho et al., "Antigenic, microbicidal and antiparasitic properties of an l-amino acid oxidase isolated from *Bothrops jararaca* snake venom," *Toxicon*, vol. 53, no. 3, pp. 330–341, 2009.

[79] O. H. Lowry, N. J. Rosebrrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of Biological Chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[80] G. Oshima and S. Iwanaga, "Occurrence of glycoproteins in various snake venoms," *Toxicon*, vol. 7, no. 3, pp. 235–238, 1969.

[81] A. De Kok and C. Veeger, "Studies on L-amino acid oxidase I. Effects and the competitive inhibitors," *Biochimica and Biophysica Acta*, vol. 33, p. 345, 1968.

[82] A. de Kok and A. B. Rawitch, "Studies on L-amino acid oxidase. II. Dissociation and characterization of its subunits," *Biochemistry*, vol. 8, no. 4, pp. 1405–1411, 1969.

[83] S. A. Ali, F. Hamid, A. Abbasi, Z. H. Zaidi, and D. Shehnaz, "Pharmacological effects of the leaf-nosed viper snake (*Eristocophis macmahoni*) venom and its HPLC fractions," *Toxicon*, vol. 37, no. 8, pp. 1095–1107, 1999.

[84] M. C. Dos Santos, L. C. L. Ferreira, W. D. Da Silva, and M. F. D. Furtado De, "Caracterización de las actividades biológicas de los venenos "amarillo" y "blanco" de *Crotalus durissus ruruima* comparados con el veneno de *Crotalus durissus terrificus*. Poder neutralizante de los antivenenos frente a los venenos de *Crotalus durissus ruruima*," *Toxicon*, vol. 31, no. 11, pp. 1459–1470, 1993.

[85] Y. Sakurai, H. Takatsuka, A. Yoshioka et al., "Inhibition of human platelet aggregation by L-amino acid oxidase purified from *Naja naja kaouthia* venom," *Toxicon*, vol. 39, no. 12, pp. 1827–1833, 2001.

[86] H. Zhang, M. Teng, L. Niu et al., "Purification, partial characterization, crystallization and structural determination of AHP-LAAO, a novel L-amino-acid oxidase with cell apoptosis-inducing activity from *Agkistrodon halys pallas* venom," *Acta Crystallographica D*, vol. 60, no. 5, pp. 974–977, 2004.

[87] B. G. Stiles, F. W. Sexton, and S. A. Weinstein, "Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian king brown or mulga snake) venom," *Toxicon*, vol. 29, no. 9, pp. 1129–1141, 1991.

[88] B. G. Fry, W. Wüster, S. F. R. Ramjan, T. Jackson, P. Martelli, and R. M. Kini, "Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: evolutionary and toxinological implications," *Rapid Communications in Mass Spectrometry*, vol. 17, no. 18, pp. 2047–2062, 2003.

[89] R. D. G. Theakston, D. A. Warrell, and E. Griffiths, "Report of a WHO workshop on the standardization and control of antivenoms," *Toxicon*, vol. 41, no. 5, pp. 541–557, 2003.

[90] N.-H. Tan, K.-K. Lim, and M. I. N. Jaafar, "An investigation into the antigenic cross-reactivity of *Ophiophagus hannah* (king cobra) venom neurotoxin, phospholipase A2, hemorrhagin and L-amino acid oxidase using enzyme-linked immunosorbent assay," *Toxicon*, vol. 31, no. 7, pp. 865–872, 1993.

[91] R. A. Harrison, W. Wüster, and R. D. G. Theakston, "The conserved structure of snake venom toxins confers extensive immunological cross-reactivity to toxin-specific antibody," *Toxicon*, vol. 41, no. 4, pp. 441–449, 2003.

[92] R. G. Stábeli, L. M. Pimenta Magalhães, H. S. Selistre-De-Araujo, and E. B. Oliveira, "Antibodies to a fragment of the *Bothrops* moojeni L-amino acid oxidase cross-react with snake venom components unrelated to the parent protein," *Toxicon*, vol. 46, no. 3, pp. 308–317, 2005.

[93] S. Torii, M. Naito, and T. Tsuruo, "Apoxin I, a novel apoptosis-inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom," *Journal of Biological Chemistry*, vol. 272, no. 14, pp. 9539–9542, 1997.

[94] A. A. Raibekas and V. Massey, "Primary structure of the snake venom L-amino acid oxidase shows high homology with the mouse B cell interleukin 4-induced Fig1 protein," *Biochemical and Biophysical Research Communications*, vol. 248, no. 3, pp. 476–478, 1998.

[95] S. C. França, S. Kashima, P. G. Roberto et al., "Molecular approaches for structural characterization of *Bothrops* l-amino acid oxidases with antiprotozoal activity: cDNA cloning, comparative sequence analysis, and molecular modeling," *Biochemical and Biophysical Research Communications*, vol. 355, no. 2, pp. 302–306, 2007.

[96] Phylogenetic analyses, http://www.Phylogeny.fr/.

[97] R. C. Edgar, "MUSCLE: multiple sequence alignment with high accuracy and high throughput," *Nucleic Acids Research*, vol. 32, no. 5, pp. 1792–1797, 2004.

[98] J. Castresana, "Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis," *Molecular Biology and Evolution*, vol. 17, no. 4, pp. 540–552, 2000.

[99] S. Guindon and O. Gascuel, "A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood," *Systematic Biology*, vol. 52, no. 5, pp. 696–704, 2003.

[100] M. Anisimova and O. Gascuel, "Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative," *Systematic Biology*, vol. 55, no. 4, pp. 539–552, 2006.

[101] J. D. Thompson, D. G. Higgins, and T. J. Gibson, "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice," *Nucleic Acids Research*, vol. 22, no. 22, pp. 4673–4680, 1994.

[102] P. D. Pawelek, J. Cheah, R. Coulombe, P. Macheroux, S. Ghisla, and A. Vrielink, "The structure of L-amino acid oxidase reveals the substrate trajectory into an enantiomerically conserved active site," *The EMBO Journal*, vol. 19, no. 16, pp. 4204–4215, 2000.

[103] S. A. Ali, S. Stoeva, A. Abbasi et al., "Isolation, structural, and functional characterization of an apoptosis-inducing L-amino acid oxidase from leaf-nosed viper (*Eristocophis macmahoni*) snake venom," *Archives of Biochemistry and Biophysics*, vol. 384, no. 2, pp. 216–226, 2000.

[104] N. Guex and M. C. Peitsch, "SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling," *Electrophoresis*, vol. 18, no. 15, pp. 2714–2723, 1997.

[105] A. Bdolah, "Comparison of venoms from two subspecies of the false horned viper (*Pseudocerastes persicus*)," *Toxicon*, vol. 24, no. 7, pp. 726–729, 1986.

[106] J. Bradley, B. J. Berger, and A. R. Bhatti, "Snake venom components and their cross-reactivity: a review," *Biochemistry and Cell Biology*, vol. 67, no. 9, pp. 597–601, 1989.

[107] N.-H. Tan and G. Ponnudurai, "A comparative study of the biological properties of some sea snake venoms," *Comparative Biochemistry and Physiology B*, vol. 99, no. 2, pp. 351–354, 1991.

[108] M. Y. Ahn, B. M. Lee, and Y. S. Kim, "Characterization and cytotoxicity of L-amino acid oxidase from the venom of king cobra (*Ophiophagus hannah*)," *International Journal of Biochemistry and Cell Biology*, vol. 29, no. 6, pp. 911–919, 1997.

[109] I. Nathan, A. Dvilansky, and T. Yirmiyahu, "Impairment of platelet aggregation by *Echis colorato* venom mediated by L-amino acid oxidase or H_2O_2," *Thrombosis and Haemostasis*, vol. 48, no. 3, pp. 277–282, 1982.

[110] Z.-Y. Li, T.-F. Yu, and E. C.-Y. Lian, "Purification and characterization of L-amino acid oxidase from king cobra (*Ophiophagus hannah*) venom and its effects on human platelet aggregation," *Toxicon*, vol. 32, no. 11, pp. 1349–1358, 1994.

[111] J. Wang, Q. Huang, M. Teng, and L. Niu, *Purification, Cloning and Characterization of the L-Amino Acid Oxidase in Snake Trimeresurus Stejnegeri*, School of Life Sciences, University of Science and Technology, 2003.

[112] Y. Sakurai, M. Shima, T. Matsumoto et al., "Anticoagulant activity of M-LAO, L-amino acid oxidase purified from *Agkistrodon halys blomhoffii*, through selective inhibition of factor IX," *Biochimica et Biophysica Acta*, vol. 1649, no. 1, pp. 51–57, 2003.

[113] R. Fernandez-Gomez, H. Zerrouk, F. Sebti, M. Loyens, A. Benslimane, and M. Ali Ouaissi, "Growth inhibition of *Trypanosoma cruzi* and *Leishmania donovani infantum* by different snake venoms: preliminary identification of proteins from *Cerastes cerastes* venom which interact with the parasites," *Toxicon*, vol. 32, no. 8, pp. 875–882, 1994.

[114] A. R. Gonçalves, M. J. Soares, W. De Souza, R. A. DaMatta, and E. W. Alves, "Ultrastructural alterations and growth inhibition of *Trypanosoma cruzi* and *Leishmania major* induced by *Bothrops jararaca* venom," *Parasitology Research*, vol. 88, no. 7, pp. 598–602, 2002.

[115] W. Peters and R. Killick-Kendrick, *The Leishmaniasis in Biology and Medicine*, Academic Press, New York, NY, USA, 1987.

[116] T. M. Buttke and P. A. Sandstrom, "Oxidative stress as a mediator of apoptosis," *Immunology Today*, vol. 15, no. 1, pp. 7–10, 1994.

[117] M. L. Pessatti, J. D. Fontana, M. F. D. Furtado et al., "Screening of *Bothrops* snake venoms for l-amino acid oxidase activity," *Applied Biochemistry and Biotechnology*, vol. 51-52, no. 1, pp. 197–210, 1995.

[118] H. W. Murray, "Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages," *Journal of Experimental Medicine*, vol. 153, no. 5, pp. 1302–1315, 1981.

[119] R. C. Skarnes, "L-amino-acid oxidase, a bactericidal system," *Nature*, vol. 225, no. 5237, pp. 1072–1073, 1970.

[120] M. L. Lee, N. H. Tan, S. Y. Fung, and S. D. Sekaran, "Antibacterial action of a heat-stable form of l-amino acid oxidase isolated from king cobra (*Ophiophagus hannah*) venom," *Comparative Biochemistry and Physiology C*, vol. 153, no. 2, pp. 237–242, 2011.

[121] P. Marcotte and C. Walsh, "Vinylglycine and propargylglycine: complementary suicide substrates for L-amino acid oxidase and D-amino acid oxidase," *Biochemistry*, vol. 15, no. 14, pp. 3070–3076, 1976.

[122] A. G. Tempone, J. Andrade H.F., P. J. Spencer, C. O. Lourenço, J. R. Rogero, and N. Nascimento, "*Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase," *Biochemical and Biophysical Research Communications*, vol. 280, no. 3, pp. 620–624, 2001.

[123] T. Suhara, K. Fukuo, T. Sugimoto et al., "Hydrogen peroxide induces up-regulation of Fas in human endothelial cells," *Journal of Immunology*, vol. 160, no. 8, pp. 4042–4047, 1998.

[124] M. M. De Vieira Santos, C. D. Sant'Ana, J. R. Giglio et al., "Antitumoural effect of an L-amino acid oxidase isolated from *Bothrops jararaca* snake venom," *Basic and Clinical Pharmacology and Toxicology*, vol. 102, no. 6, pp. 533–542, 2008.

[125] A. M. Dipietrantonio, T.-C. Hsieh, and J. M. Wu, "Activation of caspase 3 in HL-60 cells exposed to hydrogen peroxide," *Biochemical and Biophysical Research Communications*, vol. 255, no. 2, pp. 477–482, 1999.

[126] M. Y. Ahn, B. M. Lee, and Y. S. Kim, "Characterization and cytotoxicity of L-amino acid oxidase from the venom of king cobra (*Ophiophagus hannah*)," *The International Journal of Biochemistry and Cell Biology*, vol. 29, no. 6, pp. 911–919, 1997.

[127] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[128] A. M. DiPietrantonio, T. Hsieh, and J. M. Wu, "Activation of caspase 3 in HL-60 cells exposed to hydrogen peroxide," *Biochemical and Biophysical Research Communications*, vol. 255, no. 2, pp. 477–482, 1999.

[129] Y. J. Zhang, J. H. Wang, W. H. Lee et al., "Molecular characterization of *Trimeresurus stejnegeri* venom L-amino acid oxidase with potential anti-HIV activity," *Acta Crystallography*, vol. 60, pp. 974–977, 2004.

[130] Y. Kitani, K. Toyooka, M. Endo et al., "Intra-tissue localization of an antibacterial L-amino acid oxidase in the rockfish *Sebastes schlegeli*," *Developmental and Comparative Immunology*, vol. 39, pp. 456–459, 2013.

[131] F. S. Markland, "Snake venoms and the hemostatic system," *Toxicon*, vol. 36, no. 12, pp. 1749–1800, 1998.

[132] C.-H. Cheng, C.-A. Yang, S.-Y. Liu, C.-T. Lo, and K.-C. Peng, "L-Amino acid oxidase-induced apoptosis in filamentous *Botrytis cinerea*," *Analytical Biochemistry*, vol. 420, no. 1, pp. 93–95, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 195356, 13 pages
http://dx.doi.org/10.1155/2014/195356

Research Article

Purification and Biochemical Characterization of Three Myotoxins from *Bothrops mattogrossensis* Snake Venom with Toxicity against *Leishmania* and Tumor Cells

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Received 16 September 2013; Accepted 3 December 2013; Published 3 March 2014

Academic Editor: Fernando Albericio

Bothrops mattogrossensis snake is widely distributed throughout eastern South America and is responsible for snakebites in this region. This paper reports the purification and biochemical characterization of three new phospholipases A_2 (PLA_2s), one of which is presumably an enzymatically active Asp49 and two are very likely enzymatically inactive Lys49 PLA_2 homologues. The purification was obtained after two chromatographic steps on ion exchange and reverse phase column. The 2D SDS-PAGE analysis revealed that the proteins have pI values around 10, are each made of a single chain, and have molecular masses near 13 kDa, which was confirmed by MALDI-TOF mass spectrometry. The N-terminal similarity analysis of the sequences showed that the proteins are highly homologous with other Lys49 and Asp49 PLA_2s from *Bothrops* species. The PLA_2s isolated were named BmatTX-I (Lys49 PLA_2-like), BmatTX-II (Lys49 PLA_2-like), and BmatTX-III (Asp49 PLA_2). The PLA_2s induced cytokine release from mouse neutrophils and showed cytotoxicity towards JURKAT (leukemia T) and SK-BR-3 (breast adenocarcinoma) cell lines and promastigote forms of *Leishmania amazonensis*. The structural and functional elucidation of snake venoms components may contribute to a better understanding of the mechanism of action of these proteins during envenomation and their potential pharmacological and therapeutic applications.

1. Introduction

Snake venoms contain a complex mixture of components with a wide range of biological and pharmacological activities. More than 90% of their dry weight is composed of proteins, including a variety of enzymes, such as phospholipases A_2, proteases (metallo and serine), L-amino acid oxidases, esterases, as well as many other nonenzymatic proteins and peptides [1–3]. These proteins and peptides can be grouped into a small number of superfamilies based on remarkable similarities in their primary, secondary, and tertiary structures, while showing distinct pharmacologic effects [3].

One important protein superfamily present in all snake venoms is phospholipase A_2 (PLA_2, E.C. 3.1.1.4). PLA_2s are a class of heat-stable and highly homologous enzymes, which

catalyze the hydrolysis of the 2-acyl bond of cell membrane phospholipids releasing free fatty acids such as arachidonic acid and lysophospholipids. PLA_2s have been characterized as the major component of snake venoms, being responsible for several pathophysiological effects caused by snake envenomation, such as neurotoxic, cardiotoxic, myotoxic, cytotoxic, hypotensive, and anticoagulant activities triggering an intense inflammatory reaction with the release of cytotoxins and eicosanoids [4–6]. PLA_2's involvement in a variety of inflammatory diseases and accidents caused by venomous animals has raised medical and scientific interest in this enzyme [7, 8].

Myotoxic PLA_2s from the *Bothrops* species are composed of approximately 110 to 135 amino acid residues and can be divided into two groups: "classical", which contain an aspartate residue at position 49 (Asp49) and catalyze ester bond hydrolysis at the glycerophospholipid *sn*-2 position in a Ca^{2+}-dependent manner; and "variant", which contain a lysine residue at the same position (Lys49). This substitution affects the ability of these proteins to bind to Ca^{2+}, which is an essential cofactor for catalysis, leading to decreased or no catalytic activity [1–5, 7, 9, 10].

The *Bothrops mattogrossensis* snake belongs to the *Bothrops neuwiedi* complex [11]. This snake is found in the eastern region of South America including Bolivia, Brazil, Southeast Peru, Paraguay, Uruguay, and Argentina [12]. The present study describes for the first time the isolation, identification, and functional characterization of three myotoxic phospholipases A_2, named: BmatTX-I (Lys49 PLA_2-like), BmatTX-II (Lys49 PLA_2-like), and BmatTX-III (Asp49 PLA_2) and evaluates their activity against *Leishmania* and tumor cells.

2. Materials and Methods

2.1. Venom. The venom from the *Bothrops mattogrossensis* snake was acquired from Serpentário de Proteínas Bioativas, Batatais-SP, Brazil. This study was authorized by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis—IBAMA, Instituto Chico Mendes de Conservação da Biodiversidade—ICMBio (number: 27131-1) and Conselho de Gestão do Patrimônio Genético—CGEN/Brazil (number 010627/2011-1).

2.2. Animals. Swiss male mice (18–20 g) were used. These animals were housed in temperature-controlled rooms and received water and food *ad libitum* until used. Animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and was approved by the Committee of Ethics on Animal Utilization in Research from the Institute of Research of Tropical Pathologies (IPEPATRO/FIOCRUZ-Rondônia) (protocol number 2012/1).

2.3. Bothrops mattogrossensis Venom Fractioning. Around 250 mg of *B. mattogrossensis* venom was eluted in 1.2 mL of sterile deionized water and centrifuged at 1,530 ×g for 10 minutes and then submitted to cation exchange chromatography in an Akta purifier 10 (GE-Healthcare) using a CM-Sepharose column (27 × 300 mm, GE Healthcare), equilibrated with 50 mM ammonium bicarbonate buffer, pH 8.0, and eluted with a linear gradient of 0–100% 500 mM ammonium bicarbonate, pH 8.0 at a flow rate of 5.0 mL/minute. The fractions were monitored at an absorbance of 280 nm, collected manually, identified, lyophilized, and stored at −20°C. All fractions were submitted to SDS-PAGE and enzymatic activity analyses. The isolation of the three PLA_2s was obtained by liquid chromatography using a Discovery C18 column (25 × 4.6 mm, Supelco) equilibrated with deionized water with 0.1% trifluoroacetic acid (v/v) and eluted with ten volumes of a linear gradient of 0–100% acetonitrile with 0.1% trifluoroacetic acid (v/v) at a flow rate of 1.0 mL/minute. The elution was monitored at an absorbance of 280 nm, manually collected, lyophilized, and stored at −20°C. In order to determine protein concentration, the Bradford method (Bio-Rad) was used with bovine serum albumin (BSA) as a standard [14].

2.4. Monodimensional Electrophoresis (SDS-PAGE). Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was conducted using the method described by Laemmli [15]. The electrophoresis was carried out at 15 mA and 5 W using a conventional molecular weight standard (BioLabs) and a Lys49-PLA_2 homologue isolated from *B. jararacussu* snake venom (BthTX-I) as markers. The gel was stained with Coomassie Brilliant Blue (CBB) G-250 and images of the gel were obtained using *Image Scanner* (GE Healthcare).

2.5. Bidimensional Electrophoresis (2D-SDS-PAGE) and pI Determination. The protein fractions were submitted to electrofocusing in 13 cm strips with pHs ranging from 3 to 10 in a nonlinear form, using an Ettan IPGphor 3 IEF System (GE Healthcare). At the end of the electrofocusing, the strips containing the PLA_2s were equilibrated and transferred to a 12.5% polyacrylamide gel where they were separated according to molecular mass. The electrophoretic run was performed at 25 mA and 100 W during 5.5 hours and at the end of the protein separation the gel was stained with Coomassie Brilliant Blue (CBB) G-250 and images of the gel were captured using *Image Scanner* (Amershan Bioscience).

2.6. Mass Spectrometry (MALDI-TOF). The samples (1 μL) were mixed with a matrix solution composed of sinapinic acid and acetonitrile with 0.1% trifluoroacetic solubilized at a 1 : 1 ratio. The average mass of the protein was obtained in a MALDI-TOF system (Shimatzu Biotech), operated in linear mode using insulin (5,734.5 Da), cytochrome C (12,361.9 Da), apomyoglobin (16,952.2 Da), aldolase (39,212.2 Da), and albumin (66,430.0 Da) as calibrators. The mass spectra obtained were submitted to automatic baseline subtraction.

2.7. N-Terminal Sequencing and Similarity Search. The amino terminal sequence of each isolated PLA_2 previously determined by automated Edman degradation was used to search for sequence similarity. Approximately 50 μg of each isolated PLA_2 corresponding to approximately 2 nmols/mL sample

was submitted for N-terminal amino acid sequencing using the automated Edman degradation method [13] on a PPSQ-33A (Shimadzu) automatic sequencer. A sequence similarity search and multiple sequence alignment were performed in the SWISS-PROT/TREMBL database using the programs FASTA, BLAST, and CLUSTALW2.

2.8. Functional Characterization

2.8.1. Phospholipase Activity with 4-nitro-3(octanoyloxy)benzoic (4N3OBA). Phospholipase activity of the three PLA_2s was assayed following the protocols described by Holzer and Mackessy [16], modified for 96-well plates. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl plus 10 mM $CaCl_2$, and 100 mM NaCl, pH 8.0), 20 μL of 4-nitro-3(octanoyloxy)benzoic (4N3OBA – Biomol, EUA), 20 μL of deionized water, and 20 μL of PLA_2 (5 μg). After the addition of the PLA_2s, the mixture was incubated for 30 minutes at 37°C and the absorbance was determined at 425 nm using an Eon (Biotek) microplate spectrophotometer, for 3-minute intervals. The enzymatic activity was expressed as the reaction's initial velocity (V_o) calculated based on the increase in absorbance.

2.8.2. Phospholipase Activity with Fluorescent Substrate. Phospholipase A_2's (PLA_2) enzymatic activity was evaluated through the hydrolysis of synthetic fluorescent phospholipid, using the fluorescent substrate Acyl 6 : 0 NBD phospholipid, NBD-phosphatidylcholine (NBD-PC) (Avanti Polar Lipids Inc., Alabaster, AL, USA). The assay was performed in a spectrofluorometer (Shimadzu, RF-5301PC, software RFPC) with excitation and emission wavelengths of 460 and 534, respectively. The enzymatic activity of each *B. mattogrossensis* chromatographic fraction (7, 12, 15, 16, 17, and 20) was evaluated over 250 seconds, after the addition of substrate (3.3 μg/mL, final concentration) in a reaction media containing 50 mM Tris-HCl and 8 mM $CaCl_2$ at pH 7.5 at room temperature.

2.8.3. Platelet Aggregation. A platelet aggregation assay was carried out according to the process described by Fuly et al. [17], with modifications. Platelet aggregation was monitored in an Aggregometer (Chrono Log model 490 2D, Havertown, USA) using Platelet-Rich-Plasma (PRP). PRP was obtained from human whole blood of health volunteers (CAAE: 14204413.5.0000.0011). BmatTX-III (rechromatography fraction 15) was incubated with PRP for five minutes at 37°C being stirred constantly, and then, platelet aggregation was triggered by the addition of ADP (15 μM) or collagen (16 μg/mL). Assays were performed at 37°C in siliconized glass cuvettes in a final volume of 300 μL. Control experiments were performed by adding agonists in the absence of peak 15. One hundred percent (100%) platelet aggregation was obtained with a supramaximal concentration of each agonist and determined 6 minutes after the addition of each, while PRP's light transmittance showed 0% aggregation.

2.8.4. Hemorrhage Activity. Mice were injected intradermally in the dorsal region with 50 μg of crude venom dissolved in 50 μL of physiological solution [18]. Controls received 50 μL of physiological solution in identical conditions. After 3 hours, the animals were euthanized through cervical dislocation and the skin was removed. The hemorrhagicactivitywas expressed as size of thehemorrhagic area on the inner surface measured in mm^2.

2.8.5. Coagulation Activity and Minimal Coagulation Dose (MCD) Determination. Coagulation activity was tested using a methodology previously described by Gené et al. [19]. In brief, 200 μL of plasma from mice was distributed in a 96-well plate, and 10 μL of crude venom containing different concentrations (0.312, 0.625, 1.25, 2.5, 5, and 10 μg) of proteins was added. The plate was placed in a thermostatically controlled environment and the optical density was measured every 3 seconds at 600 nm using an Eon microplate spectrophotometer (Biotek) in order to evaluate the smallest concentration of venom able to coagulate 200 μL of plasma/minute.

2.8.6. Proteolytic Activity. Proteolytic activitywas ascertained using azocasein (Sigma) as a substrate according to the procedure described by Charney and Tomarelli [20]. Azocasein solubilized in distilled water (150 μL) was added to 7 μL of the crude venom in different protein concentrations (0.312, 0.625, 1.25, 2.5, 5, and 10 μg) and then the mixture was incubated in a water bath at 37°C for 1 hour. The reaction stopped when 150 μL of 20% (m/v) trichloroacetic acid was added, which was followed by incubation for 30 min and centrifugation at 10,000 ×g for 10 more minutes. Then, 100 μL of the supernatant was transferred to a multiple-well plate and the absorbance was measured at 440 nm using an Eon microplate spectrometer (Biotek) and one enzymatic unit (U) was defined as the amount of enzyme necessary to increase the absorbance by 0.05.

2.8.7. Myotoxic Activity. Myotoxic activity was determined by measuring the creatine kinase (CK) activity in the plasma [21]. Groups of mice were injected in the gastrocnemius muscle with 25 μg of isolated myotoxins diluted in 50 μL of phosphate buffered saline (PBS). Negative controls received an injection of 50 μL of PBS. Three hours after the injections, aliquots of mice blood were collected from the caudal vein, in heparinized capillaries and centrifuged at 1,530 ×g for 20 minutes. Creatine kinase's enzymatic activity was determined using the CK-NAC kinetic kit (Bioclin, Brazil) according to the manufacturer's protocol. Absorbance was measured for 3 minutes at 37°C, in a spectrophotometer at 340 nm. Enzymatic activity was expressed in units/liter (U/L) and each unit consists of the result of the phosphorylation of one nanomol of creatine per minute.

2.8.8. Neutrophil Viability. Neutrophils were collected 6 hours after the intraperitoneal (IP) injection of 1.5 mL of 3% thioglycollate sterile solution according to the method previously described by Call et al. [22]. The animals were euthanized in order to collect the cells and the peritoneal cavity was washed with 3 mL of PBS. The predominance of

neutrophils in the liquid obtained was confirmed by microscopic analysis with glass slides stained with a panoptic dye. The peritoneal neutrophils obtained were suspended in an RPMI culture medium (Gibco-BRL) supplemented with gentamicin (100 μg/mL), L-glutamine (2 mM), and 10% bovine fetal serum (SFB) in order to obtain 2×10^5 cells/100 μL. Next, cellular viability was assayed over 1, 12, and 24 h at 37°C, and 5% CO_2 in which the cells were incubated in a 96-well plate with the previously isolated PLA_2s at a concentration of 12 μg/mL using RPMI as a negative control. After this, the samples were centrifuged and the supernatant removed. Cellular viability was determined using the MTT method [23].

2.8.9. Quantification of Cytokine. EIA was used to evaluate IL-1β cytokine as described by Schumacher et al. [24]. Briefly, the neutrophils (2×10^5 cells/200 μL) were either incubated with the isolated proteins at 3, 6, and 12 μg/mL concentrations (experimental group) or with PMA (positive control group) or with RPMI (negative control) and kept for 12 and 24 hours at 37°C in a humid atmosphere with 5% CO_2. After that, 96-well plates were coated with 100 μL of the capture monoclonal antibody anti-IL-1β and incubated for 18 hours at 37°C. The plate was then washed with washer buffer (PBS/Tween20). After that, 200 μL of blocking buffer, containing 5% bovine serum albumin (BSA) in PBS/Tween20, was added to the wells and the plates were incubated for 1 hour at 37°C. Following this, the wells were washed and 50 μL of either samples or standard were dispensed into each well and the plates were incubated for 2 hours at 37°C. After this, the plate was washed and 100 μL of detection antibody anti-IL-1β was added for 2 hours at 37°C. After incubation and washing, 100 μL of streptavidin-peroxidase was added, followed by incubation and addition of the substrate (100 μL/mL 3,3′,5,5′-tetramethylbenzidine). Finally, sulfuric acid (50 μL) was added to stop the reaction. Absorbances were recorded at 540 and 450 nm and concentration of IL-1β was estimated from standard curves prepared with recombinant cytokine. The results were expressed as pg/mL of IL-1β.

2.8.10. Antitumor Activity. Cytotoxic activity of isolated PLA_2s on human T-cell leukemia (JURKAT) and breast adenocarcinoma (SK-BR-3) lines obtained from the American Culture Collection of Cells (ATCC, American Type Culture Collection, Rockville, MD, USA) were investigated (Figure 6). This activity was assayed by MTT staining as described by Mosmann [25] and adapted by Stábeli et al. [21]. Cells were dispensed in 96-well plates at a density of 5×10^5 cells/mL. After 24 h of incubation, the medium was removed and fresh medium, with or without different concentrations of PLA_2s (BmatTX-I, BmatTX-II, BmatTX-III, or methotrexate), was added to the wells and incubated for another 24 hours. The evaluation of the cytotoxic activity was measured in a spectrophotometer using an interference filter of 570 nm and expressed as a percentage.

2.8.11. Anti-Leishmania Activity. Promastigote forms of *Leishmania amazonensis* (IFLA/BR/67/PH8) were dispensed in a 96-well plate with 1×10^5 cells/well. Different concentrations (3.12, 6.25, 12.5, 25, 50, and 100 μg/mL) of the crude venom of *B. mattogrossensis* and the isolated PLA_2s (BmatTX-I and BmatTX-II) were added to each well. 100 mg/mL of pentamidine was used as a positive control. After an incubation period of 48 h, 10 μL of a 5 mg/mL MTT solution was added. Then, the plates were placed in the oven at 33°C with 5% CO_2 for 4 hours of incubation after which 50 μL of SDS (20%, w/v) was added. Absorbance was monitored at 570 nm. Results were expressed in toxicity percentage following the equation: 1 − (D.O. sample/D.O. control) × 100.

Table 1: Activities induced by *B. mattogrossensis* snake venom.

Effect[a]	Activity
Phospholipase activity[b] (U/mg)	1,864.05
Proteolytic activity[c] (U/10 μg)	3.0 ± 0.1
Hemorrhagic halo[d] (cm^2)	3.33 ± 0.05
Coagulation activity[e] (MCD, μg)	0.325

[a]All experiments were carried out in triplicate. [b]Activity using NOB stained substrate. [c]One enzymatic unit (U) was defined as the quantity of enzyme needed to increase the absorbance by 0.05 UA/440 nm. [d]Values 3 h after incubation with crude venom of *B. mattogrossensis* (50 μg). [e]MCD: the minimum coagulation dose was the dose capable of coagulating 200 μL of citrated plasma in less than a minute.

2.9. Statistical Analysis. Results were expressed as mean +/− standard deviation. An ANOVA test was used to evaluate the significance of the differences observed with P value ≤ 0.05 considered to be significant.

3. Results and Discussion

3.1. Crude Venom Biological Activities. B. mattogrossensis snake venom induced hemorrhage, coagulation, proteolytic, and phospholipase activities *in vitro* (Table 1). The hemorrhagic activity of *B. mattogrossensis* crude venom was evaluated based on the dimensions of the average hemorrhagic halo which was 3.33 cm^2. This result is in agreement with the results recently obtained with *B. atrox* [26].

Coagulation activity was confirmed after incubation of different concentrations of *B. mattogrossensis* crude venom with plasma in which the minimum coagulation dose capable of promoting coagulation in less than 1 min was 0.325 μg of protein. Analysis of the proteolytic activity of *B. mattogrossensis* crude venom demonstrated a concentration-dependent response (data not shown). Regarding phospholipase, proteolytic, coagulating, and hemorrhagic activities of *B. mattogrossensis* crude venom, assays confirmed the presence and activity of proteases and phospholipases. The presence of metalloproteases was evidenced by the important formation of an extensive hemorrhagic halo *in vivo*. The presence of serine proteases was evidenced by coagulating activity present even in low concentrations of the venom (325 μg/mL).

Phospholipase activity of *B. mattogrossensis* crude venom assayed with 4N3OBA synthetic substrate was 1,864.05 U/mg measured by the number of moles of chromophores released

per minute (n°mols/min or U) for a given quantity of protein in milligrams (mg).

The properties of snake venom components observed in this study are characteristic of accidents caused by snakes of the *Bothrops sp* genus; symptoms such as pain, edema, hemorrhage, and necrosis and, additionally, systemic disturbances are characteristic and corroborate the literature that describes that proteases of snake venom proteins are closely related to interference in the hemostatic system promoting blood coagulation, fibrinolysis, and platelet aggregation [27–30].

3.2. Isolation and Biochemical Characterization of Phospholipases. The present study showed, for the first time, the isolation of the three phospholipases A_2 from *B. mattogrossensis* snake venom, obtained by two chromatographic steps. First, ionic exchange chromatography was performed on a CM-Sepharose column with an ammonium bicarbonate gradient (50 to 500 mM, pH 8.0). The elution of absorbed proteins with a linear gradient of concentrated buffer resulted in thirteen fractions (Figure 1(a)), of which fraction nine (9) was related to PLA_2s because it showed phospholipase activity of 221.09 U/mg on artificial substratum. All fractions were lyophilized and submitted to unidimensional electrophoresis revealing many protein bands.

Fraction 9 was submitted to the second chromatographic step in a reverse column phase on a Discovery C18 column, using 0.1% Trifluoroacetic (TFA) and 99.9% Acetonitrile (ACN) as solvents for the separation of other venom components. The elution of absorbed proteins with a linear gradient of concentrated buffer resulted in twenty-two (22) fractions (Figure 1(b)). The PLA_2s were highly purified with approximately 40% ACN. Similar results were observed in the isolation of other PLA_2s from snake venoms in high performance liquid chromatography on reverse phase columns where the elution profile of these proteins occurs between 30 and 40% ACN [5].

The association of chromatographic techniques such as ionic exchange and reverse phase has commonly been used, and many snake venoms have been fractioned this way, highlighting the phospholipase purification of a species belonging to the old complex "*Bothrops neuwiedi*", as well as the target species studied in this research. Two PLA_2 basic isoforms from *B. (neuwiedi) pauloensis* venom were purified by Rodrigues et al. [31], using biochemical techniques similar to the ones used in this study, with the combination of ionic exchange (cationic) and reverse phase chromatographies.

Other PLA_2s have also been purified using simplified procedures based in CM-Sepharose and/or reverse phase, as for example, venom PLA_2s from *Bothrops moojeni* [21, 32, 33], *B. pirajai* [34, 35], *B. jararacussu* [36, 37], *B. alternatus* [8, 38], *Cerastes cerastes* [39], and *Elaphe climacophora* [40].

Fractions 13 and 14 were related to enzymatically inactive PLA_2s, whereas fraction 15 was related to enzymatically active PLA_2s. The phospholipase activity of the collected fractions were analyzed with synthetic NOB and NBD-PC substrates (Figures 2(a) and 2(b)). The amount of activity was compared to BthTX-II, a basic enzymatically active PLA_2 (Asp49), and BthTX-I, a basic enzymatically inactive PLA_2 (Lys49), both

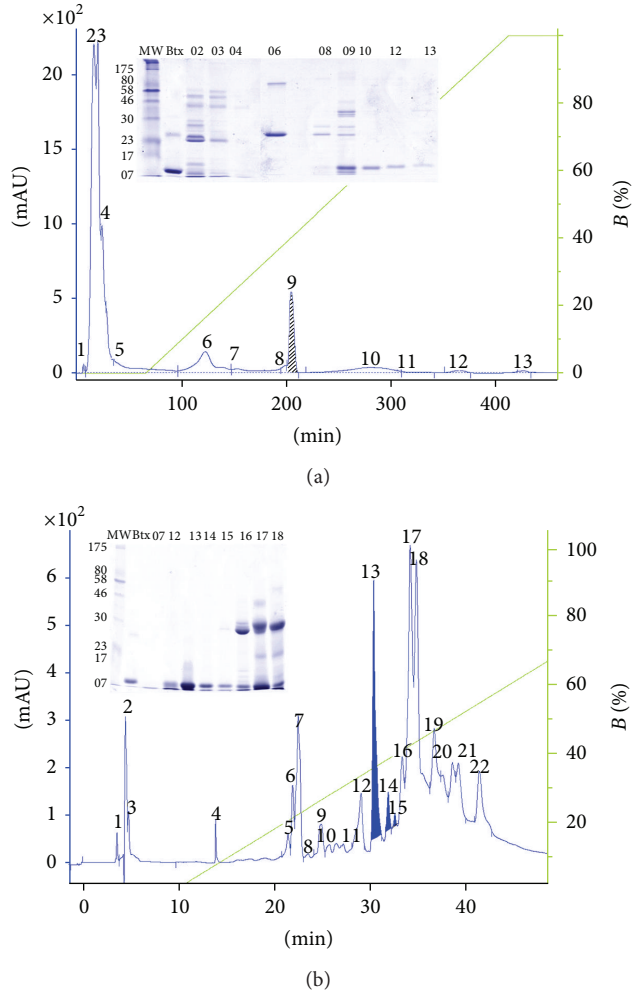

Figure 1: Chromatographic and electrophoretic profile of *Bothrops mattogrossensis* venom fractioning. (a) CM-Sepharose columns equilibrated with solvent A (50 mM ammonium bicarbonate, pH 8.0) and eluted with a 0–100% concentration gradient of solvent B (500 mM ammonium bicarbonate, pH 8.0) at a 5.0 mL/minute flow. Emphasis on peak 9, rechromatographed. (b) Rechromatography of fraction 9 on Discovery C18 column equilibrated with solvent A (0.1% TFA) and eluted with a concentration gradient of 0–100% of solvent B (99.9% acetonitrile and 0.1% trifluoroacetic acid) and a 1.0 mL/min flow. Emphasized in blue are fractions 13, 14, and 15 characterized as phospholipases BmatTX-I, BmatTX-II, and BmatTX-III, respectively. Controls: MW: molecular weight standard; BTx: BthTX-I a basic enzymatically inactive PLA_2 (Lys49) (10 μg) isolated from *Bothrops jararacussu* venom. Absorbances read at 280 nm. Electrophoresis gel made with 12.5% (w/w) acrylamide/bis-acrylamide.

previously isolated from *Bothrops jararacussu* venom [36, 41]. The BmatTX-III PLA_2s (Asp49) were not able to induce platelet aggregation and did not inhibit collagen or ADP induced platelet aggregation (data not shown).

The degree of purity of the isolated proteins was further demonstrated by SDS-PAGE, mass spectrometry (Figure 3), and N-terminal sequencing (Figures 4(a) and 4(b)). The purified PLA_2s were named BmatTX-I, BmatTX-II, and

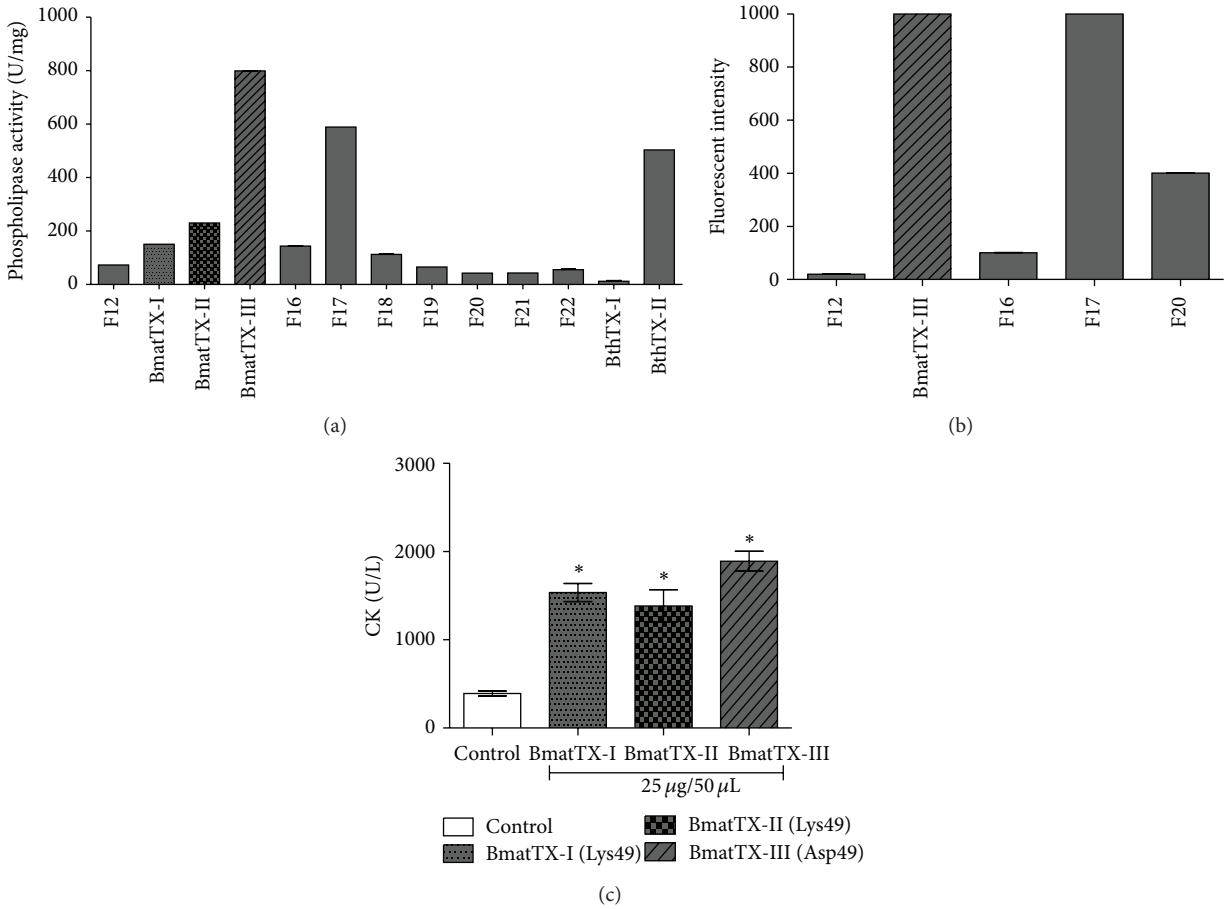

Figure 2: Enzymatic activity and myotoxic activity of PLA_2s isolated from the venom of *B. mattogrossensis*. (a) Phospholipase activity of the fractions collected from the rechromatography of fraction 9 done in C18 column assayed using an NOB stained substrate. This activity was assessed through the measurement of the number of moles of the released chromophore per minute (n°mols/min or U) per milligram of protein. (b) Phospholipase activity of the fractions collected from the rechromatography of fraction 9 done in C18 column assayed using a fluorescent substrate. (c) Myotoxic activity evaluated for inoculation of PLA_2s (25 μg/50 μL) or PBS (control) done intramuscularly, in the gastrocnemius muscle of mice. After 3 hours, the creatine kinase (CK) level, an important marker of muscular lesion, was assayed in the animal's plasma. Each bar represents the average +/− SD of three independent groups. $^*P < 0.05$ compared to the control. F13: BmatTX-I (Lys49); F14: BmatTX-II (Lys49); F15: BmatTX-III (Asp49); Controls: BthTX-I (Lys49) and BthTX-II (Asp49).

BmatTX-III. They were characterized as single polypeptide chains, with isoelectric points around 10 (data not shown). This result agrees with data from published literature, where most basic PLA_2s, with or without catalytic activity on artificial substrates [32, 42], show an isoelectric point between 8 and 10.

The average molecular mass defined by mass spectrometry was 13,304 Da for BmatTX-I, 13,623 Da for BmatTX-II, and 13,681 Da for BmatTX-III (Figure 3). This molecular mass is consistent with most isolated PLA_2s from snake venoms which are around 13 to 16 kDa [27, 36, 43].

BmatTX-I sequencing showed a lysine (Lys) at position 49 and although only the first 28 amino acid residues of BmatTX-II have been sequenced, both are highly similar to the PLA_2 Lys49 homologue subgroup. The N-terminal sequence alignment of BmatTX-I and BmatTX-II has revealed that these basic proteins are PLA_2s similar to homologous Lys49 and other Lys49 myotoxins from snake venoms (Figure 4(a)). BmatTX-I showed 94% similarity with MjTX-I present in *B. moojeni* venom, 92% with BthTX-I and BOJU-I present in *B. jararacussu* venom, and 94% similarity with MTX-II present in *B. brazili* venom.

Additionally, multiple sequence alignment of BmatTX-III showed a PLA_2-Asp49 basic myotoxin with another Asp49 of the *Bothrops* genera (Figure 4(b)). It can be observed that BmatTX-III presented 78% similarity with BmjeTX-I and 75% with BmoTX-I, both from *B. moojeni* venom, 71% similarity with BthTX-II isolated from *B. jararacussu* venom, and 65% similarity with PrTX-III from *B. pirajai* venom.

In the analysis of the sequences of the PLA_2s isolated from *B. mattogrossensis* in this study, highly preserved constituent residues of the α-helix structure, characteristic of phospholipases were identified, as well as the presence of many cysteine residues, which suggests the existence of many disulfide bridges, important for the stabilization of PLA_2's molecular structure [21, 44].

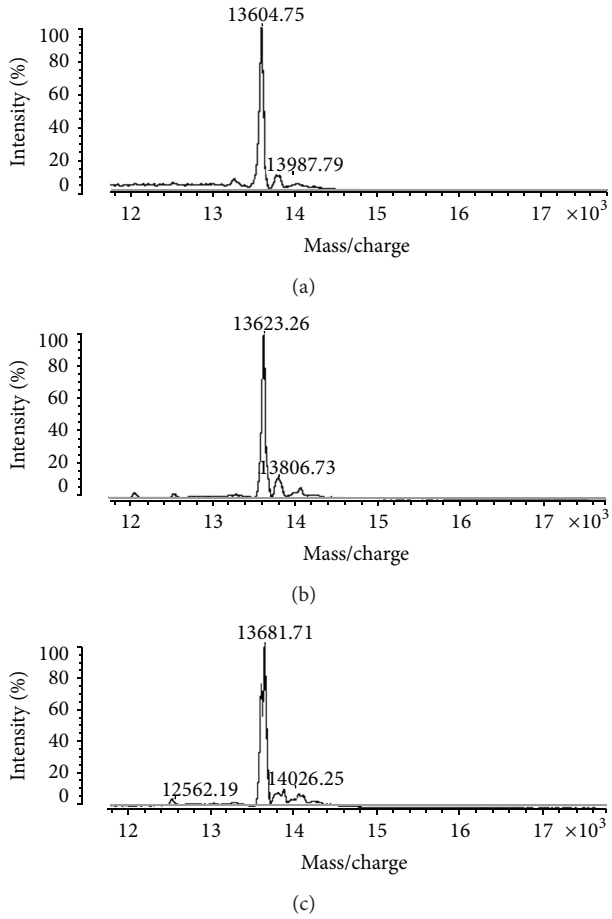

FIGURE 3: Mass determination by spectrometry. The average protein masses of BmatTX-I (a), BmatTX-II (b), and BmatTX-III (c) were obtained in a MALDI-TOF system, operated in linear mode using external standards for calibration. The resulting mass spectra were submitted to automatic baseline subtraction.

Some studies regarding the amino acid composition of PLA_2s demonstrate that these proteins are rich in basic and hydrophobic amino acids containing three long α-helixes, two beta sheets, and a Ca^{2+} binding site [45–47]. Calcium is absolutely necessary for hydrolysis; therefore, almost all PLA_2s have a highly preserved region for a Ca^{2+} bond (XCGXGG) and a catalytic site (DXCCXXHD) [48]. It is observed in BmatTX-III that the calcium binding site ($_{27}$Y**CG**W**GG**$_{32}$) is preserved, indicating that it is a catalytically active PLA_2 belonging to the PLA_2 Asp49 subgroup.

3.3. Biological Activities of Phospholipases A_2. The main objective for the isolation and characterization of components of snake venom is to better understand the participation of each component in different pathophysiological processes resulting from envenomation. Local lesions can be attributed to proteases, phospholipase activity, and hemorrhagic factors of these venoms, followed by the release of vasoactive agents causing hemorrhaging in various organs and tissues [5, 44, 49–52].

The PLA_2s isolated from *B. mattogrossensis* venom, BmatTX-I, BmatTX-II, and BmatTX-III, showed high myotoxic activity (Figure 2(c)). At a concentration of 25 μg/50 μL, the PLA_2s induced a significant release of CK, an important muscular lesion marker, when compared to the control. Myotoxicity is the characteristic presented by most basic phospholipases A_2 from snake venoms. Several studies demonstrate that the myotoxic effect begins quickly by direct action of the myotoxic PLA_2s on the plasma membrane of muscle cells or it is mediated by metalloproteases, due to consequent degeneration and ischemia [27, 28, 44].

Regarding the Lys49-PLA_2 myotoxins, it is evident that they lyse the plasma membrane of the muscle cell infected *in vivo*; however the exact mechanism has not been described yet. Furthermore, it is not known if the toxin is internalized before, during, or after the initial lysis or if it is not internalized. Although myotoxicity can be induced by the production of fatty acids, there is a second mechanism that seems to be independent of the enzymatic activity and is mediated by the C-terminal region at sites 115–129 of the Lys49 molecules [43, 53, 54].

In an attempt to better understand the development of the inflammatory process unleashed by the protein complex present in snake venoms, many studies have been done, such as edema induction [55], leukocyte participation [56, 57], mast cells degranulation [55], participation of various cytotoxins in the inflammatory system [58, 59], participation of cyclooxygenases [60, 61], and the participation of venom PLA_2s in the inflammatory process [6, 51, 56, 62].

Many studies about PLA_2 activity in macrophages have already been done [8, 63, 64]. Little is known about PLA_2's effect on neutrophils however. Escocard et al. [65] described an influx of inflammatory cells including many neutrophils into the peritoneal cavity of mice after the injection of *Bothrops atrox* venom. The induction of reactive oxygen species (ROS), cytokines like IL-6 and IL-1β, was seen in these neutrophils. These data were also observed in a study carried out by Souza et al. [26] where besides the influx of neutrophils into the peritoneal cavity of mice after injection of the venom of *B. atrox*, there was also induction of superoxide by these cells, mast cell degranulation, and phagocytosis by macrophages. Regarding the activity of PLA_2 Gambero et al. [66] have observed the ability of some myotoxins (bothropstoxin-I,-II and piratoxin-I) to induce neutrophil chemotaxis in a concentration-dependent manner.

In order to evaluate the activation of leukocytes, the toxicity of *B. mattogrossensis* myotoxins on neutrophil cells was investigated. The cells were incubated with different concentrations (3, 6, and 12 μg/mL) of BmatTX-I (Lys49), BmatTX-II (Lys49), and BmatTX-III (Asp49) myotoxins during 1, 12, and 24 hours (data not shown). These myotoxins did not affect the neutrophils' viability, which agrees with Zuliani et al. [64], showing low toxicity on thioglycollate elicited mice macrophages.

Nonetheless, our data showed that neutrophils incubated with BmatTX-I and BmatTX-II myotoxins induced the release of IL-1β (Figures 5(a) and 5(b)). Moreover, these results suggest that phospholipid hydrolysis is not essential for the activity observed and argue with the hyphotesis that

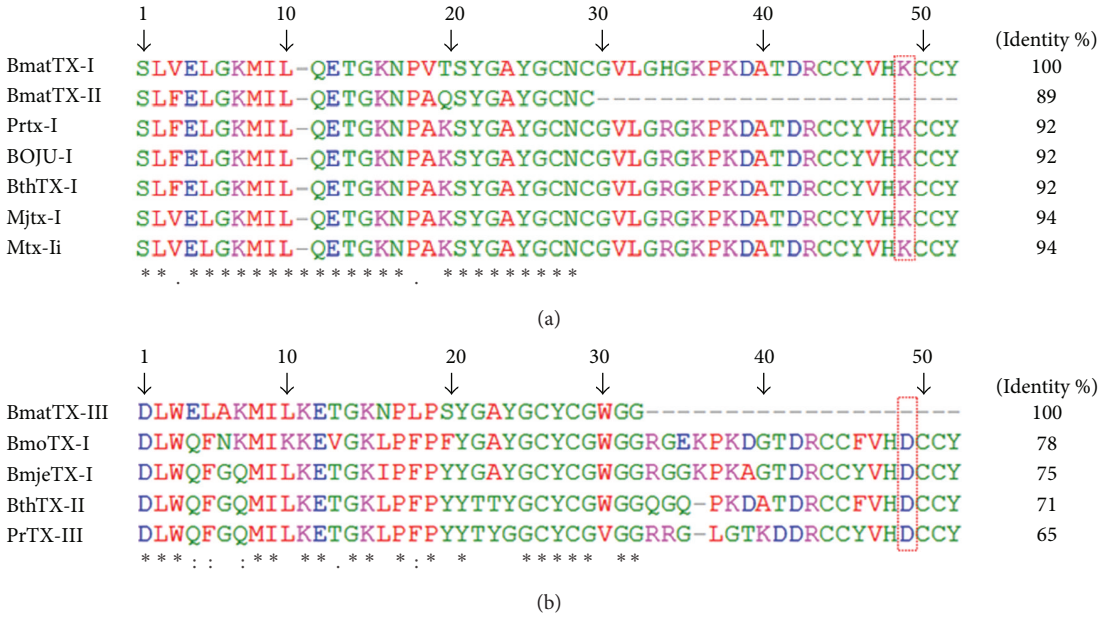

Figure 4: Comparison of the N-terminal sequence of the snake's PLA_2s with the PLA_2s isolated from *B. mattogrossensis* venom. N-terminal sequencing of the PLA_2s obtained by Edman [13] degradation and alignment done with CLUSTALW2 expressed as % of similarity. (a) BmatTX-I, BmatTX-II with Lys49 residues in marked sequences. (b) BmatTX-III with Asp49 residues in marked sequences. Sequences used for the alignment and their respective access numbers: BthTX-I (gi:51890398); MjTX-I (gi:17368325); Mtx-II (gi:390981003); PrTX-I (gi:190016174); BOJU-I (gi:209572966); BthTX-II (gi:1171971); PrTX-III (gi:90016174); BmjeTX-I (gi:313471399); BmoTX-I (gi:221272396). BthTX-I (Lys49), BthTX-II (Asp49), and BOJU-I (Lys49): isolated from the venom of *B. jararacussu*; MjTX-I (Lys49), BmoTX-I (Asp49), and BmjeTX-I (Asp49): isolated from the venom of *B. moojeni*; Mtx-II (Lys49): isolated from the venom of *B. brazili*; PrTX-I (Lys49) and PrTX-III (Asp49): isolated from the venom of *B. pirajai*; the "∗" was used to indicate the amino acid residues that are the same between the sequences.

other molecular regions distinct from the active site may be involved in this effect.

PLA_2s are multifunctional proteins that can be used as mediators in several areas of medicine, such as in the treatment of rheumatoid arthritis, as a new class of HIV inhibitors by blocking the host cell invasion, as a potential treatment against malaria, and as an antibiotic by inducing cytotoxicity via the disruption of bacterial membranes [21, 23, 30, 67, 68]. A study by Costa et al. [67] with PLA_2s isolated from *B. brazili*, MTX-I and II, demonstrated cytotoxic activity against Jurkat tumor cells as well as antimicrobial activity against *E. coli* and *C. albicans* and antiparasitic activity against *Leishmania sp*.

In the present study we evaluated the cytotoxic activity of PLA_2s isolated from *B. mattogrossensis* on JURKAT (T leukemia) and SK-BR-3 (breast adenocarcinoma), both of which are human tumor cell lines. Like MTX-II of *B. brazili* [67] the cytotoxic activity of PLA_2s BmatTX-I and BmatTX-II on JURKAT cells was independent of their catalytic activity, since these are characterized as Lys49 PLA_2s and are therefore catalytically inactive. BmatTX-III, characterized as Asp49, despite being enzymatically active also showed a lower level of toxicity. Some authors propose that the cytotoxic activity on tumor cell lines is associated with the induction of apoptosis, considering the fact that PLA_2 promotes alterations in the cell membrane. And some studies involving Lys49 PLA_2s isolated from *B. asper* demonstrated that the C-terminal region comprised of amino acids 115–129 is concerned with the cytotoxic and bactericidal activities of this protein [69–71]. The same observation was made by Costa et al. [67] with synthetic peptides derived from the C-terminal portion of MTX-I and II PLA_2s.

We also evaluated the antiparasitic activity of the crude venom and isolated $PLA_2$of *B. mattogrossensis* on promastigote forms of *L. amazonensis*. After the analysis, it was observed that the crude venom of *B. mattogrossensis* presents increasing toxic activity (approximately 50 to 80%) against promastigote forms of *L. amazonensis* after 48 h of incubation (Figure 7(a)). The PLA_2s isolated from *B. mattogrossensis,* at 100 μg/mL, characterized as BmatTX-I (Lys49) and BmatTX-III (Asp49) presented toxic activity between 25% and 30%, respectively, even with values close to those presented after incubation of the protozoan with Pentamidine, a drug used as a positive control (Figures 7(b) and 7(c)).

When compared, the cytotoxicity values of PLA_2s against promastigote forms of *L. amazonensis* show similar activity between the Lys49-PLA_2 and the Asp49-PLA_2. Comparison of this activity with the crude venom (70%) showed that PLA_2s are responsible for almost half of the observed effect. Nonetheless, notably, the results suggest that other toxins present in the venom contribute to the parasite's death.

Similar to the results obtained in the present study, Stábeli et al. [21] demonstrated that MjTX-II, a Lys49-PLA_2 homologue isolated from *B. moojeni* venom, in different

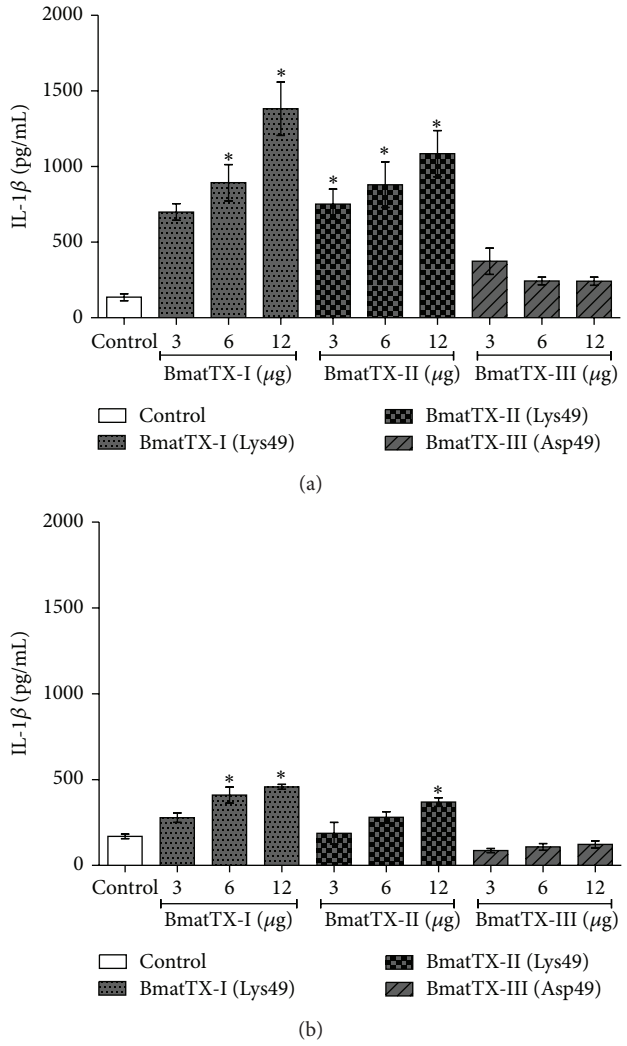

FIGURE 5: Production of IL-1β by mice neutrophils. These cells were incubatedwith PLA_2 (3, 6, and 12 μg/mL) or RPMI (control) for 12 hours (a) and 24 hours (b) at 37°C in a humid atmosphere with 5% CO_2. Quantification was done by ELISA as described in 2.9. Each bar represents the average +/− SD of three independent groups. $^*P < 0.05$ compared to the control.

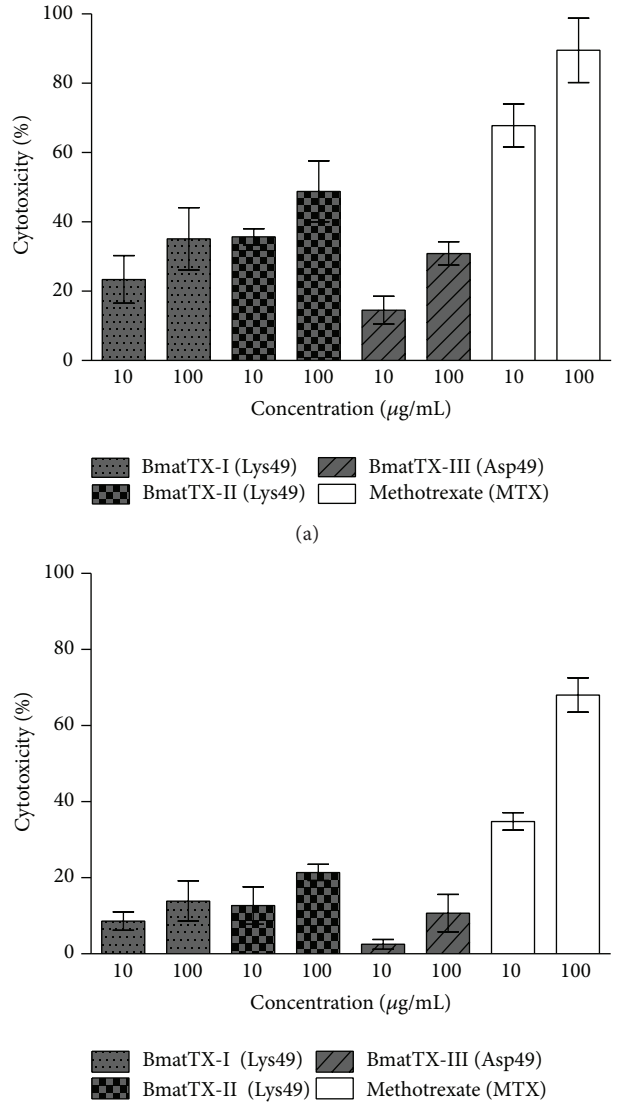

FIGURE 6: Antitumoral activity of PLA_2s from *B. mattogrossensis*. (a) Antitumoral activity on human acute T-cell leukemia (JURKAT) lines. (b) Antitumoral activity on human breast adenocarcinoma (SK-BR-3). Different concentrations of the PLA_2s were incubated with cell lines. Methotrexate was used as the positive control. Results are presented as mean +/− SD ($n = 3$).

concentrations (5, 25 and 75 μg) was effective as a parasiticide agent against *Schistosoma mansoni* and promastigote forms of *Leishmania* (*L. amazonensis, L. braziliensis, L. donovani,* and *L. major*).

The action of the PLA_2s, BmatTX-I (Lys49), and BmatTX-III (Asp49), on promastigote forms of *L. amazonensis,* was independent of its catalytic activity, since catalytically inactive Lys49 myotoxin also demonstrated toxicity against *Leishmania*. It is believed that the observed cytotoxic activity might be related to the C-terminal regions of these phospholipase-homologues that are able to promote a disturbance in the cellular membranes independent of their catalytic activity [67, 72]. However, more studies are necessary to define the exact mechanism of action of these enzymes on parasites.

Growing interest in the comprehension of the structure and function of snake venom components, especially PLA_2, contributes to a better understanding of the mechanism of action of their enzymatic and toxic activities. It opens the path to better understand the intoxication caused by envenomation and the physiopathology behind its side effects. Future studies will potentially improve serum therapy and help develop the pharmaceutical potential that molecules isolated from animal venoms can have, such as the PLA_2s isolated from the *B. mattogrossensis* venom which show anti-*Leishmania* and antitumor activities.

4. Conclusion

In conclusion, the venom of *Bothrops mattogrossensis* has a qualitatively similar toxicological profile to previously

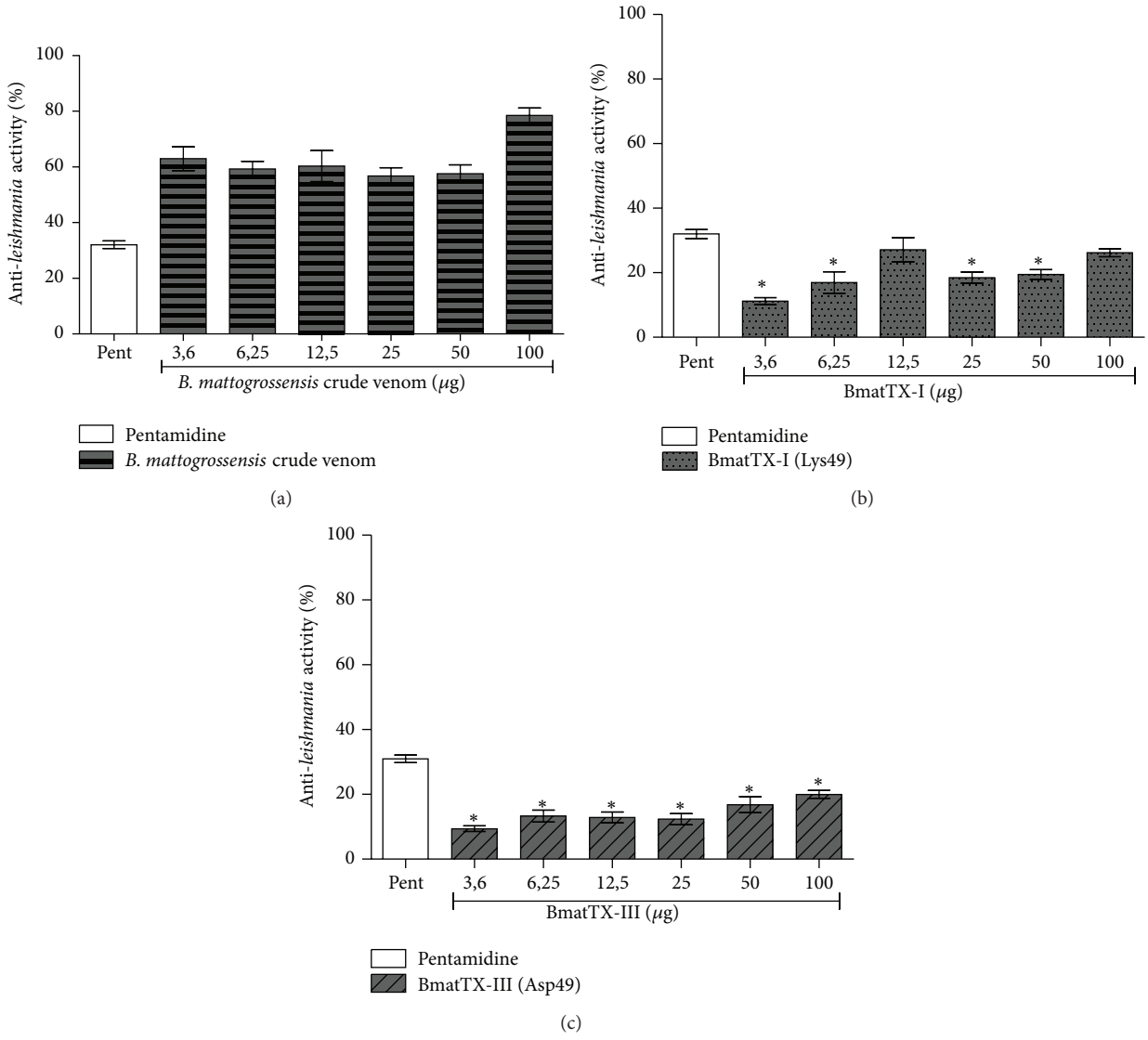

FIGURE 7: Antileishmanial activity of the crude venom of and PLA_2s from *B. mattogrossensis*. The promastigote forms of *L. amazonensis* were plated with 1×10^5 cells/well. Then, different concentrations of crude venom and isolated PLA_2s were added to each well. The experiment was done in a 48 h period, with the (a) crude venom and the PLA_2enzymes, (b) BmatTX-I, and (c) BmatTX-III. MTT was added and after the incubation period at 33°C, the formazan crystal formed was dissolved in SDS. Readings were done in a spectrophotometer at 570 nm. Each bar represents the average +/− SD of the three independent experimental groups, sixfold total. $^*P < 0.05$ compared to the control.

studied snake venoms of the *Bothrops sp.* genera despite the observation of quantitative variations. Of the three basic PLA_2s from *B. mattogrossensis* venom, now isolated for the first time, two are characterized as Lys49-PLA_2 homologues, BmatTX-I and -II, and the other as an Asp49-PLA_2, named BmatTX-III. This showed high phospholipase activity. The PLA_2s isolated induced myotoxic effects as well as the release of proinflammatory cytokines by neutrophils. BmatTX-I and -III PLA_2s were cytotoxic to human tumor cell lines JURKAT and SK-BR-3 and showed activity against promastigote forms of *L. amazonensis*.

Conflict of Interests

The authors state that there is no conflict of interests.

Acknowledgments

The authors are grateful to the Ministry of Science and Technology (MCTI), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Tecnologia do Acre (FUNTAC/FDCT), Coordenação de Aperfeiçoamento de Nível Superior (CAPES)—Projeto NanoBiotec, Rede de Biodiversidade e Biotecnologia da Amazônia Legal (BIONORTE/CNPq/MCTI), Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT-INPeTAm/CNPq/MCTI) and Instituto Nacional para Pesquisa em Toxinas (INCT-Tox), and Secretary of Development of Rondonia State (SEPLAN/PRONEX/CNPq), for financial support. We are grateful to

Sr. Guilherme G. Galassi (Mantenedouro da Fauna Silvestre, autorização IBAMA 02027.008991/2001-95, Americana-SP) for their helpful technical collaboration in provide the snake venom. Amy Grabner provided the English editing of the paper.

References

[1] Y. Angulo and B. Lomonte, "Biochemistry and toxicology of toxins purified from the venom of the snake *Bothrops asper*," *Toxicon*, vol. 54, no. 7, pp. 949–957, 2009.

[2] J. J. Calvete, P. Juárez, and L. Sanz, "Snake venomics. Strategy and applications," *Journal of Mass Spectrometry*, vol. 42, no. 11, pp. 1405–1414, 2007.

[3] T. S. Kang, D. Georgieva, N. Genov et al., "Enzymatic toxins from snake venom: structural characterization and mechanism of catalysis," *The FEBS Journal*, vol. 278, no. 23, pp. 4544–4576, 2011.

[4] C. Z. Oliveira, *Caracterização funcional e estrutural de inibidores de fosfolipases A_2 isolados do plasma de serpente Bothrops jararacussu [Ph.D. thesis]*, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Ribeirão Preto, Brazil, 2009.

[5] R. G. Stábeli, R. Simões-Silva, A. M. Kayano et al., "Purification of phospholipases A_2 from American snake venoms," in *Chromatography—The Most Versatile Method of Chemical Analysis*, chapter 1, pp. 1–34, InTech, Vienna, Austria, 2012.

[6] J. P. Zuliani, C. M. Fernandes, S. R. Zamuner, J. M. Gutiérrez, and C. F. P. Teixeira, "Inflammatory events induced by Lys-49 and Asp-49 phospholipases A_2 isolated from *Bothrops asper* snake venom: role of catalytic activity," *Toxicon*, vol. 45, no. 3, pp. 335–346, 2005.

[7] S. Marcussi, R. G. Stabeli, N. A. Santos-Filho et al., "Genotoxic effect of *Bothrops* snake venoms and isolated toxins on human lymphocyte DNA," *Toxicon*, vol. 65, pp. 9–14, 2013.

[8] S. S. Setúbal, A. S. Pontes, J. L. Furtado et al., "Action of two phospholipases A_2 purified from *Bothrops alternatus* snake venom on macrophages," *Biochemistry*, vol. 78, no. 2, pp. 194–203, 2013.

[9] J. M. Gutiérrez, L. A. Ponce-Soto, S. Marangoni, and B. Lomonte, "Systemic and local myotoxicity induced by snake venom group II phospholipases A_2: comparison between crotoxin, crotoxin B and a Lys49 PLA_2 homologue," *Toxicon*, vol. 51, no. 1, pp. 80–92, 2008.

[10] A. Q. Rueda, I. G. Rodríguez, E. C. Ara et al., "Biochemical characterization, action on macrophages, and superoxide anion production of four basic phospholipases A_2 from Panamanian *Bothrops asper* snake venom," *BioMed Research International*, vol. 2013, Article ID 789689, 9 pages, 2013.

[11] V. X. da Silva and M. T. Rodrigues, "Taxonomic revision of the *Bothrops neuwiedi* complex (Serpentes, Viperidae) with description of a new species," *Phyllomedusa*, vol. 7, no. 1, pp. 45–90, 2008.

[12] J. A. Campbell and W. W. Lamar, *The Venomous Reptiles of the Western Hemisphere*, Comstock, Ithaca, NY, USA, 2004.

[13] P. Edman, "Method for determination of the amino acid sequence in peptides," *Acta Chemica Scandinavica*, vol. 4, pp. 283–293, 1950.

[14] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[15] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[16] M. Holzer and S. P. Mackessy, "An aqueous endpoint assay of snake venom phospholipase A_2," *Toxicon*, vol. 34, no. 10, pp. 1149–1155, 1996.

[17] A. L. Fuly, A. L. P. de Miranda, R. B. Zingali, and J. A. Guimarães, "Purification and characterization of a phospholipase A_2 isoenzyme isolated from *Lachesis muta* snake venom," *Biochemical Pharmacology*, vol. 63, no. 9, pp. 1589–1597, 2002.

[18] T. Nikai, N. Mori, M. Kishida, H. Sugihara, and A. T. Tu, "Isolation and biochemical characterization of hemorrhagic toxin *f* from the venom of *Crotalus atrox* (Western Diamondback Rattlesnake)," *Archives of Biochemistry and Biophysics*, vol. 231, no. 2, pp. 309–319, 1984.

[19] J. A. Gené, A. Roy, G. Rojas, J. M. Gutierrez, and L. Cerdas, "Comparative study on coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of Costa Rican crotaline snake venoms and their neutralization by a polyvalent antivenom," *Toxicon*, vol. 27, no. 8, pp. 841–848, 1989.

[20] M. S. Charney and R. M. Tomarelli, "A colorimetric method for the determination of the proteolytic activity of duodenal juice," *The Journal of Biological Chemistry*, vol. 171, pp. 501–505, 1947.

[21] R. G. Stábeli, S. F. Amui, C. D. Sant'Ana et al., "*Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A_2 homologue: an example of function versatility of snake venom proteins," *Comparative Biochemistry and Physiology C*, vol. 142, no. 3-4, pp. 371–381, 2006.

[22] D. R. Call, J. A. Nemzek, S. J. Ebong, G. L. Bolgos, D. E. Newcomb, and D. G. Remick, "Ratio of local to systemic chemokine concentrations regulates neutrophil recruitment," *The American Journal of Pathology*, vol. 158, no. 2, pp. 715–721, 2001.

[23] P. G. Roberto, S. Kashima, S. Marcussi et al., "Cloning and identification of a complete cDNA coding for a bactericidal and antitumoral acidic phospholipase A_2 from *Bothrops jararacussu* venom," *The Protein Journal*, vol. 23, no. 4, pp. 273–285, 2004.

[24] J. H. Schumacher, A. O'Garra, B. Shrader et al., "The characterization of four monoclonal antibodies specific for mouse IL-5 and development of mouse and human IL-5 enzyme-linked immunosorbent," *The Journal of Immunology*, vol. 141, no. 5, pp. 1576–1581, 1988.

[25] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[26] C. A. T. Souza, A. M. Kayano, S. S. Setúbal et al., "Local and systemic biochemical alterations induced by *Bothrops atrox* snake venom in mice," *Journal of Venom Research*, vol. 3, pp. 28–34, 2012.

[27] J. M. Gutiérrez and B. Lomonte, "Phospholipase A_2 myotoxins from *Bothrops* snake venoms," *Toxicon*, vol. 33, no. 11, pp. 1405–1424, 1995.

[28] J. M. Gutiérrez and A. Rucavado, "Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage," *Biochimie*, vol. 82, no. 9-10, pp. 841–850, 2000.

[29] T. Matsui, Y. Fujimura, and K. Titani, "Snake venom proteases affecting hemostasis and thrombosis," *Biochimica et Biophysica Acta*, vol. 1477, no. 1-2, pp. 146–156, 2000.

[30] A. M. Soares, M. R. M. Fontes, and J. R. Giglio, "Phospholipase A_2 myotoxins from *Bothrops* snake venoms: structure-function

relationship," *Current Organic Chemistry*, vol. 8, no. 17, pp. 1677–1690, 2004.

[31] V. M. Rodrigues, S. Marcussi, R. S. Cambraia et al., "Bactericidal and neurotoxic activities of two myotoxic phospholipases A_2 from *Bothrops neuwiedi pauloensis* snake venom," *Toxicon*, vol. 44, no. 3, pp. 305–314, 2004.

[32] N. A. Santos-Filho, L. B. Silveira, C. Z. Oliveira et al., "A new acidic myotoxic, anti-platelet and prostaglandin I_2 inductor phospholipase A_2 isolated from *Bothrops moojeni* snake venom," *Toxicon*, vol. 52, no. 8, pp. 908–917, 2008.

[33] A. M. Soares, S. H. Andrião-Escarso, Y. Ângulo et al., "Structural and functional characterization of myotoxin I, a Lys49 phospholipase A_2 homologue from *Bothrops moojeni* (Caissaca) snake venom," *Archives of Biochemistry and Biophysics*, vol. 373, no. 1, pp. 7–15, 2000.

[34] A. M. Soares, S. H. Andrião-Escarso, R. K. Bortoleto et al., "Dissociation of enzymatic and pharmacological properties of piratoxins-I and -III, two myotoxic phospholipases A_2 from *Bothrops pirajai* snake venom," *Archives of Biochemistry and Biophysics*, vol. 387, no. 2, pp. 188–196, 2001.

[35] S. S. Teixeira, L. B. Silveira, F. M. N. da Silva et al., "Molecular characterization of an acidic phospholipase A_2 from *Bothrops pirajai* snake venom: synthetic C-terminal peptide identifies its antiplatelet region," *Archives of Toxicology*, vol. 85, no. 10, pp. 1219–1233, 2011.

[36] S. H. Andrião-Escarso, A. M. Soares, V. M. Rodrigues et al., "Myotoxic phospholipases A_2 in *Bothrops* snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*," *Biochimie*, vol. 82, no. 8, pp. 755–763, 2000.

[37] S. H. Andrião-Escarso, A. M. Soares, M. R. M. Fontes et al., "Structural and functional characterization of an acidic platelet aggregation inhibitor and hypotensive phospholipase A_2 from *Bothrops jararacussu* snake venom," *Biochemical Pharmacology*, vol. 64, no. 4, pp. 723–732, 2002.

[38] M. E. G. Denegri, O. C. Acosta, S. Huancahuire-Vega et al., "Isolation and functional characterization of a new acidic PLA_2 Ba SpII RP4 of the *Bothrops alternatus* snake venom from Argentina," *Toxicon*, vol. 56, no. 1, pp. 64–74, 2010.

[39] R. Zouari-Kessentini, J. Luis, A. Karray et al., "Two purified and characterized phospholipases A_2 from *Cerastes cerastes* venom, that inhibit cancerous cell adhesion and migration," *Toxicon*, vol. 53, no. 4, pp. 444–453, 2009.

[40] R. Shirai, M. Toriba, K. Hayashi, K. Ikeda, and S. Inoue, "Identification and characterization of phospholipase A_2 inhibitors from the serum of the Japanese rat snake, *Elaphe climacophora*," *Toxicon*, vol. 53, no. 6, pp. 685–692, 2009.

[41] M. I. Homsi-Brandenburgo, L. S. Queiroz, H. Santo-Neto, L. Rodrigues-Simoni, and J. R. Giglio, "Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin," *Toxicon*, vol. 26, no. 7, pp. 615–627, 1988.

[42] G. P. Fernández, A. Segura, M. Herrera et al., "Neutralization of *Bothrops mattogrossensis* snake venom from Bolivia: experimental evaluation of llama and donkey antivenoms produced by caprylic acid precipitation," *Toxicon*, vol. 55, no. 2-3, pp. 642–645, 2010.

[43] C. L. Ownby, H. S. S. de Araujo, S. P. White, and J. E. Fletcher, "Lysine 49 phospholipase A_2 proteins," *Toxicon*, vol. 37, no. 3, pp. 411–445, 1999.

[44] A. M. Soares, W. P. Sestito, S. Marcussi et al., "Alkylation of myotoxic phospholipases A_2 in *Bothrops moojeni* venom: a promising approach to an enhanced antivenom production," *International Journal of Biochemistry & Cell Biology*, vol. 36, no. 2, pp. 258–270, 2004.

[45] B. Lomonte, J. M. Gutiérrez, M. F. Furtado et al., "Isolation of basic myotoxins from *Bothrops moojeni* and *Bothrops atrox* snake venoms," *Toxicon*, vol. 28, no. 10, pp. 1137–1146, 1990.

[46] L. C. Mancuso, M. M. Correa, C. A. Vieira et al., "Fractionation of *Bothrops pirajai* snake venom: isolation and characterization of piratoxin-I, a new myotoxic protein," *Toxicon*, vol. 33, no. 5, pp. 615–626, 1995.

[47] H. S. Selistre, L. S. Queiroz, O. A. B. Cunha, G. E. P. de Souza, and J. R. Giglio, "Isolation and characterization of hemorrhagic, myonecrotic and edema-inducing toxins from *Bothrops insularis* (jararaca ilhoa) snake venom," *Toxicon*, vol. 28, no. 3, pp. 261–273, 1990.

[48] E. A. Dennis, J. Cao, Y.-H. Hsu, V. Magrioti, and G. Kokotos, "Phospholipase A_2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention," *Chemical Reviews*, vol. 111, no. 10, pp. 6130–6185, 2011.

[49] A. G. T. Cardoso, *Ação do veneno de Bothrops moojeni e sua fração L-aminoácido oxidase, submetida ao tratamento com raios gama de ^{60}CO, em Leishmania spp [M.S. thesis]*, IPEN-CNEN/SP, São Paulo, Brazil, 1999.

[50] M. Gallacci and W. L. G. Cavalcante, "Understanding the *in vitro* neuromuscular activity of snake venom Lys49 phospholipase A_2 homologues," *Toxicon*, vol. 55, no. 1, pp. 1–11, 2010.

[51] S. S. Teixeira, *Caracterização funcional e estrutural de uma fosfolipase A_2 ácida isolada do veneno de Bothrops pirajai [M.S. thesis]*, Faculdade de Ciências Farmacêuticas de Ribeirão Preto/USP, Ribeirão Preto, Brazil, 2009.

[52] E. Valentin and G. Lambeau, "Increasing molecular diversity of secreted phospholipases A_2 and their receptors and binding proteins," *Biochimica et Biophysica Acta*, vol. 1488, no. 1-2, pp. 59–70, 2000.

[53] J. M. Gutiérrez and B. Lomonte, "Phospholipases A_2 from Viperidae snake venoms: how do they induce skeletal muscle damage?" *Acta Chimica Slovenica*, vol. 58, no. 4, pp. 647–659, 2011.

[54] J. M. Gutiérrez, C. L. Ownby, and G. V. Odell, "Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle," *Toxicon*, vol. 22, no. 1, pp. 115–128, 1984.

[55] N. G. Nascimento, M. C. Sampaio, R. A. Olivo, and C. Teixeira, "Contribution of mast cells to the oedema induced by *Bothrops moojeni* snake venom and a pharmacological assessment of the inflammatory mediators involved," *Toxicon*, vol. 55, no. 2-3, pp. 343–352, 2010.

[56] C. F. P. Teixeira, E. C. T. Landucci, E. Antunes, M. Chacur, and Y. Cury, "Inflammatory effects of snake venom myotoxic phospholipases A_2," *Toxicon*, vol. 42, no. 8, pp. 947–962, 2003.

[57] S. R. Zamuner and C. F. P. Teixeira, "Cell adhesion molecules involved in the leukocyte recruitment induced by venom of the snake *Bothrops jararaca*," *Mediators of Inflammation*, vol. 11, no. 6, pp. 351–357, 2002.

[58] F. Chaves, C. F. P. Teixeira, and J. M. Gutiérrez, "Role of TNF-α, IL-1β and IL-6 in the local tissue damage induced by *Bothrops asper* snake venom: an experimental assessment in mice," *Toxicon*, vol. 45, no. 2, pp. 171–178, 2005.

[59] C. D. F. P. Teixeira, C. M. Fernandes, J. P. Zuliani, and S. F. Zamuner, "Inflammatory effects of snake venom metalloproteinases," *Memórias do Instituto Oswaldo Cruz*, vol. 100, supplement 1, pp. 181–184, 2005.

[60] R. D. A. Olivo, C. F. P. Teixeira, J. L. Wallace, J. M. Gutierrez, and S. R. Zamuner, "Role of cyclooxygenases in oedema-forming activity of bothropic venoms," *Toxicon*, vol. 49, no. 5, pp. 670–677, 2007.

[61] L. B. Silveira, D. P. Marchi-Salvador, N. A. Santos-Filho et al., "Isolation and expression of a hypotensive and anti-platelet acidic phospholipase A_2 from *Bothrops moojeni* snake venom," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 73, pp. 35–43, 2013.

[62] A. Rucavado, T. Escalante, C. F. P. Teixeira, C. M. Fernándes, C. Díaz, and J. M. Gutiérrez, "Increments in cytokines and matrix metalloproteinases in skeletal muscle after injection of tissue-damaging toxins from the venom of the snake *Bothrops asper*," *Mediators of Inflammation*, vol. 11, no. 2, pp. 121–128, 2002.

[63] E. Leiguez, J. P. Zuliani, A. M. Cianciarullo, C. M. Fernandes, J. M. Gutiérrez, and C. Teixeira, "A group IIA-secreted phospholipase A_2 from snake venom induces lipid body formation in macrophages: the roles of intracellular phospholipases A_2 and distinct signaling pathways," *Journal of Leukocyte Biology*, vol. 90, no. 1, pp. 155–166, 2011.

[64] J. P. Zuliani, J. M. Gutiérrez, L. L. Casais e Silva, S. C. Sampaio, B. Lomonte, and C. D. F. P. Teixeira, "Activation of cellular functions in macrophages by venom secretory Asp-49 and Lys-49 phospholipases A_2," *Toxicon*, vol. 46, no. 5, pp. 523–532, 2005.

[65] R. D. C. M. Escocard, M. M. Kanashiro, J. H. Petretski et al., "Neutrophils regulate the expression of cytokines, chemokines and nitric oxide synthase/nitric oxide in mice injected with *Bothrops atrox* venom," *Immunobiology*, vol. 211, no. 1-2, pp. 37–46, 2006.

[66] A. Gambero, E. C. T. Landucci, M. H. Toyama et al., "Human neutrophil migration *in vitro* induced by secretory phospholipases A_2: a role for cell surface glycosaminoglycans," *Biochemical Pharmacology*, vol. 63, no. 1, pp. 65–72, 2002.

[67] F. L. S. Costa, R. S. Rodrigues, L. F. M. Izidoro et al., "Biochemical and functional properties of a thrombin-like enzyme isolated from *Bothrops pauloensis* snake venom," *Toxicon*, vol. 54, no. 6, pp. 725–735, 2009.

[68] J. C. Villalobos, R. Mora, B. Lomonte, J. M. Gutiérrez, and Y. Angulo, "Cytotoxicity induced in myotubes by a Lys49 phospholipase A_2 homologue from the venom of the snake *Bothrops asper*: evidence of rapid plasma membrane damage and a dual role for extracellular calcium," *Toxicology in Vitro*, vol. 21, no. 8, pp. 1382–1389, 2007.

[69] B. S. Cummings, J. McHowat, and R. G. Schnellmann, "Phospholipase A_2s in cell injury and death," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 294, no. 3, pp. 793–799, 2000.

[70] S. R. Panini, L. Yang, A. E. Rusinol, M. S. Sinensky, J. V. Bonventre, and C. C. Leslie, "Arachidonate metabolism and the signaling pathway of induction of apoptosis by oxidized LDL/oxysterol," *Journal of Lipid Research*, vol. 42, no. 10, pp. 1678–1686, 2001.

[71] L. Páramo, B. Lomonte, J. Pizarro-Cerdá, J.-A. Bengoechea, J.-P. Gorvel, and E. Moreno, "Bactericidal activity of Lys49 and Asp49 myotoxic phospholipases A_2 from *Bothrops asper* snake venom: synthetic Lys49 myotoxin II-(115-129)-peptide identifies its bactericidal region," *European Journal of Biochemistry*, vol. 253, no. 2, pp. 452–461, 1998.

[72] B. Lomonte, Y. Angulo, and E. Moreno, "Synthetic peptides derived from the C-terminal region of Lys49 phospholipase A_2 homologues from viperidae snake venoms: biomimetic activities and potential applications," *Current Pharmaceutical Design*, vol. 16, no. 28, pp. 3224–3230, 2010.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 595186, 13 pages
http://dx.doi.org/10.1155/2014/595186

Research Article

Isolation and Biochemical Characterization of a New Thrombin-Like Serine Protease from *Bothrops pirajai* Snake Venom

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Received 9 July 2013; Revised 20 September 2013; Accepted 1 December 2013; Published 26 February 2014

Academic Editor: Edward G. Rowan

This paper presents a novel serine protease (SP) isolated from *Bothrops pirajai*, a venomous snake found solely in Brazil that belongs to the Viperidae family. The identified SP, named BpirSP-39, was isolated by three chromatographic steps (size exclusion, bioaffinity, and reverse phase chromatographies). The molecular mass of BpirSP-39 was estimated by SDS-PAGE and confirmed by mass spectrometry (39,408.32 Da). The protein was able to form fibrin networks, which was not observed in the presence of serine protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF). Furthermore, BpirSP-39 presented considerable thermal stability and was apparently able to activate factor XIII of the blood coagulation cascade, unlike most serine proteases. BpirSP-39 was capable of hydrolyzing different chromogenic substrates tested (S-2222, S-2302, and S-2238) while Cu^{2+} significantly diminished BspirSP-39 activity on the three tested substrates. The enzyme promoted platelet aggregation and also exhibited fibrinogenolytic, fibrinolytic, gelatinolytic, and amidolytic activities. The multiple alignment showed high sequence similarity to other thrombin-like enzymes from snake venoms. These results allow us to conclude that a new SP was isolated from *Bothrops pirajai* snake venom.

1. Introduction

Snake venoms have proteolytic enzymes that can be divided into two main groups: metalloproteases and serine proteases, which affect the hemostatic system through different mechanisms [1, 2]. Serine proteases are abundant in snake venoms, particularly of the Viperidae family, and may constitute up to 20% of total venom proteins. This class of enzymes

has a highly conserved catalytic triad (His57, Asp102, and Ser195) [3]. Besides venomous snakes, these enzymes are often found in many organisms such as viruses, bacteria, and higher mammals. Serine proteases can participate in several biological activities, such as complementing system activation, cell differentiation and homeostasis and even prey digestion [4–7]. This class of proteases affects different steps of the coagulation cascade, often nonspecifically, by proteolytic degradation. Selectively, they can activate or inactivate specific coagulation factors involved in platelet aggregation, coagulation, and fibrinolysis [8].

In snake venoms, one class of serine proteases, named snake venom thrombin-like enzymes (svTLEs), possesses coagulant activity similar to human thrombin. They convert fibrinogen to fibrin by the cleavage of the Aα and Bβ chains [2]. Some of these enzymes are able to cleave only the α or β chains or both chains of the fibrinogen and are therefore known as svTLE-A, svTLE-B, or svTLE-AB, respectively [9]. New serine proteases are constantly being described and/or characterized [10–13].

The aim of the present study is the isolation and biochemical characterization of a new serineprotease from *Bothrops pirajai* snake venom. Because it is such a rare snake and lives in a small area of the world, little about its venom has been described to date; these studies are limited to the purification of (i) phospholipase A_2: piratoxin-I [14], piratoxin-II and -III [15], MP-III 4R [16], and BpirPLA_2-I [17], (ii) C-type lectin: BPL [18], (iii) LAAO: BpirLAAO-I [19] and (iv) two serine proteases: BpirSP27 and BpirSP41 [20].

2. Materials and Methods

2.1. Isolation and Molecular Mass Determination. The identified serineprotease (BpirSP-39) was isolated after chromatographic fractionation of *B. pirajai* venom by size exclusion, followed by bioaffinity and reverse phase chromatographies. So, about 40 mg of crude venom was solubilized in 1 mL of 20 mM Tris-HCl pH 7.6 and centrifuged at 9000 ×g for 10 min at room temperature. The clear supernatant was applied to a Superdex G-75 (70 × 0.9 cm) column (GE Healthcare), preequilibrated with 20 mM Tris-HCl pH 7.6, and the chromatography was carried out at a flow of 0.75 mL/min, collecting fractions of 1 mL/tube. The elution of proteins was monitored at 280 nm.

Fractions with coagulant activity were lyophilized, suspended in 50 mM Tris-HCl pH 7.4 plus 0.5 M NaCl, and applied to a Hitrap benzamidine *Fast Flow* column (GE Healthcare), previously equilibrated with 50 mM Tris-HCl pH 7.4 plus 0.5 M NaCl. The elution of proteins was performed using 0.5 M NaCl plus 10 mM HCl at a flow of 1 mL/min. The collected samples (1 mL) were desalted and lyophilized.

The fraction of interest was dissolved in 0.1% trifluoroacetic acid (TFA) and a reversed-phase high-performance chromatography was performed using a C2/C18 column (10 mm × 4.6 mm, 3 μm, 120 Å) (GE Healthcare), preequilibrated with 0.1% TFA. The elution was carried out using a linear gradient of 0–100% (99% acetonitrile plus 0.1% TFA) at a flow rate of 0.75 mL/min. All chromatographic steps were performed in an Akta Purifier 10 system (GE Healthcare).

The molecular mass was estimated by 12.5% SDS-PAGE [21] and determined by mass spectrometry in an AXIMA TOF^2 system. The mass spectrum was acquired in linear mode, using a saturated solution of sinapinic acid as ionization matrix.

2.2. Enzyme Activities

2.2.1. Determination of Coagulant Activity. The minimum coagulant dose (MCD), or the amount of enzyme capable of coagulating 200 μL of plasma in 60 sec, was determined visually using different concentrations of isolated protein (0.5–3.0 μg) and citrated human plasma [22]. The time needed to form fibrin networks was measured by a chronometer and the results were expressed in percentage of seconds ($1/\Delta \times 100$), where Δ is the average time in seconds. The action of protease inhibitors on the purified enzyme was evaluated determining coagulation activity after the incubation of 2 μg serineprotease with heparin, citrate, ethylenediaminetetraacetic acid (EDTA), and phenylmethanesulfonyl fluoride (PMSF) for 20 minutes at room temperature. The thermal stability of the protease was verified by measuring coagulation activity after preincubation of 2 μg protein at different temperatures (−70°C–85°C) for 30 minutes. The assays were carried out in duplicate with $n = 3$.

2.2.2. Activation of Factor XIII of the Clotting Cascade. After centrifugation of heparinized blood samples at 2205 ×g for 15 minutes, 400 μL plasma was incubated with (i) 2 μg BpirSP-39 (40 μL), or (ii) 2 μg BjussuSP-I (a serineprotease from *B. jararacussu* that is not able to activate factor XIII), or (iii) 40 μL of water, dilution the sample solution (negative controls) and (iv) blood collected without anticoagulant (positive control) in order to evaluate the stability of the formed fibrin network [23]. Aiming at evaluating the activation of coagulation factor XIII, 200 μL of 10 M urea solution was added to the clots and the samples were incubated for 48 h at 37°C. The assays were carried out in duplicate with $n = 3$.

2.2.3. Activity on Synthetic Substrates. The ability of SP in hydrolyzing chromogenic substrates (0.1 mM, final concentration) S-2238 (that is suitable for thrombin-like enzymes), S-2222 (for factor Xa) and S-2302 (for plasma kallikrein, factor XIa and XIIa), was analyzed using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA, USA). The enzymatic reaction was monitored for 20 min. at 37°C and A405 nm. The effective concentration (EC) was determined as the concentration of SP (μg/mL) able to produce an increase of 0.3/5 minutes. SP was pre-incubated either with EDTA (20 mM), PMSF (2 mM), benzamidine (15 mM), and O-Phe (0.3 mM) for 60 min at 37°C or with 10 mM divalent cations (Cu^{2+}, Mn^{2+}, Ba^{2+}, and Ca^{2+}) for 30 min at 37°C, and then the reaction was started by adding substrates [24, 25].

2.2.4. Platelet Aggregation Assays. Washed rabbit platelets (WRP) were prepared according to the procedure described by Fuly and coworkers [26]. Collected blood plus 5 mM EDTA was centrifuged at 360 ×g for 12 min at room temperature, and the Platelet Rich Plasma (PRP) obtained was further centrifuged at 1370 ×g for 20 minutes. The platelet pellets were suspended in a calcium-free Tyrode's solution containing 0.35% (w/v) bovine serum albumin (BSA) plus 0.1 mM EGTA (final concentration) pH 6.5 and washed twice by centrifugation. The final pellet was then suspended in Tyrode-BSA pH 7.5, without EGTA. The suspension was adjusted to 3-4 $\times 10^5$ platelets/mL and platelet aggregation was measured by turbidimetry using a dual Whole Blood Lumi-Aggregometer (model 490 2D, Chrono-Log Corporation). The assays were performed at 37°C in siliconized glass cells using 200 μL of WRP, under stirring conditions, and aggregation was triggered after preincubation for 2 min with aliquots of SP in the presence of 1.0 mM $CaCl_2$ (final concentration).

2.2.5. Fibrinogenolytic Activity. The fibrinogenolytic activity of SP was determined according to Cominetti and coworkers [27] with modifications. Samples of bovine fibrinogen (10 mg/mL) were incubated with different concentrations of enzyme (0.5–3 μg) at 37°C for 2 hours. The reactions were stopped by adding 0.5 mM Tris-HCl pH 8.0, 20% glycerol (v/v), 4% SDS (v/v), 0.05% bromophenol blue (w/v), and 0.3% DL-dithiothreitol (w/v), in a 1 : 1 proportion. After overnight incubation, the digested fibrinogen was analyzed using 10% SDS-PAGE.

2.2.6. Fibrinolytic Activity. Fibrinolytic activity was evaluated according to the method described by Cominetti and coworkers [27] and Chudzinski-Tavassi and Modesto [23]. First enough agarose to prepare a 0.9% gel was solubilized in 50 mM Tris-HCl pH 7.4 plus 100 mM $CaCl_2$ and heated until melted. At a temperature of 37°C, 0.3% bovine fibrinogen, dissolved in 50 mM Tris-HCl pH 7.4 plus 100 mM $CaCl_2$, and 1.2 U/mL bovine thrombin were added to the agarose solution. Afterwards, the mixture was polymerized in a Petri dish (0.9 cm × 15 cm) and the SP (5 and 10 μg in PBS), crude venom (3 μg, positive control), and PBS (negative control) were incubated at 37°C overnight in orifices as previously done. The halos that formed indicating fibrinolytic activity were analyzed by comparison to negative and positive controls. An activity unit was defined as the quantity of protein capable of producing a 1 mm halo on fibrin gel. The result was expressed in millimeters.

2.2.7. Gelatinolytic Activity Assay. Gelatinolytic activity was assessed according to the procedure described by Cominetti and coworkers [27]. SDS-PAGE was carried out on 12.5% gel containing 0.3% gelatin as a copolymerized substrate under nonreducing conditions [21]. After electrophoresis, the gel was washed twice in 0.5% Triton X-100 (v/v) for 30 min to remove SDS and incubated in 50 mM Tris-HCl pH 8.0 plus 5 mM $CaCl_2$ at 37°C for 20 h. Then, the gel was stained with Coomassie blue R-250 and gelatinolytic activity was observed by the presence of clear proteolytic zones.

2.2.8. Amidolytic Activity on Substrate BApNA. Amidolytic activity was measured after incubation at 37°C for 5 h of 10 μg BpirSP-39 in 500 μL solution containing 1% N^{α}-Benzoyl-DL-Arginyl *p*-nitroanilide (BA*p*NA) in 100 mM Tris-HCl pH 8.0. The reaction's product was analyzed at 405 nm using a value of 8800 $M^{-1} \cdot cm^{-1}$ as the molar extinction coefficient of p-nitroanilide. The negative control was carried out using water plus BA*p*NA. A unit of enzymatic activity was defined as the quantity of enzyme capable of releasing 1 μmol p-nitroanilide/min, corresponding to the increase of 0.009 absorbance units measured at A405 nm.

2.3. Sequencing Determination

2.3.1. Solution Digestion. The protein was reduced by treatment with a solution of 20 mM DTT in 50 mM NH_4HCO_3 for 1 h at 30°C and alkylated with a solution of 150 mM iodine acetamide in 50 mM NH_4HCO_3 for 1 h at 30°C. The sample was then digested overnight at 37°C with trypsin (sequencing grade modified, Promega). Afterwards, tryptic peptides were cleaned up with a Proxeon Stage tip and eluted with 70% acetonitrile/0.1% trifluoroacetic acid. The eluted peptides were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC-MS/MS analysis. Mass spectrometry was performed in a nanoAcquity (Waters) HPLC coupled to an Orbitrap Velos mass spectrometer (Thermo Scientific). An aliquot of the tryptic digest was injected and separated in a C18 reverse phase column (75 μmOi, 10 cm, nanoAcquity, 1.7 μm BEH column, Waters). Bound peptides were eluted with the following gradients: 1 to 40% B in 20 minutes, followed by 40 to 60% B in 5 min; flow was 250 nL/min (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile). Eluted peptides were ionized in an emitter needle (PicoTipTM, New Objective). Spray voltage applied was 1900 V. Peptide masses (*m/z*: 300–1700) were measured in full scan in the Orbitrap at a resolution of 60,000 at 400 *m/z*. Up to the 5 most abundant peptides (minimum intensity of 1500 counts) were selected from each MS scan and fragmented in the HCD collision cell using a normalized collision energy (NCE) of 40% with nitrogen as the collision gas. Fragments were detected in the Orbitrap with a resolution of 7500 FWHM at 400 *m/z*. Raw data were collected using Thermo Xcalibur (v.2.1.0.1140).

2.3.2. Database Search. Raw data were analyzed using Proteome Discoverer (v.1.3.0.339) software. A search was run with the search engine MASCOT against the NCBInr Serpentes database. Also, an .mgf file was generated in Proteome Discoverer and this file was used to search with PEAKS Studio (v.5.3.) against the same database. After that the homology search tool SPIDER* was used to run a tag homology search. The search parameters were Database/Taxonomy: NCBInr Serpentes; missed cleavage: 2; fixed modifications: carbamidomethyl of cysteine; variable modifications: oxidation of methionine and pyro-Glu (N-term Glutamine);

peptide tolerance: 10 ppm for MS spectra and 0.05 Da for MS/MS spectra; and enzyme: trypsin.

The Percolator node was used in the Proteome Discoverer Mascot search in order to discriminate correct from incorrect peptide spectrum matches using the q-value (FDR) to improve the number of confidently identified peptides at a given false discovery rate. The results have been filtered so only high confidence peptides (FDR $\leq$ 0.01) are considered for identification results.

2.4. BpirSP-39 Molecular Modeling and Determination of N-Glycosylation Sites. The structural model of the BpirSP-39 from *Bothrops pirajai* was generated employing the threading modeling method [28–30], which was performed using the HHpred software [31] available at http://toolkit.tuebingen.mpg.de/hhpred. Initially, HHpred generated 112 alignments for BpirSP-39. The alignments were obtained using the global mode and the gaps resulting from LC-MS/MS sequencing were filled by homology with a thrombin-like enzyme from *Agkistrodon halys* venom (PDB ID: 4E7N) [32] (selected to construct the model of BpirSP-39). The first two gaps were confirmed by Edman's degradation (data not showed), and the third and fourth gaps are justified by the large amount of lysine which generated small fragments not detected by LC-MS/MS. The chosen template showed the best alignment score (286.73) and the identity between the studied protein sequence and the template was 67%. Potential N-glycosylation sites of serineprotease were predicted employing NetNGlyc v.1.0, [33] available at http://www.cbs.dtu.dk/.

2.4.1. Simulation of Molecular Dynamics. After the construction of the initial model, we performed simulations of molecular dynamics (MD) of the studied protein. All the MD parameters were equally set to the two generated models. The MD simulations were performed employing GROMACS (GROningenMAchine for Chemical Simulation) v.4.5.4 software [34, 35] in Intel Xeon processor with 8 GB RAM, operating in a CentOS 5.5 Linux operational system. The simple point charge (SPC) model was used to represent explicit water molecules. Protonation states of charged groups were set according to pH 7.0 and counter ions were added to neutralize the system. GROMOS force field [36] was chosen to perform the MD simulation. These simulations were performed at constant temperature and pressure in a periodic truncated cubic box, and the minimum distance between any atom of the protein and the box wall was 1.0 nm.

Initially, an energy minimization using the steepest descent algorithm was performed. After that 20 ps of MD simulation with position restraints applied to the protein was performed at 298 K to relax the system. And finally, an unrestrained MD simulation was performed at 298 K for 10 ns to assess the stability of the structures. During the simulation, temperature and pressure (1.0 bar) were maintained by an external bath controlling heat and isotropic pressure.

2.4.2. Structural Analysis and Validation. The model generated after the MD simulation was checked using several GROMACS structural analyses, as well as the analysis of Ramachandran plot generated with Rampage [37]. The pseudo-energy profile of the models was analyzed with Verify 3D [38, 39] available at <http://nihserver.mbi.ucla.edu/Verify_3D/> and ProSA-web [40, 41].

3. Results

The purification of BpirSP-39 was performed using three consecutive chromatographic steps. The first step of the *B. pirajai* venom fractionation, performed by size-exclusion molecular chromatography on Superdex G-75, resulted in five fractions (P1–P5) (Figure 1(a)). The peaks P-1 and P-2 were capable of coagulating the citrated plasma and promoting proteolytic activity, when the chromogenic substrate BA*p*NA was used. Since P-1 demonstrated the highest coagulant and proteolytic activities, it was fractioned by affinity chromatography using a benzamidine Sepharose column resulting in two peaks (Figure 1(b)). The coagulant fraction was applied to a C2/C18 column, and after elution enzymatic activity was observed in the first fraction (Figure 1(c)).

The relative molecular mass of SP, estimated by SDS-PAGE 12.5%, was approximately 49 kDa (Figure 1(a)) but when determined by mass spectrometry it was 39.408,32 Da (Figure 1(d)). Knowing that mass spectrometry is a more accurate method than polyacrylamide electrophoresis, the new identified serineprotease was called BpirSP-39.

BpirSP-39 is a serineprotease that presents coagulant activity in citrated plasma in a concentration-dependent manner, with a minimum coagulant dose (MCD) determined to be 1.7 μg of the protein (Figure 2(a)).

In contrast to the majority of snake venom serine proteases [42], BpirSP-39 is apparently able to activate the clotting cascade factor XIII and, as observed in the positive control, the fibrin network showed stability after 48 h incubation. The clot induced by BjussuSP-I was dissolved in less than 120 seconds which indicates that factor XIII was not activated. The second negative control (40 μL of water) was not able to induce a coagulation process, proving that thrombin was neutralized by heparin and does not participate in the coagulation induced by BpirSP-39, though for a definite conclusion, it needs to be tested with purified factor XIII.

BpirSP-39 clotting activity was not influenced by different thrombin inhibitors (citrate, heparin, and EDTA), which distinguishes it from most svTLEs (see Table 1). However, BpirSP-39 clotting activity was significantly reduced after incubation with PMSF (Figure 2(b)). BpirSP-39 also proved to be a thermo-stable enzyme (Figure 2(c)), exhibiting highest activity at room temperature (25°C).

The enzyme possesses high catalytic activity on different chromogenic substrates tested (S-2238, S-2222, and S-2302) (Figure 2(d)); however, when incubated with Cu^{2+}, its catalytic activity was diminished significantly on the three tested substrates. While Mn^{2+} influenced the activity on substrates S-2222 (for factor Xa) and S-2302 (for plasma kallikrein, factor XIa and XIIa), Ba^{2+} and Ca^{2+} had no influence on the catalytic activity on substrate S-2238 (that is suitable for thrombin-like enzymes) but modified the enzyme's activity on substrates S-2222 and S-2302. The protein was also

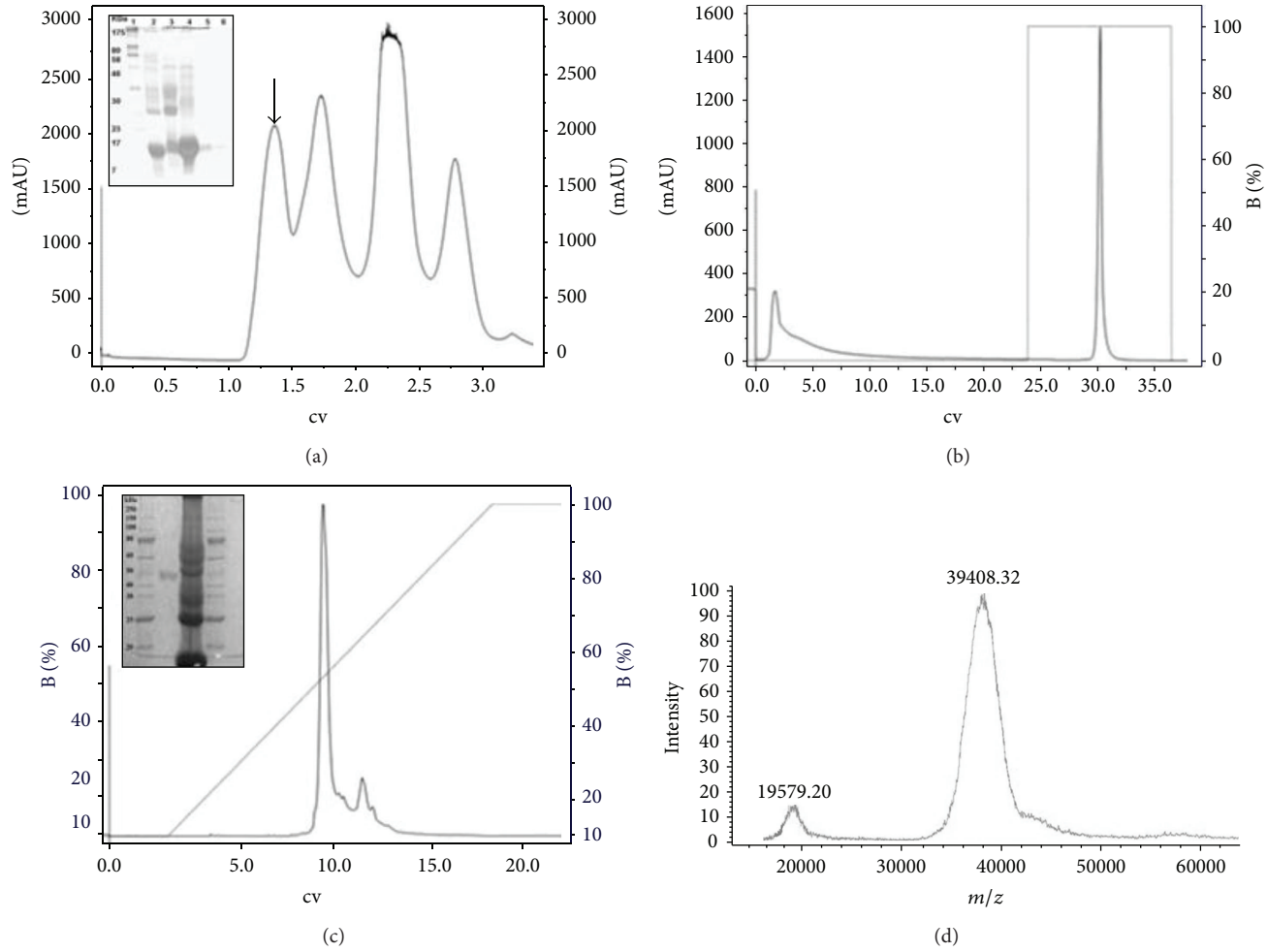

Figure 1: Purification profile of the serineprotease BpirSP-39 from *Bothrops pirajai* crude venom. The detached arrow (a) indicates the fraction with the highest coagulation activity, fraction 1 of 12.5% SDS-PAGE in denaturing conditions. Line 1: molecular mass standard, Color Plus Prestained Protein Marker, Broad Range (7–175 kDa) (P7709S New England Biolabs), lines 2–6: Fractions 1–5 obtained after chromatography. (b) Affinity chromatography of fraction 1 on benzamidine sepharose column. (c) High performance liquid chromatography using the C2/C18 column (10 mm × 4.6 mm, 3 μm, 120 Å) and 12.5% SDS-PAGE of BpirSP-39 and *B. pirajai* crude venom. Lines 1 and 4: molecular mass standard: Protein Ladder (10–250 kDa) (P7703S New England Biolabs); 2- BpirSP-39 in denaturing conditions showing a band of approximately 49 kDa; 3-crude venom of *B. pirajai* in denaturing conditions. (d) Mass spectrum of BpirSP-39 determined by AXIMA TOF^2. The identified protein presented a molecular mass of 39,408.32 Da. The peak at 19,579.02 Da indicates the double charge of the protein. The absorbance was monitored at A280 nm.

capable of promoting platelet aggregation in a concentration-dependent manner (Figure 2(f)).

The proteolytic activity of BpirSP-39 on fibrin (Figure 3(b)) demonstrates that the purified serineprotease is a fibrinogenolytic enzyme similar to other svTLEs [43]. Furthermore, BpirSP-39 showed gelatinolytic activity (Figure 3(c)) and amidolytic activity on BA*p*NA (Figure 3(d)).

The amino acid sequence of BpirSP-39 was determined by MS/MS and showed a multiple sequence alignment between the enzyme and other serine proteases (Figure 4). Figure 5(a) displays the root mean squared deviation (RMSD) of the backbone during the MD simulation and we can see that the structure of the BpirSP-39 model was clearly stabilized after 7500 ps. From these results, it is possible to say that the MD simulations were important to minimize the system. From the RMS fluctuation plot between 7500 and 10000 ps of MD simulation (Figure 5(b)), we can note that only the loop regions had deviation high values. The average fluctuation of the protein structure is around 0.6 Å and the maximum fluctuation is around 1.4 Å, indicating a high level of stability. These structural findings confirm the quality of the generated model. Figure 5(c) shows the alignment between the final model and the chosen template, indicating that the predicted tertiary structure was preserved during the MD simulation.

After the MD simulation, the final model presented Verify3D scores above zero for all residues, suggesting that the conformation of individual residues was adequate. Analyzing the Ramachandran plot of the final model, the BpirSP-39 structure shows 81.3% of the residues located at allowed regions and only 4.3% in outlier regions (Figure 6(a)). Also,

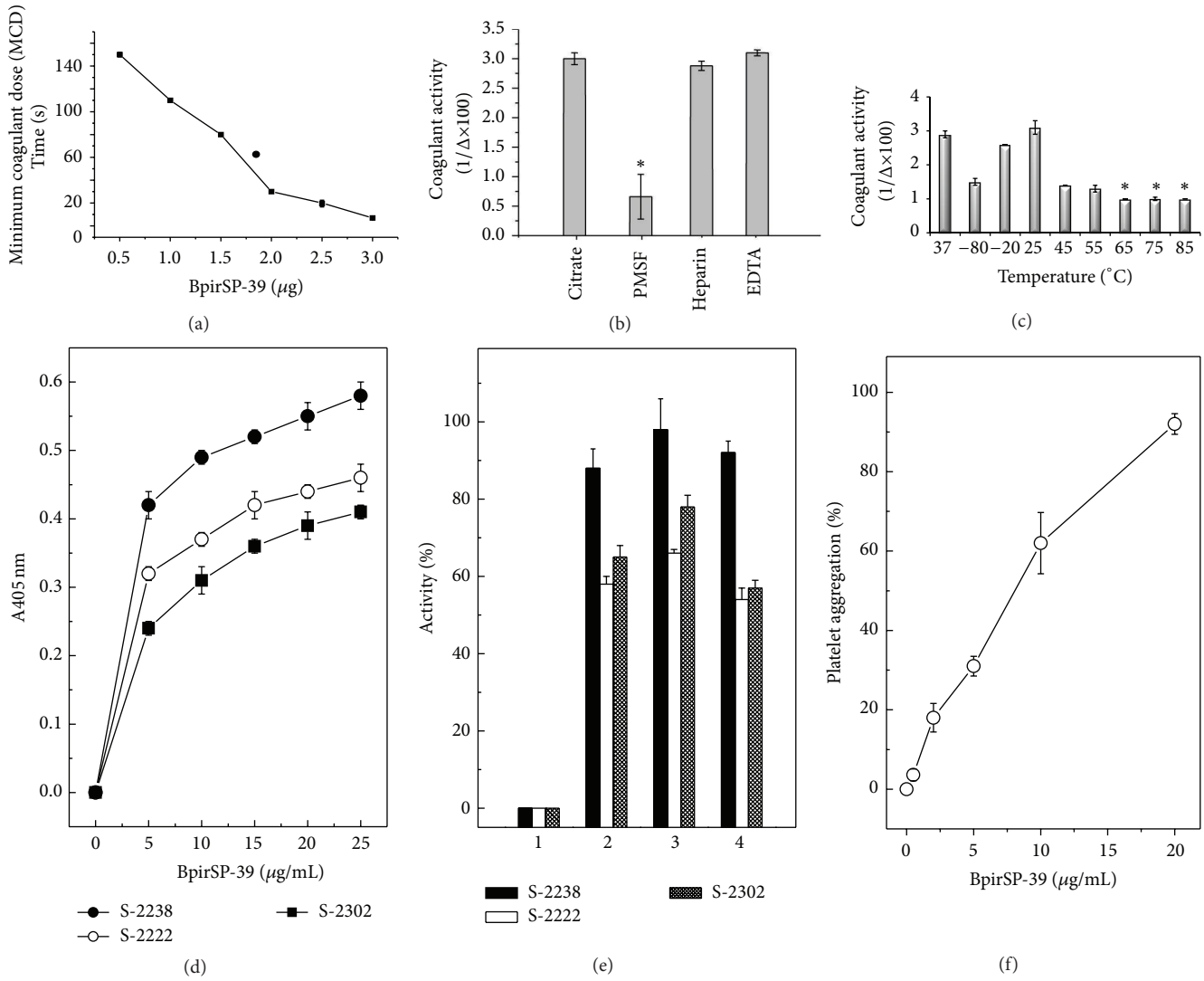

Figure 2: Enzymatic characterization of the protein. (a) Determination of minimum coagulant dose (MCD) of BpirSP-39 using a concentration-response curve. The time of formation of fibrin network was measured by chronometer and the samples were evaluated visually. (b) Evaluation of inhibitors' action on the coagulation activity of BpirSP-39. (c) Evaluation of BpirSP-39 thermostability on the clotting of human citrated plasma. (d) Effect of protein on different chromogenic substrates. (e) Effect of metals on the enzymatic activity of serineprotease (column 1, Cu^{2+}; column 2, Mn^{2+}; column 3, Ba^{2+}; and column 4, Ca^{2+}). (f) Effect of serineprotease on platelet aggregation. Results are expressed as means ± SD of two individual experiments ($n = 3$).

all disordered predicted residues were located at loop regions (Figure 6(b)), suggesting that the conformation of the final model has good stereochemical quality.

Finally, we validated the final model using the energy profile from ProSA web server. The initial model contained a high energy region and the final model has no region with energy higher than 0, indicating that the MD simulation was important in improving the model's quality (Supplementary Material—Figure 8, available on line at http://dx.doi.org/10.1155/2014/595186). The binding site of the modeled serineprotease is composed of a histidine, an asparagine, and a serine (His42, Asp86, and Ser194). Figure 7 displays the obtained model with disulfide bonds in yellow and the catalytic triad in red. The final model presented the same number of disulfide bonds as other snake venom serine proteases.

Based on these results, the new identified serineprotease mimics several thrombin characteristics (Table 2).

4. Discussion

The present report details the isolation and biochemical characterization of BpriSP-39, a new thrombin-like enzyme from *Bothrops pirajai* snake venom, with common procedures for the isolation of snake venom serine proteases [44–50].

The divergences observed between the relative and absolute mass of BpirSP-39 were also detected with other thrombin-like proteins purified from snake venoms [51–54].

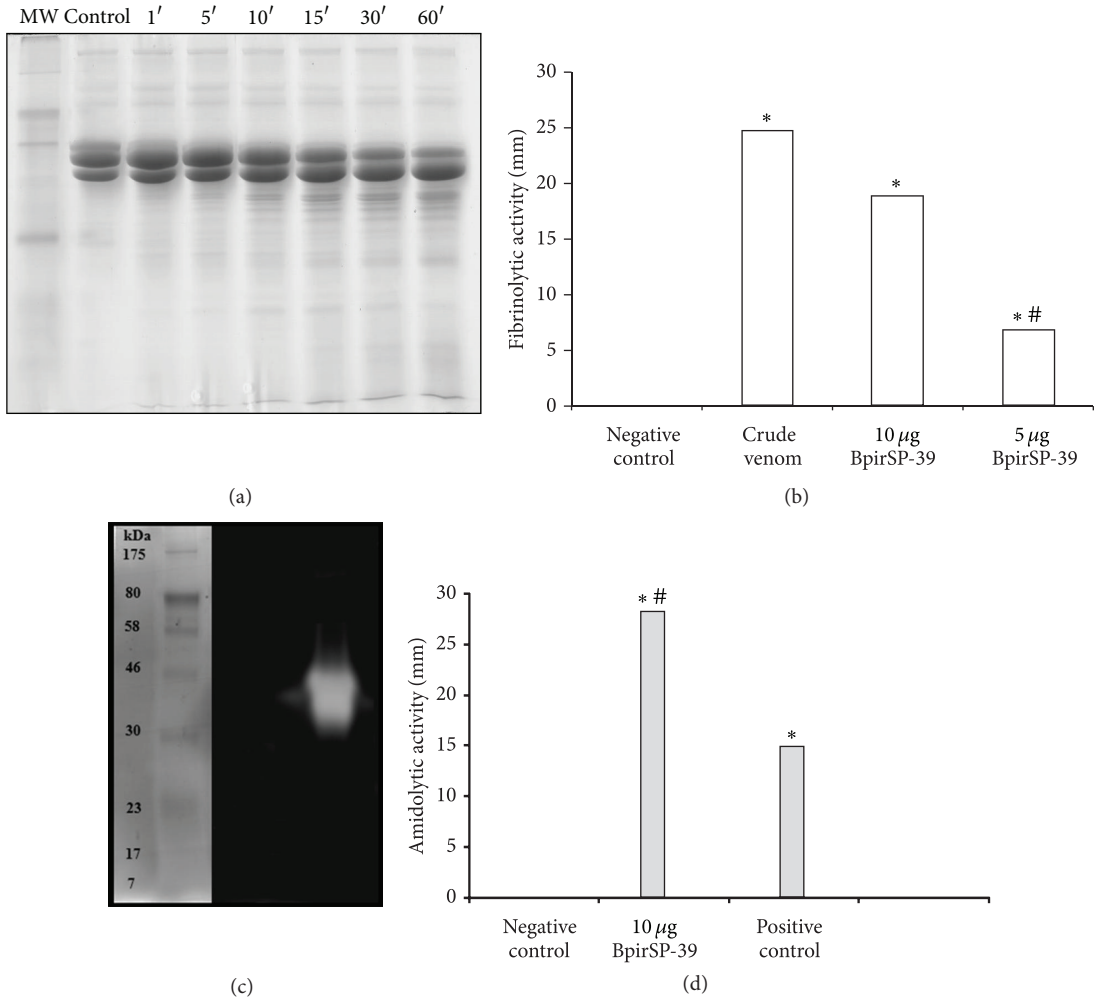

Figure 3: Enzymatic characterization of BpirSP-39. (a) Fibrinogenolytic activity demonstrating the degradation of the α and β chains of bovine fibrinogen. (b) Fibrinolytic activity. The data are expressed in millimeters. (c) Gelatinase activity stained with Coomassie R-250. (d) Amidolytic activity on chromogenic substrate BA*p*NA. The crude venom of *B. pirajai* was used as positive control and the dilution buffer of the sample as negative control. (∗) Values significantly different from the negative control ($P \leq 0.05$) and (#) values significantly different from positive control ($P \leq 0.05$).

Like the majority of serine proteases [12, 13, 20, 44–48], these differences indicate that BpirSP-39 seems to be a glycosylated protein. The difference detected during electrophoretic migration was probably caused by the carbohydrate microheterogeneity of the enzyme since this fraction can vary the weight of the serine protease up to 30%. Castro and coworkers [9] suggest that the glycidyl domain can aid in structural stabilization and participate in the recognition of substrates by the enzyme.

The MCD of BpirSp-39 was 1.5 μg which is similar to BjussuSP-I, a serineprotease isolated from the venom of *Bothrops jararacussu*, a venomous snake phylogenetically similar to *Bothrops pirajai* [48]. When compared to two other serineprotease isoforms isolated and characterized from the same species by Menaldo and coworkers [20] (BpirSP27 and BpirSP41, with MCDs of ~3.5 μg and ~20 μg, resp.), BpirSP-39 presented a higher coagulant potential. Other purified serine proteases such as PA-BJ and Jararassin-I from *Bothrops jararaca* venom show considerably reduced coagulant activity, with MCDs of 5 of 10 μg, respectively [55, 56].

The new isoform of serineprotease from *Bothrops pirajai* is apparently able to activate factor III in XIIIa. It is known that factor XIII is a protransglutaminase activated by thrombin at the end of blood cascade system [57]. In plasma, factor XIII presents two subunits. While subunit A is the active form of the enzyme, subunit B plays the role of a carrier protein [58]. Factor XIIIa modifies the structure of the clot by forming cross-links between the fibrin by a link ε (γ-glutamyl) lysine [59] leading to increased resistance to fibrinolysis. Factor XIIIa is also able to maintain its structure when exposed to denaturing agents.

BpirSP-39 has clotting activity and its action was not influenced by different thrombin inhibitors. However, this proclotting activity was significantly reduced after incubation

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B_pirajai_45-seq1                                  VIGGDECNINEHR FLVALYGKRSRGYFC
V_STEJN | Q71QI1.1              MVLIRVLANLLILQLSYAQRSSELVIGGDECNINEHR FLVALYK  SGRFRC
B_insularis | Q8QG86.1          MVLIRVIANLLILQVSYAQKSSELVVGGDECDINEHP FLAFLY-  SHGYFC
B_ALTER | Q6IWF1.1              ------------------------VIGGDECDINEHR FLAFLY-  PGRFFC
A_acutus | 1OP2_A | PDBID       ------------------------VIGGNECDINEHR FLVAFFN  TTGFFC
D_acutus | AAK52506.2           ----------------------MVIGGDECDINEHR FLVAFFN  TTGFFC
B_JUSSU | Q2PQJ3.1               ------------------------VLGGDECDINEHP FL-AFLY  SHGYFC

B_pirajai_45-seq1               GLTLINQEWVLTAAHCDR KNFRIY LGIHTR KVLNEDEQTR ------FLCPN
V_STEJN | Q71QI1.1              GGTLINQEWVLTAAHCDR RNMEIK LGMHSK NVPNEDEQRR VPKEKF-FCDS
B_insularis | Q8QG86.1          GLTLINQEWVLTAAHCDR RFMRIY LGIHAR SVANDDEVIR YPKEKF-ICPN
B_ALTER | Q6IWF1.1              SGTLINQEWVLTVAHCDT ISMRIY LGLHTR SVPNDDEEIR YPMEKF-KCPN
A_acutus | 1OP2_A | PDBID       GGTLINPEWVVTAAHCDS TNFQMQ LGVHSK KVLNEDEQTR NPKEKF-ICPN
D_acutus | AAK52506.2           GGTLINPEWVVTAAHCDS TNFQMQ LGVHSK KVLNEDEQTR NPKEKF-ICPN
B_JUSSU | Q2PQJ3.1               GLTLINQEWVVTAAHCDS TNFQMQ LGVHSK KVLNEDEQTR NPKEKF-ICPN

B_pirajai_45-seq1               GK KDDVLDKDIMLIR LDSPVS NSEHIAPLSLPSSPPSVGSVCR IMGWGTI
V_STEJN | Q71QI1.1              NK NYTQWNKDIMLIR LNSPVN NSTHIAPLSLPSNPPIVGSVCR IMGWGTI
B_insularis | Q8QG86.1          KN MSDEKDKDIMLIR LNRPVK NSTHIAPISLPSNPPSVGSVCR VMGWGSI
B_ALTER | Q6IWF1.1              RK RSYIKDKDIMLIR LNRPVN DSPHIAPLSLPSNPPSVGSVCH VMGWGTT
A_acutus | 1OP2_A | PDBID       KN NNEVLDKDIMLIK LDKPIS NSKHIAPLSLPSSPPSVGSVCR IMGWGSI
D_acutus | AAK52506.2           KN NNEVLDKDIMLIK LDSPVN NSAHIAPISLPSNPPSVGSVCR VMGWGSI
B_JUSSU | Q2PQJ3.1               KN S-EVLDKDIMLIK LDKPIS NSKHIAPLSLPSNPPSVGSVCR IMGWGSI

B_pirajai_45-seq1               SPTK  TNPDVPHCAN INLLDDAVCR AAYPELPAEYR   TLCAGILQGGI
V_STEJN | Q71QI1.1              TSPN ETYPDVPHCAN INLFNYTVCH GAHAGLPATSR   TLCAGVLEGGK
B_insularis | Q8QG86.1          TIPN DTYPDVPHCAN INLVNDTVCR GAYKRFPAKSR   TLCAGVLQGGK
B_ALTER | Q6IWF1.1              SPSK ATYPDVPHCAN INLVNDTMCH GAYNGLPVTSR   KFCAGVLQGGI
A_acutus | 1OP2_A | PDBID       TPVK ETFPDVPYCAN INLLDHAVCQ AGYPELLAEYR   TLCAGIVQGGK
D_acutus | AAK52506.2           TSPN VTIPGVPHCAN INILDYEVCR ATKPELPAKSR   TLCAGILEGGK
B_JUSSU | Q2PQJ3.1               TIPN ETYPDVPYCAN INLVDYEVCQ GAYNGLPAKT-   TLCAGVLEGGK

B_pirajai_45-seq1               DSCK -------LCNGQFQG LLSWGSKVCAQP RLKPALYTK VSDYTEWIK SIIAG
V_STEJN | Q71QI1.1              DTCK GDSGGPLICNGQFQG FVSWGGDPCAQP REPGVYTK  VFDHLDWIQ NIIAG
B_insularis | Q8QG86.1          DTCV GDSGGPLICNGTFQG IVSWGGKVCARP RKPALYTK  VFDYLPWIQ SIIAG
B_ALTER | Q6IWF1.1              DTCV GDSGGPLICNGQFQG IVSWGGKVCARL PRPALYTK  VFEYLPWIQ SIIAG
A_acutus | 1OP2_A | PDBID       DTCG GDSGGPLICNGQFQG IVSYGAHPCGQG PKPGIYTN  VFDYTDWIQ RNIAG
D_acutus | AAK52506.2           DTCG GDSGGPLICNGQFQG IVSYGAHPCGQG PKPGIYTN  VFDYTDWIQ RNIAG
B_JUSSU | Q2PQJ3.1               DTCV GDSGGPLICNGQFQG IVSYGAHSCGQG PKPGIYTN  VFDYTDWIQ RNIAG

B_pirajai_45-seq1               NTDVTCPP
V_STEJN | Q71QI1.1              NTTATCPL
B_insularis | Q8QG86.1          NKTATCPP
B_ALTER | Q6IWF1.1              NTTATCPL
A_acutus | 1OP2_A | PDBID       NTDATCPP
D_acutus | AAK52506.2           NTSATCPP
B_JUSSU | Q2PQJ3.1               NTDATCPP
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Figure 4: Multiple sequence alignment between BpirSP-39 and other serine proteases.

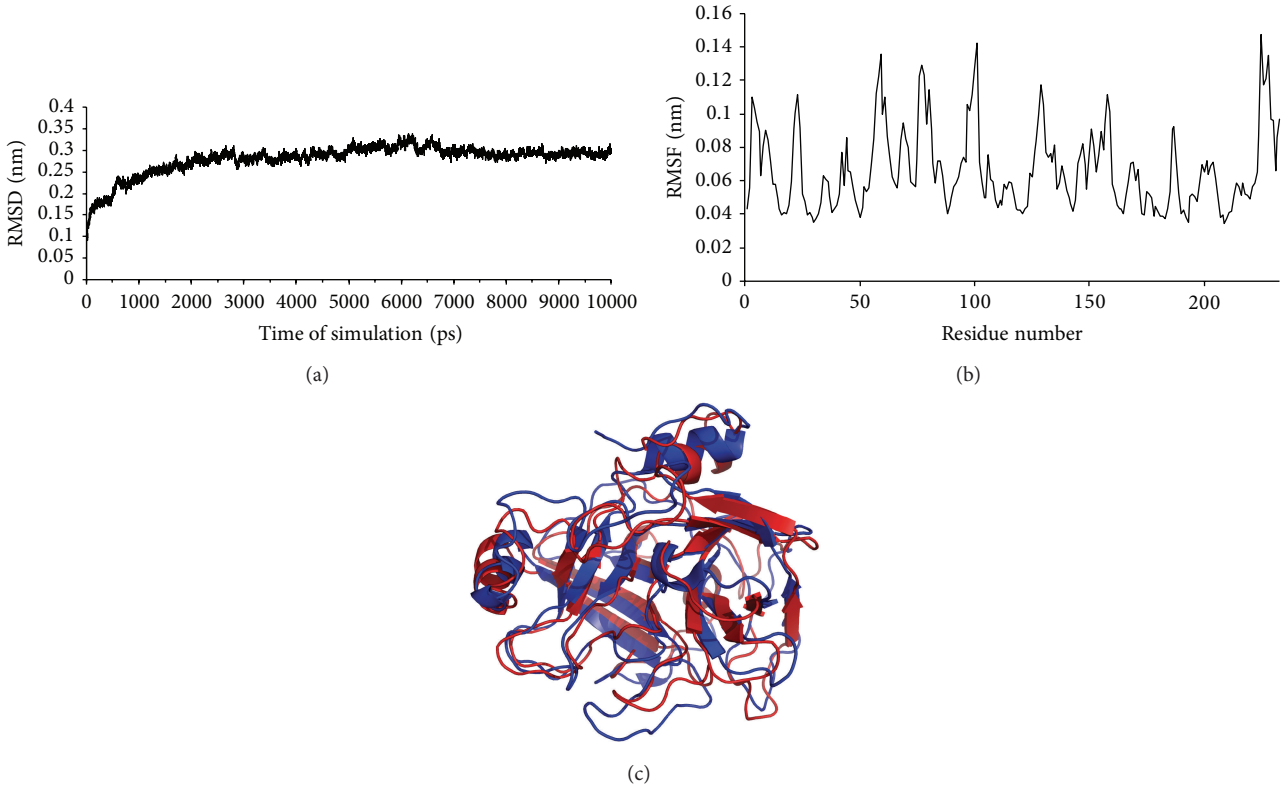


Figure 5: (a) RMSD versus MD simulation time for the generated model; (b) root mean squared fluctuation (RMSF) of average structure of BpirSP-39 (between 7.5 and 10 ns of MD simulation); (c) alignment between BpirSP-39 (red) model and crystallographic template (blue).

with PMSF, indicating that serine residues present in the enzyme's catalytic site participate in the proteolytic activity, since PMSF binds covalently to reactive serine residues present in the catalytic site [60]. Because the clotting activity was not inhibited by heparin, a direct thrombin inhibitor, it can be proposed that the identified protein acts as a thrombin-like enzyme, and not as a prothrombin activator, as is true of some snake serine proteases. If BpirSP-39 acted indirectly, activating prothrombin, the resulting thrombin would be inactivated by heparin preventing fibrin network formation. In this same way, the clotting activity of Agacutase, a recent thrombin-like enzyme isolated from *Deinagkistrodon acutus* [12], was not influenced by heparin or hirudin, which is different from BjussuSP-I, a serineprotease from *Bothrops jararacussu* whose clotting ability was reduced by heparin [48].

The BpirSP-39 showed a high thermostability similar to BpirSP27 and BpirSP41 [20], BjussuSP-I [48], and Barnettobin, a coagulant thrombin-like enzyme isolated and characterized from *Bothrops barnetti* venom [10]. This data confirms the expected results of an enzyme belonging to this class, which possesses considerable thermal stability, differing from metalloproteases which are quickly inactivated when exposed to extreme variations in temperature and pH [43].

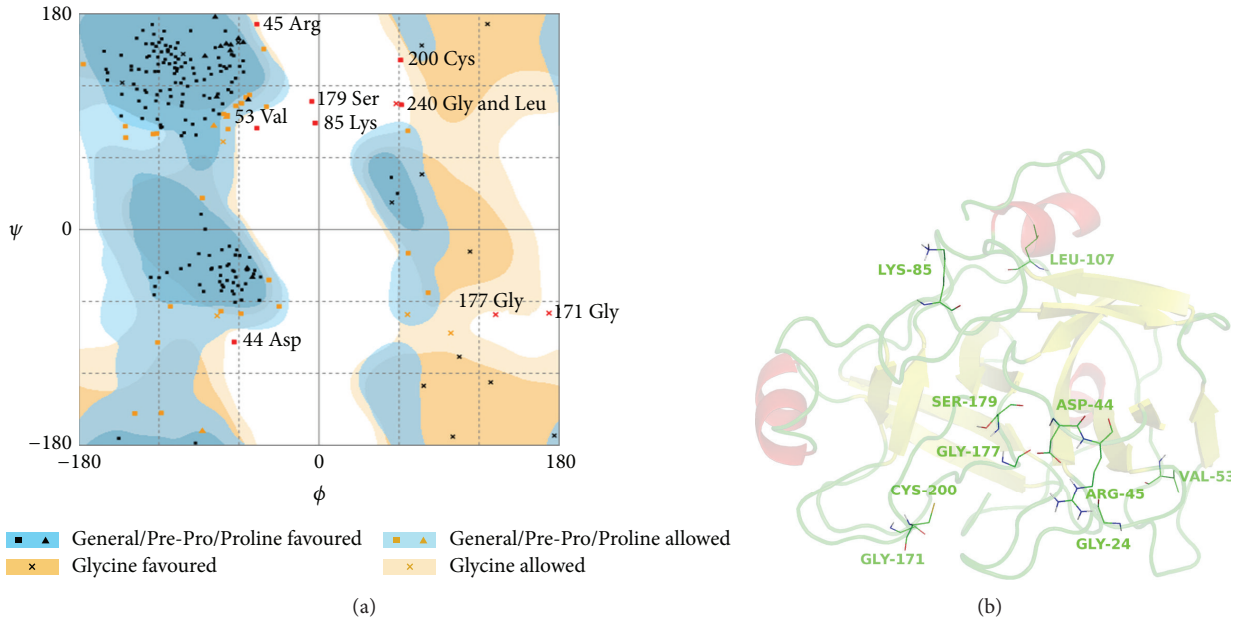
The results obtained related to the substrate S-2238 for BpirSP-39 are similar to the data from BpirSP27 and BpirSP41 [20]. These isoforms showed reduced thrombin-like activity when incubated with Cu^{2+}, BpirSP27 activity was influenced by Mn^{2+}, and neither was modified when incubated with Ba^{2+} and Ca^{2+} (Figure 2(e)).

Concerning the enzyme's capacity to promote platelet aggregation, BpirSP-39 seems to be more active compared to other isolated isoforms of the same species [20]. The new isolated serineprotease was able to degrade fibrinogen and induce fibrin network formation, as well as cleave the α and β chains of bovine fibrinogen (Figure 3(a)). This is

Table 1: Effect of inhibitors on enzymatic activity of SP.

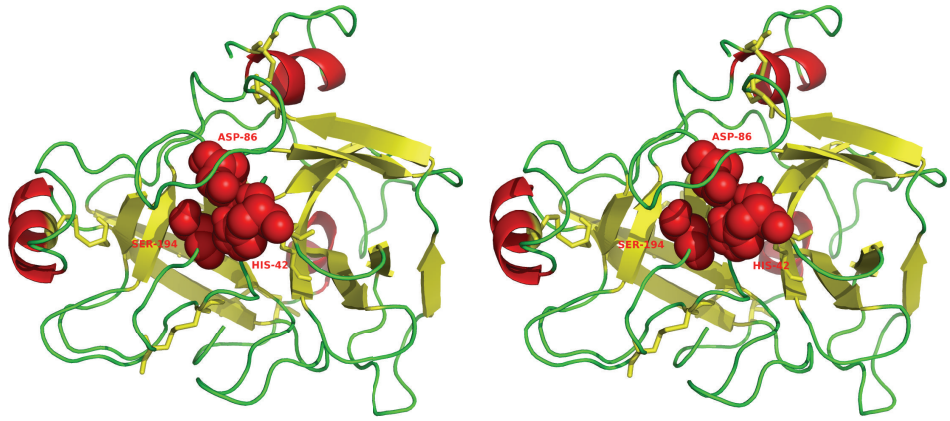
Inhibitors	% inhibition of		
	S-2238	S-2222	S-2303
Benzamidine	62 ± 4	61 ± 2	47 ± 1
PMSF	55 ± 3	52 ± 3	45 ± 3
EDTA	7.5 ± 1	15 ± 2	5 ± 2
O-Phe	3.9 ± 2	3 ± 1	4.5 ± 2

The inhibitors in final concentration, benzamidine (15 mM), PMSF (2 mM), EDTA (20 mM), or O-Phe (0.3 mM) were preincubated with SP (20 μg/mL) for 60 min at 37°C; then the reaction was initiated by adding chromogenic substrates (0.1 mM, final concentration). The reaction was monitored for 5 min, as described in the Section 2 and % inhibition was measured. 100% of the SP enzymatic activity was obtained in the absence of inhibitors for each substrate. Results are expressed as means ± SD of two individual experiments ($n = 2$).



(a) (b)

Figure 6: Residues located at outlier regions predicted by Ramachandran plot.



(a) (b)

Figure 7: Stereoview of the final 3D model for BpirSP-39.

in contrast to other thrombin-like enzymes, which cleave preferentially either the α or β fibrinogen chains, occasioning an increase in fibrinopeptides A or B and consequently generating abnormal blood clots [62, 63].

As for BpirSP-39's proteolytic activity upon fibrin, gelatin, and the amidolytic substrate BA*p*NA, the purified serine-protease demonstrates enzymatic activities similar to other svTLEs [43]. It is known that the proteolytic action on BA*p*NA occurs between the amino acids Arg and Gly. This confirms that BpirSP-39 possesses fibrinogenolytic action on the α and β chains of fibrinogen, since the *in vivo* conversion of fibrinogen to fibrin carried out by thrombin is obtained by the cleavage of four peptide bonds in the amino terminal regions of the polypeptide chains 2Aα and 2Bβ, which occur between the amino acids Arg^{14}-Gly^{17} of the Aα chains and Arg^{14}-Gly^{15} of the Bβ chains [64].

The best template found for molecular modeling was the structure in the PDB registered under the number 4E7N, corresponding to a thrombin-like enzyme isolated from the venom of the snake *Agkistrodon halys*. This template has 67% identity with the sequence of *B. pirajai* and a similarity score of about 286.7. The literature says that templates with percent identities above 30% are sufficient to predict the three-dimensional structure between template-protein and target-protein.

Two potential glycosylation sites on BpirSP-39 were identified at positions Asn05 and Asn74 using the software NetNGlyc (results not shown). The presence of these sites

Table 2: Comparison between thrombin and BpirSP-39 activities*.

Activities	Thrombin	BpirSP-39
Aggregation of platelet disaggregation	+	Not tested
Clot retraction	+	Not tested
Fibrinogen clotting	+	+
Factor XIII activation	+	+
Degradation of fibrinogen (α and β)	+	+
Hydrolysis of BAPNA	+	+
Inhibition by heparin	+	−
Inhibition by PMSF	+	+
Inhibition by citrate	+	−
Inhibition by EDTA	+	−

*Adapted from Niewiarowski et al., 1979 [61].
The presented data represents a summary of thrombin and BpirSP-39 activities.

is conserved in SVSPs. BpiSP-39 also showed the presence of twelve cysteine residues, ten of which form five disulfide bonds. The other two cysteines form a unique bridge conserved among SVSPs, in this case, involving Cys226 found in the C-terminal extension [55].

Medical and scientific interest in thrombin-like enzymes has increased considerably because of their specificity when compared to thrombin, a multifunctional enzyme [65]. These serine proteases seem to be promising defibrinogenation agents. The enzymes ancrod (Arwin), isolated from the venom of *Calloselasma rhodostoma*, and batroxobin (Defibrase), isolated from *B. moojeni*, are being used in patients suffering from thrombosis, myocardial infarction, peripheral vascular diseases, acute ischemia, and renal transplant rejection [66, 67]. Ancrod has also been used as a treatment for heparin-induced thrombocytopenia [68] without any impact on platelets [69]. Besides that, batroxobin (isolated from *Bothrops atrox*) and gyroxin, a serineprotease described by Bacila [70] and purified by Alexander and coworkers [71] from the venom of *Crotalus durissus terrificus*, are used to prepare fibrin sealants that can be utilized in different medical situations [72].

The fibrin sealants made by fibrinogen extracted from large animals and thrombin-like enzymes extracted from snake venoms were tested in both animals and humans and have diverse advantages, such as quick, easy, and cheap production; they have a large diversity of applications; they are safe, since they do not produce notable adverse reactions; and they do not use human blood or present risk of infectious disease transmission [73].

In summary, a novel isoform of serineprotease was isolated and characterized from the crude venom of the *Bothrops pirajai* snake. BpirSP-39 is a thrombin-like protein. Based on its characteristics, the enzyme could be an alternative to thrombin in the production of fibrin sealants, such as autologous fibrinogen. The enzyme, by itself, does not induce viral contamination and it also shows promising use in the treatment of clotting dysfunction.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

The authors express their gratitude to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Instituto Nacional de Ciência e Tecnologia em Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT-INPeTAm), Instituto Nacional de Ciência e Tecnologia em Toxinas (INCT-Tox), Secretaria de Estado do Planejamento e Coordenação Geral (CNPq-SEPLAN-RO), CICYT (CTQ2012-30930), and the *Generalitat de Catalunya* (2009SGR 1024) for the financial support and to Conselho de Gestão do Patrimônio Genético (CGEN/MMA) for the authorization number 010627/2011-1. Amy Grabner provided the English editing of the paper.

References

[1] J. White, "Snake venoms and coagulopathy," *Toxicon*, vol. 45, no. 8, pp. 951–967, 2005.

[2] T. Matsui, Y. Fujimura, and K. Titani, "Snake venom proteases affecting hemostasis and thrombosis," *Biochimica et Biophysica Acta*, vol. 1477, no. 1-2, pp. 146–156, 2000.

[3] S. M. T. Serrano and R. C. Maroun, "Snake venom serine proteinases: sequence homology versus substrate specificity, a paradox to be solved," *Toxicon*, vol. 45, no. 8, pp. 1115–1132, 2005.

[4] J. A. F. P. Villar, F. T. D. Lima, C. L. Veber et al., "Synthesis and evaluation of nitrostyrene derivative compounds, new snake venom phospholipase A2 inhibitors," *Toxicon*, vol. 51, no. 8, pp. 1467–1478, 2008.

[5] J. D. O. Costa, K. C. Fonseca, M. S. Garrote-Filho et al., "Structural and functional comparison of proteolytic enzymes from plant latex and snake venoms," *Biochimie*, vol. 92, no. 12, pp. 1760–1765, 2010.

[6] J. D. O. Costa, K. C. Fonseca, C. C. Neves Mamede et al., "Bhalternin: functional and structural characterization of a new thrombin-like enzyme from Bothrops alternatus snake venom," *Toxicon*, vol. 55, no. 7, pp. 1365–1377, 2010.

[7] E. Longo, F. M. L. G. Stamato, R. Ferreira, and O. Tapia, "The catalytic mechanism of serine proteases II: the effect of the protein environment in the α-chymotrypsin proton relay system," *Journal of Theoretical Biology*, vol. 112, no. 4, pp. 783–798, 1985.

[8] S. Braud, C. Bon, and A. Wisner, "Snake venom proteins acting on hemostasis," *Biochimie*, vol. 82, no. 9-10, pp. 851–859, 2000.

[9] H. C. Castro, R. B. Zingali, M. G. Albuquerque, M. Pujol-Luz, and C. R. Rodrigues, "Snake venom thrombin-like enzymes: from reptilase to now," *Cellular and Molecular Life Sciences*, vol. 61, no. 7-8, pp. 843–856, 2004.

[10] D. E. Vivas-Ruiz, G. A. Sandoval, J. Mendoza et al., "Coagulant thrombin-like enzyme (barnettobin) from *Bothrops barnetti* venom: molecular sequence analysis of its cDNA and biochemical properties," *Biochimie*, vol. 95, no. 494, pp. 1476–1486, 2013.

[11] A. K. Mukherjee and S. P. Mackessy, "Biochemical and pharmacological properties of a new thrombin-like serine protease (Russelobin) from the venom of Russell's Viper (*Daboia russelii russelii*) and assessment of its therapeutic potential," *Biochimica et Biophysica Acta*, vol. 1830, no. 495, pp. 3476–3488, 2013.

[12] S. S. Tang, X. H. Wang, J. H. Zhang et al., "Biochemical properties and comparative pharmacology of a coagulant from *Deinagkistrodon acutus* snake venom," *European Journal of Pharmaceutical Sciences*, vol. 49, no. 496, pp. 90–98, 2013.

[13] Y. Zheng, F. P. Ye, J. Wang et al., "Purification, characterization and gene cloning of Da-36, a novel serine protease from *Deinagkistrodon acutus* venom," *Toxicon*, vol. 67, no. 497, pp. 1–11, 2013.

[14] L. C. Mancuso, M. M. Correa, C. A. Vieira et al., "Fractionation of *Bothrops pirajai* snake venom: isolation and characterization of piratoxin-I, a new myotoxic protein," *Toxicon*, vol. 33, no. 5, pp. 615–626, 1995.

[15] M. H. Toyama, L. C. Mancuso, J. R. Giglio, J. C. Novello, B. Oliveira, and S. Marangoni, "A quick procedure for the isolation of dimeric piratoxins-I and II, two myotoxins from *Bothrops pirajai* snake venom. N-terminal sequencing," *Biochemistry and Molecular Biology International*, vol. 37, no. 6, pp. 1047–1055, 1995.

[16] M. H. Toyama, P. D. Costa, J. C. Novello et al., "Purification and amino acid sequence of MP-III 4R D49 phospholipase A2 from *Bothrops pirajai* snake venom, a toxin with moderate PLA_2 and anticoagulant activities and high myotoxic activity," *Protein Journal*, vol. 18, no. 3, pp. 371–378, 1999.

[17] S. S. Teixeira, L. B. Silveira, F. M. N. da Silva et al., "Molecular characterization of an acidic phospholipase A_2 from *Bothrops pirajai* snake venom: synthetic C-terminal peptide identifies its antiplatelet region," *Archives of Toxicology*, vol. 85, no. 10, pp. 1219–1233, 2011.

[18] A. Havt, M. H. Toyama, N. R. F. Do Nascimento et al., "A new C-type animal lectin isolated from *Bothrops pirajai* is responsible for the snake venom major effects in the isolated kidney," *International Journal of Biochemistry and Cell Biology*, vol. 37, no. 1, pp. 130–141, 2005.

[19] L. F. M. Izidoro, M. C. Ribeiro, G. R. L. Souza et al., "Biochemical and functional characterization of an l-amino acid oxidase isolated from *Bothrops pirajai* snake venom," *Bioorganic and Medicinal Chemistry*, vol. 14, no. 20, pp. 7034–7043, 2006.

[20] D. L. Menaldo, C. P. Bernardes, N. A. Santos-Filho et al., "Biochemical characterization and comparative analysis of two distinct serine proteases from *Bothrops pirajai* snake venom," *Biochimie*, vol. 94, no. 613, pp. 2545–2558, 2012.

[21] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, pp. 680–685, 1970.

[22] R. D. G. Theakston and H. A. Reid, "Development of simple standard assay procedures for the characterization of snake venoms," *Bulletin of the World Health Organization*, vol. 61, no. 6, pp. 949–956, 1983.

[23] A. M. Chudzinski-Tavassi and J. C. A. Modesto, "Venenos de serpentes e suas ações nos mecanismos de coagulação e fibrinólise," in *Métodos em Toxinologia: Toxinas de Serpentes*, H. S. Selistre-de-Araújo and D. H. F. Souza, Eds., pp. 11–23, EDUFSCar, São Carlos, Brazil, 2007.

[24] L. Romero, S. Marcussi, D. P. Marchi-Salvador et al., "Enzymatic and structural characterization of a basic phospholipase A_2 from the sea anemone *Condylactis gigantea*," *Biochimie*, vol. 92, no. 8, pp. 1063–1071, 2010.

[25] S. L. Da Silva, C. A. Dias-Junior, P. A. Baldasso et al., "Vascular effects and electrolyte homeostasis of the natriuretic peptide isolated from *Crotalus oreganus abyssus* (North American Grand Canyon rattlesnake) venom," *Peptides*, vol. 36, no. 501, pp. 206–212, 2012.

[26] A. L. Fuly, O. L. T. Machado, E. W. Alves, and C. R. Carlini, "Mechanism of inhibitory action on platelet activation of a phospholipase A_2 isolated from *Lachesis muta* (Bushmaster) snake venom," *Thrombosis and Haemostasis*, vol. 78, no. 5, pp. 1372–1380, 1997.

[27] M. R. Cominetti, C. L. S. Pontes, and D. H. F. Souza, "Métodos cromatográficos e critério de pureza," in *Métodos em Toxinologia: Toxinas de Serpentes*, H. S. Selistre-de-Araújo and D. H. F. Souza, Eds., pp. 11–23, EDUFSCar, São Carlos, Brazil, 2007.

[28] C. Chothia and A. M. Lesk, "The relation between the divergence of sequence and structure in proteins," *The EMBO Journal*, vol. 5, no. 4, pp. 823–826, 1986.

[29] L. Holm and C. Sander, "Protein structure comparison by alignment of distance matrices," *Journal of Molecular Biology*, vol. 233, no. 1, pp. 123–138, 1993.

[30] L. Holm and C. Sander, "Dali: a network tool for protein structure comparison," *Trends in Biochemical Sciences*, vol. 20, no. 11, pp. 478–480, 1995.

[31] J. Söding, A. Biegert, and A. N. Lupas, "The HHpred interactive server for protein homology detection and structure prediction," *Nucleic Acids Research*, vol. 33, no. 2, pp. W244–W248, 2005.

[32] F. Zeng, B. Shen, Z. Zhu et al., "Crystal structure and activating effect on RyRs of AhV_TL-I, a glycosylated thrombin-like enzyme from Agkistrodon halys snake venom," *Archives of Toxicology*, vol. 87, no. 647, pp. 535–545, 2013.

[33] N. Blom, T. Sicheritz-Pontén, R. Gupta, S. Gammeltoft, and S. Brunak, "Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence," *Proteomics*, vol. 4, no. 6, pp. 1633–1649, 2004.

[34] H. J. C. Berendsen, D. van der Spoel, and R. van Drunen, "GROMACS: a message-passing parallel molecular dynamics implementation," *Computer Physics Communications*, vol. 91, no. 1–3, pp. 43–56, 1995.

[35] E. Lindahl, B. Hess, and D. van der Spoel, "GROMACS 3.0: a package for molecular simulation and trajectory analysis," *Journal of Molecular Modeling*, vol. 7, no. 8, pp. 306–317, 2001.

[36] W. F. Van Gunsteren, S. R. Billeter, A. A. Eising et al., "Biomolecular simulations: the GROMOS96 manual and user guide," *VdF Hochschulverlag ETHZ*, vol. 648, 1996.

[37] S. C. Lovell, I. W. Davis, W. B. Arendall III et al., "Structure validation by Calpha geometry: phi, psi and Cbeta deviation," *Proteins*, vol. 50, pp. 437–450, 2003.

[38] J. U. Bowie, R. Luthy, and D. Eisenberg, "A method to identify protein sequences that fold into a known three-dimensional structure," *Science*, vol. 253, pp. 164–170, 1991.

[39] R. Luthy, J. U. Bowie, and D. Eisenberg, "Assesment of protein models with three-dimensional profiles," *Nature*, vol. 356, pp. 83–85, 1992.

[40] M. J. Sippl, "Recognition of errors in three-dimensional structures of proteins," *Proteins: Structure, Function and Genetics*, vol. 17, no. 4, pp. 355–362, 1993.

[41] M. Wiederstein and M. J. Sippl, "ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins," *Nucleic Acids Research*, vol. 35, pp. W407–W410, 2007.

[42] H. Pirkle, "Thrombin-like enzymes from snake venoms: an updated inventory," *Thrombosis and Haemostasis*, vol. 79, no. 3, pp. 675–683, 1998.

[43] S. Swenson and F. S. Markland Jr., "Snake venom fibrin(ogen)olytic enzymes," *Toxicon*, vol. 45, no. 8, pp. 1021–1039, 2005.

[44] J. O. Costa, C. B. Petri, A. Hamaguchi et al., "Purification and functional characterization of two fibrinogenolytic enzymes from Bothrops alternatus venom," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 13, no. 3, pp. 640–654, 2007.

[45] Y.-S. Koh, K.-H. Chung, and D.-S. Kim, "Biochemical characterization of a thrombin-like enzyme and a fibrinolytic serine protease from snake (*Agkistrodon saxatilis*) venom," *Toxicon*, vol. 39, no. 4, pp. 555–560, 2000.

[46] A. Magalhães, H. P. B. Magalhães, M. Richardson et al., "Purification and properties of a coagulant thrombin-like enzyme from the venom of *Bothrops leucurus*," *Comparative Biochemistry and Physiology*, vol. 146, no. 4, pp. 565–575, 2007.

[47] L. A. Ponce-Soto, V. L. Bonfim, J. C. Novello, R. Navarro Oviedo, A. Yarlequé Chocas, and S. Marangoni, "Isolation and characterization of a serine protease, Ba III-4, from Peruvian *Bothrops atrox* venom," *Protein Journal*, vol. 26, no. 6, pp. 387–394, 2007.

[48] C. D. Sant' Ana, F. K. Ticli, L. L. Oliveira et al., "BjussuSP-I: a new thrombin-like enzyme isolated from *Bothrops jararacussu* snake venom," *Comparative Biochemistry and Physiology*, vol. 151, no. 3, pp. 443–454, 2008.

[49] C. D. Sant'Ana, C. P. Bernardes, L. F. M. Izidoro et al., "Molecular characterization of BjussuSP-I, a new thrombin-like enzyme with procoagulant and kallikrein-like activity isolated from Bothrops jararacussu snake venom," *Biochimie*, vol. 90, no. 3, pp. 500–507, 2008.

[50] A. Vilca-Quispe, L. A. Ponce-Soto, F. V. Winck, and S. Marangoni, "Isolation and characterization of a new serine protease with thrombin-like activity (TLBm) from the venom of the snake *Bothrops marajoensis*," *Toxicon*, vol. 55, no. 4, pp. 745–753, 2010.

[51] A. Magalhaes, G. J. De Oliveira, and C. R. Diniz, "Purification and partial characterization of a thrombin-like enzyme from the venom of the bushmaster snake, *Lachesis muta noctivaga*," *Toxicon*, vol. 19, no. 2, pp. 279–294, 1981.

[52] F. S. Markland and P. S. Damus, "Purification and properties of a thrombin-like enzyme from the venom of *Crotalus adamanteus* (Eastern diamondback rattlesnake)," *Journal of Biological Chemistry*, vol. 246, no. 21, pp. 6460–6473, 1971.

[53] H. S. Selistre and J. R. Giglio, "Isolation and characterization of a thrombin-like enzyme from the venom of the snake *Bothrops insularis* (jararaca ilhoa)," *Toxicon*, vol. 25, no. 11, pp. 1135–1144, 1987.

[54] K. Stocker and G. H. Barlow, "The coagulant enzyme from *Bothrops atrox* venom (batroxobin)," *Methods in Enzymology*, vol. 45, pp. 214–223, 1976.

[55] S. M. T. Serrano, R. Mentele, C. A. M. Sampaio, and E. Fink, "Purification, characterization, and amino acid sequence of a serine proteinase PA-BJ, with platelet-aggregating activity from the venom of *Bothrops jararaca*," *Biochemistry*, vol. 34, no. 21, pp. 7186–7193, 1995.

[56] D. F. Vieira, L. Watanabe, C. D. Sant'Ana et al., "Purification and characterization of jararassin-I, a thrombin-like enzyme from *Bothrops jararaca* snake venom," *Acta Biochimica et Biophysica Sinica*, vol. 36, no. 12, pp. 798–802, 2004.

[57] L. Muszbek, V. C. Yee, and Z. Hevessy, "Blood coagulation factor XIII: structure and function," *Thrombosis Research*, vol. 94, no. 5, pp. 271–305, 1999.

[58] R. Adany, "Intracellular factor XIII: cellular distribution of factor XIII subunit a in humans," *Seminars in Thrombosis and Hemostasis*, vol. 22, no. 5, pp. 399–408, 1996.

[59] S. I. Rapaport, "The initiation of the tissue factor dependent pathway of blood coagulation," *Advances in Experimental Medicine and Biology*, vol. 281, pp. 97–103, 1991.

[60] R. Bezerra and L. B. Carvalho, "Proteases no trato digestivo de Peixes," *Biotecnologia Ciência & Desenvolvimento*, vol. 622, pp. 46–49, 2001.

[61] S. Niewiarowski, E. P. Kirby, T. M. Brudzynski, and K. Stocker, "Thrombocytin, a serine protease from Bothrops atrox venom—2. Interaction with platelets and plasma-clotting factors," *Biochemistry*, vol. 18, no. 16, pp. 3570–3577, 1979.

[62] F. S. Markland, "Snake venoms and the hemostatic system," *Toxicon*, vol. 36, no. 12, pp. 1749–1800, 1998.

[63] C. Ouyang, C.-M. Teng, and T.-F. Huang, "Characterization of snake venom components acting on blood coagulation and platelet function," *Toxicon*, vol. 30, no. 9, pp. 945–966, 1992.

[64] J. Martins-Silva, J. Carvalho de Sousa, C. Saldanha, and J. Martins-Silva, in *Fibrinogen: From Physiopathology to Clinics*, C. Perdigão, Ed., pp. 13–26, 1996.

[65] N. Marsh and V. Williams, "Practical applications of snake venom toxins in haemostasis," *Toxicon*, vol. 45, no. 8, pp. 1171–1181, 2005.

[66] W. R. Bell, "Clinical trials with ancrod," in *Hemostasis and Animal Venoms*, H. Pirkle and F. S. Markland Jr., Eds., pp. 541–551, Marcel Dekker, New York, 1988.

[67] K. Stocker and J. Meier, "Thrombin-like snake-venom enzymes," *Thrombosis and Haemostasis*, vol. 54, pp. 313–313, 1985.

[68] C. Demers, J. S. Ginsberg, P. Brill-Edwards et al., "Rapid anticoagulation using ancrod for heparin-induced thrombocytopenia," *Blood*, vol. 78, no. 9, pp. 2194–2197, 1991.

[69] J. G. Kelton, J. W. Smith, D. Moffatt, A. Santos, and P. Horsewood, "The interaction of ancrod with human platelets," *Platelets*, vol. 10, no. 1, pp. 24–29, 1999.

[70] M. Bacila, "Gyroxin, a new neurotoxin of *Crotalus durissus terrificus* venom," *Acta Physiologica Latinoamericana*, vol. 11, p. 224, 1961.

[71] G. Alexander, J. Grothusen, H. Zepeda, and R. J. Schwartzman, "Gyroxin, a toxin from the venom of *Crotalus durissus terrificus*, is a thrombin-like enzyme," *Toxicon*, vol. 26, no. 10, pp. 953–960, 1988.

[72] W. H. Dascombe, G. Dumanian, C. Hong et al., "Application of thrombin based fibrin glue and non-thrombin based batroxobin glue on intact human blood vessels: evidence for transmural thrombin activity," *Thrombosis and Haemostasis*, vol. 78, no. 2, pp. 947–951, 1997.

[73] L. C. Barros, R. S. Ferreira Jr., S. R. C. S. Barraviera et al., "A new fibrin sealant from *Crotalus durissus terrificus* venom: applications in medicine," *Journal of Toxicology and Environmental Health B*, vol. 12, no. 8, pp. 553–571, 2009.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 654170, 15 pages
http://dx.doi.org/10.1155/2014/654170

Research Article

A Novel Phospholipase A_2 (D49) from the Venom of the *Crotalus oreganus abyssus* (North American Grand Canyon Rattlesnake)

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Received 31 August 2013; Revised 25 November 2013; Accepted 6 December 2013; Published 24 February 2014

Academic Editor: Edward G. Rowan

Currently, *Crotalus viridis* was divided into two species: *Crotalus viridis* and *Crotalus oreganus*. The current classification divides "the old" *Crotalus viridis* into two new and independent species: *Crotalus viridis* (subspecies: *viridis and nuntius*) and *Crotalus oreganus* (subspecies: *abyssus, lutosus, concolor, oreganus, helleri, cerberus, and caliginis*). The analysis of a product from cDNA (E6d), derived from the gland of a specie *Crotalus viridis viridis*, was found to produce an acid phospholipase A_2. In this study we isolated and characterized a PLA_2 (D49) from *Crotalus oreganus abyssus* venom. Our studies show that the PLA_2 produced from the cDNA of *Crotalus viridis viridis* (named E6d) is exactly the same PLA_2 primary sequence of amino acids isolated from the venom of *Crotalus oreganus abyssus*. Thus, the PLA_2 from E6d cDNA is actually the same PLA_2 presented in the venom of *Crotalus oreganus abyssus* and does not correspond to the venom from *Crotalus viridis viridis*. These facts highlight the importance of performing more studies on subspecies of *Crotalus oreganus* and *Crotalus viridis*, since the old classification may have led to mixed results or mistaken data.

1. Introduction

Crotalus viridis defines a large group of snakes, also named as Western Rattlesnakes, which inhabit the eastern region of the Rocky Mountains of the United States that stretch from southern Canada to northern Mexico (Figure 1) [1, 2]. Phylogenetic analyses on mitochondrial DNA sequences of snakes classified as *Crotalus viridis* show significant taxonomic variations between individuals from different areas of USA and indicate that this species has several subspecies [3]. To understand the variations in these *subspecies*, morphological analyses were carried out based on distance analysis of whole venom profiles and based on maximum parsimony (MP) analysis of cyt *b* and ND4 [1, 4, 5].

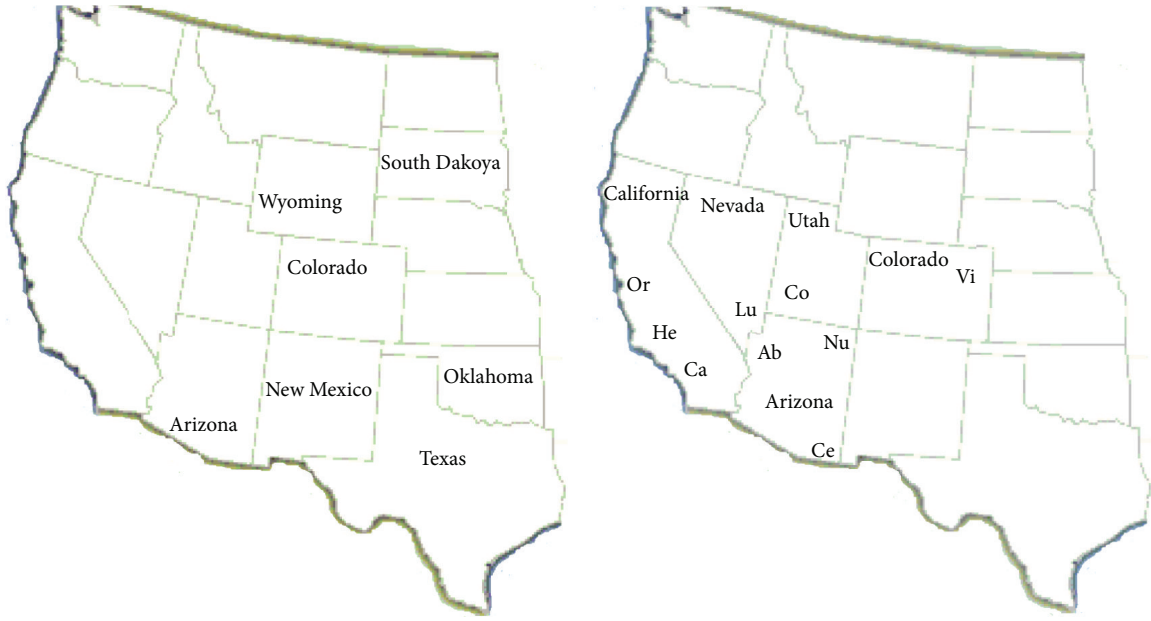


Figure 1: States inhabited by subspecies of *C. viridis* and *C. oreganus*. (a) States from which snakes used by Tsai et al. [37] were collected. (b) States and approximate locations from which snakes were collected Mackssey [2].

Initially, based only on morphology, Klauber classified these snakes into nine subspecies of *Crotalus viridis: C. v. viridis, C. v. nuntius, C. v. abyssus, C. v. lutosus, C. v. concolor, C. v. oreganus, C. v. helleri, C. v. Cerberus, and C. v. caliginis* [1]. This classification was in place until the early 2000s, when reports by Pook et al. and Asthon and de Queiroz, based on the analysis of the molecular characteristics of DNA of the nine subspecies of *C. viridis*, showed that the subspecies could be grouped into two distinct and new groups: *Crotalus viridis* and *Crotalus oreganus* [3–5]. The new classification, grouped the *C. viridis* into two new subspecies: *C. v. viridis* and *C. v. nuntius* and *Crotalus oreganus* into five new subspecies: *C. o. abyssus, C. o. lutosus, C. o. concolor, C. o. oreganus, C. o. helleri, C. o. Cerberus, and C. o. caliginis*. This is the current and official classification used for snakes previously classified as *C. viridis* (and its subspecies).

Secreted phospholipases A_2 (PLA_2s) are a family of relatively stable enzymes, with low molecular mass (13–15 kDa) and 6 (or 7) conserved disulfide bonds. PLA_2s employ calcium ions and the amino acid residues, Asp47, and His48, to catalyze the hydrolysis of the *Sn-2* of glycerophospholipid esters bonds of membranes. This hydrolysis reaction releases glycerol and proinflammatory eicosanoids [6–10].

PLA_2 are present in snake venom and the biological fluids, cells, and tissues of these species and are widely studied due to their pharmacological diversity. These enzymes can act as regulators of the membrane phospholipid membrane homeostasis [8–10] and also present physiopathological processes that can be neurotoxic (pre- or postsynaptic), cardiotoxic [11–14], hypotensive [15–17], anticoagulant and platelet aggregating [18, 19], genotoxic [20, 21], myotoxic [22, 23], antitumoral, and bacterial [24, 25]. Due to the toxic pharmacological effects produced by PLA_2, several studies have researched or developed natural or synthetic compounds to aid in the treatment of the snake bites to inhibit the toxic effects of PLA_2 [26–33]. In addition, the amino acid sequences of hundreds of PLA_2s from snake venom have been determined [34–36].

Tsai et al. studied PLA_2 from glands obtained from different samples of *C. viridis viridis,* arising from several regions of the United States (Figure 1(a)) [37]. They purified and sequenced five acidic PLA_2s sharing 78% or greater sequence identity. Interestingly, Tsai et al. observed that the product of the cDNA sequence named cvvE6d modified a PLA_2 with a molecular mass of 13782 ± 1 Da. This specific molecule of PLA_2 was found only in a unique snake from Southeastern Arizona. The authors correctly inferred and suggested that these individuals from Southeastern Arizona could actually represent a distinct population of *Crotalus viridis viridis* [37].

Recently, while studying the differences in total venoms from *C. viridis* and *C. oreganus* subspecies, Mackessy verified that all venoms display great variation, both in protein composition as well as in the activities of several enzymes, including the PLA_2 enzyme family [2]. The venom used by Mackssey was obtained from *C. viridis* and *C. oreganus* subspecies from the locations shown in Figure 1(b) [2].

According to Mackssey, as the Western Rattlesnake occurs across a broad geographical area, it represents an ideal species group to investigate variations in venom composition, and to understand how these differences evolve and how composition affects the biological role(s) of venom [2]. In this study, to further the understanding of the biological diversity of the subspecies of *C. oreganus,* we biochemically isolated and characterized a PLA_2 (D49) from *C. oreganus abyssus* venom. Moreover, we sequenced the primary structure of

PLA_2, performed pharmacological and biochemical characterization assays, and used molecular modeling to analyze the structure obtained.

2. Material and Methods

2.1. Material. All reagents were purchased from Aldrich or Sigma Co (USA). *Crotalus oreganus abyssus* (Coa), *Crotalus viridis viridis* (Cvv), and *Crotalus viridis nuntius* (Cvn) venoms were obtained from The National Natural Toxins Research Center (NNTRC) of Texas A&M University-Kingsville (Kingsville, Texas, USA). The substrate, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP), was supplied by Molecular Probes (USA). The substrate 4-nitro-3-octanoyloxy benzoic acid (NOBA) was synthesized following the methodology described by Cho et al. [38].

2.2. Isolation of the Phospholipase A_2 from Crotalus oreganus abyssus ($CoaPLA_2$). Venom from *C. o. abyssus* (200 mg) was fractioned by chromatography on a G75-Sephadex column, previously balanced with 0.05 M ammonium bicarbonate buffer (AMBIC—pH 8.0). Elution was performed using 1.0 M ammonium bicarbonate (AMBIC—pH 8.0) at a flow rate of 0.5 mL/min. Fraction II, presenting phospholipase activity, was collected and ultrafiltered using the MidJet apparatus (Ge Healthcare, USA) equipped with the UFP-10-C-MM01A cartridge (superficial area of 26 cm^2, cut off: 10,000 Da—Ge Healthcare, USA). The filtrate was lyophilized and stored frozen at −20°C.

Lyophilized fraction II (25 mg), containing PLA_2 activity, was dissolved in 250 μL of 5% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA), homogenized and centrifuged at 480 ×g for 5 min, and then subjected to a reverse phase HPLC (model 2010, Shimadzu, Japan) using an analytical C18 column (Supelco, 250 mm × 4.6 mm). The analytical C18 column was equilibrated in solvent A (5% acetonitrile, 0.1% TFA) and elution proceeded with a concentration gradient from 0 to 100% of solvent B (60% acetonitrile, 0.1% TFA), at a flow rate of 1 mL/min, for 60 min.

To help remove any other impurities that might be present, the fraction with PLA_2 activity was again subjected to ultrafiltration using the MidJet apparatus (Ge Healthcare, USA), equipped with the UFP-10-C-MM01A cartridge (superficial area of 26 cm^2, cut off: 10,000 Da—Ge Healthcare, USA). A PLA_2 named $CoaPLA_2$ was isolated, and the filtrate was lyophilized and rechromatographed to evaluate its purity, under the same conditions as described above (Figure 2). The fractions were monitored by spectrophotometry at 280 nm. The purity level of the $CoaPLA_2$ was also evaluated using native polyacrylamide gel (PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18, 21–23].

2.3. Biochemical Characterization of $CoaPLA_2$

2.3.1. SDS-PAGE and PAGE Electrophoresis. Electrophoresis analysis was performed to evaluate the purity and estimate molecular mass of $CoaPLA_2$, under reducing and nonreducing conditions. The standard molecular weight proteins were purchased from BioRad Co. (Phosphorylase b—97,400; Serum albumin—66,200; Ovalbumin—45,000; Carbonic anhydrase—30,000; Trypsin inhibitor—20,100; Lysozyme—14,400 MW). $CoaPLA_2$ pI was determined by isoelectric focusing, according to a previously described method [24–26].

2.3.2. Phospholipase A_2 Activity. Enzymatic activity was measured by two methods using two different substrates; a nonmicellar (4-nitro-3-octanoyloxy benzoic acid—NOBA) and a micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol—HPGP).

(1) Phospholipase A_2 Activity Measured Using a Nonmicellar Substrate (4-nitro-3-octanoyloxy benzoic acid—NOBA). The phospholipase A_2 activity of $CoaPLA_2$ (both in the isolated protein and in total venoms) was measured using the assay described by Holzer and Mackessy [43], but modified for 96-well plates [17, 31–34]. The standard assay mixture contained 200 μL of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 μL of substrate (3 mM 4-nitro-3-octanoyloxy benzoic acid), 20 μL of water, and 20 μL of PLA_2 (10 mg/mL) in a final volume of 260 μL. After adding PLA_2 (or total venom) (20 μg), the mixture was incubated for up to 40 min at 37°C, with the reading absorbance at intervals of 10 min until 60 min. Enzyme activity, expressed as the initial velocity of the reaction (V_0), was calculated based on the of absorbance at 20 min. After this time, the velocity did not change (maximum velocity was achieved). Enzyme activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate.

(2) Phospholipase A_2 Activity Measured Using a Micellar Substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol—HPGP). The measurements of enzymatic activity using the substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP) were carried out using a microtiter plate assay [10, 20, 44]. One hundred μL of solution A in assay buffer (27 μM bovine serum albumin, 50 mM KCl, 1 mM $CaCl_2$, 50 mM Tris-HCl pH 8.0) were added to a 96-well microtiter plate. Solution B presented the same composition as Solution A but with PLA_2 (0.5 μg/mL) or total venom (1.0 μg/mL) and was delivered in 100 μL portions to four wells, except for the first one. As a control, instead of Solution B, an additional 100 μL of Solution A was added to the first of the four wells in the assay. Solution B was prepared immediately prior to each set of assays to avoid loss of enzymatic activity. After the addition of Solution B, the assay was rapidly initiated by the addition of 100 μL of Solution C (420 mM 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol vesicles in assay buffer) with a repeating pipette to all four wells. The fluorescence (excitation = 342 nm, emission = 395 nm) was read with a microtiter plate spectrophotometer (Fluorocount, Packard Instruments). Enzyme activity, expressed as the initial velocity of the reaction (V_0) was calculated based on the absorbance at 20 min. After this time, the velocity did not

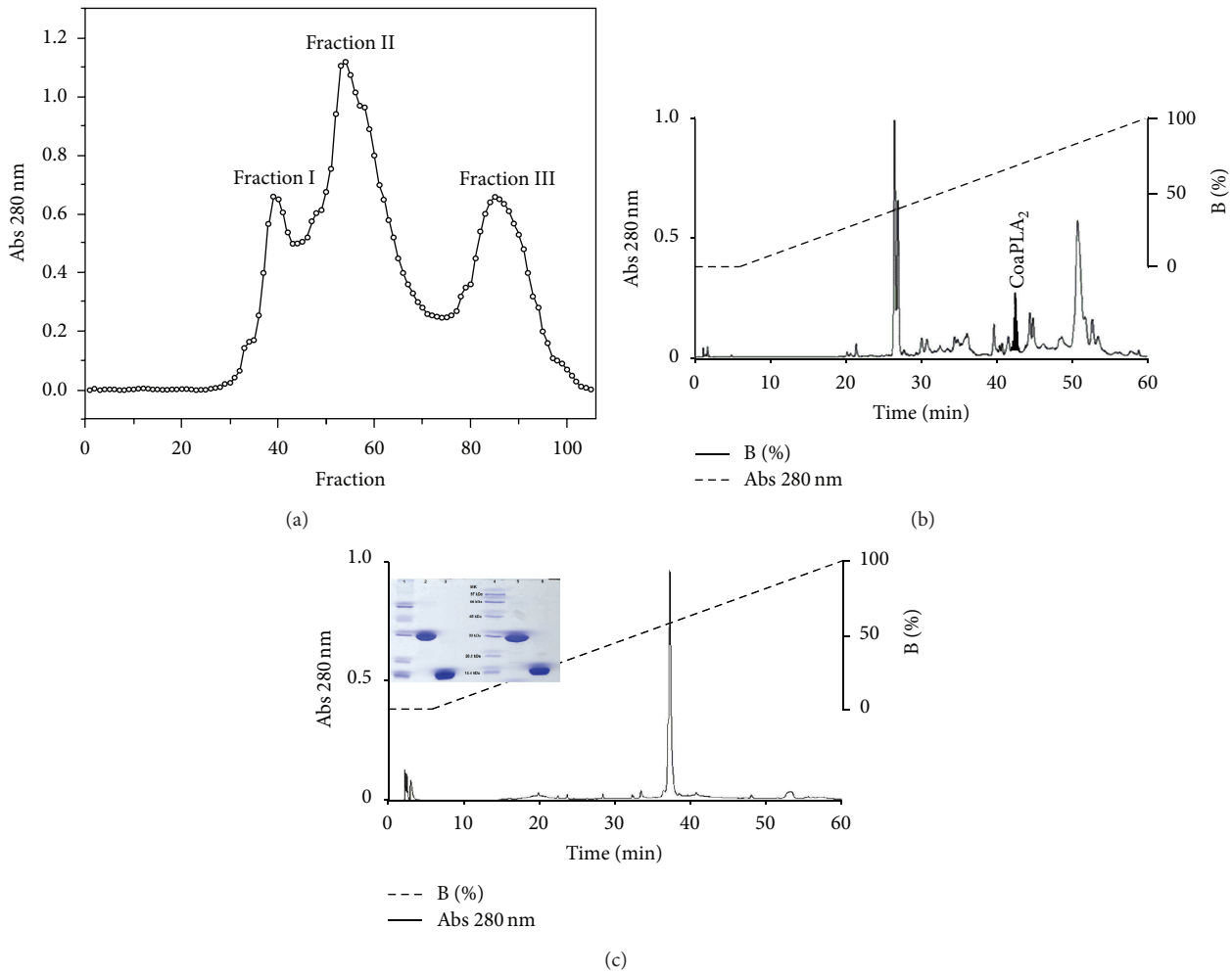


Figure 2: Isolation of $CoaPLA_2$ from *Crotalus oreganus abyssus* venom. (a) Profile obtained by gel chromatography on a G75-Sephadex column. Fraction II, presenting phospholipase activity. (b) Lyophilized fraction II was homogenized and centrifuged and subjected to reverse phase HPLC using an analytical C18 column. (c) Assessment of purity of RP-HPLC under the same conditions used in (b). Electrophoretic analysis shows the homogeneity of the $CoaPLA_2$ isolated from the venom of *Crotalus Oreganus abyssus*: Lines 1 and 4: standard molecular weight; Lines 2 and 5: PAGE under nonreduced conditions; Lines 3 and 6: PAGE under reduced conditions (SDS-PAGE).

change (the maximum velocity was achieved). Enzyme activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate.

2.3.3. Optimal pH and Temperature Determination of the Enzymatic Activity. Optimal pH and optimal temperature of the PLA_2 activity (using methodology described in Section 2.3.2(1)) of the $CoaPLA_2$ were determined by incubating the enzyme in four buffers of different pH values (4–10) and at different temperatures (25, 30, 35, 40, and 45°C), respectively, as described above (Section 2.3.2). The effect of substrate concentration (10, 5, 2.5, 1.25, 0.625, and 0.312 μM) on enzyme activity was determined by measuring the increase of absorbance after 20 min and absorbance values at 425 nm were measured with a VersaMax 190 multiwell plate reader (Molecular Devices, S., CA). Enzyme activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate.

2.3.4. Determination of Influence of Ca^{2+} (and Other Ions) on PLA_2 Activity. Three experiments were carried out to determine the influence of calcium ions on $CoaPLA_2$ activity (using methodology described in Section 2.3.2(1)). The activity was described above (Section 2.3.2). Initially, Ca^{2+} concentrations of 0, 1, and 10 mM were used. After this procedure, the other three experiments were carried out: (1) without Ca^{2+}, but in the presence of 10 mM of Mg^{2+}, Cd^{2+}, and Mn^{2+}; (2) 1 mM of Ca^{2+} in the presence of 10 mM of Mg^{2+}, Cd^{2+}, and Mn^{2+}, and (3) 10 mM of Ca^{2+} in the presence of 10 mM of Mg^{2+}, Cd^{2+}, and Mn^{2+}. The influences of the ions on the enzyme activity were measured by determining absorbances at 425 nm with a VersaMax 190 multiwell plate reader (Molecular Devices, S., CA). Enzyme activity was expressed as

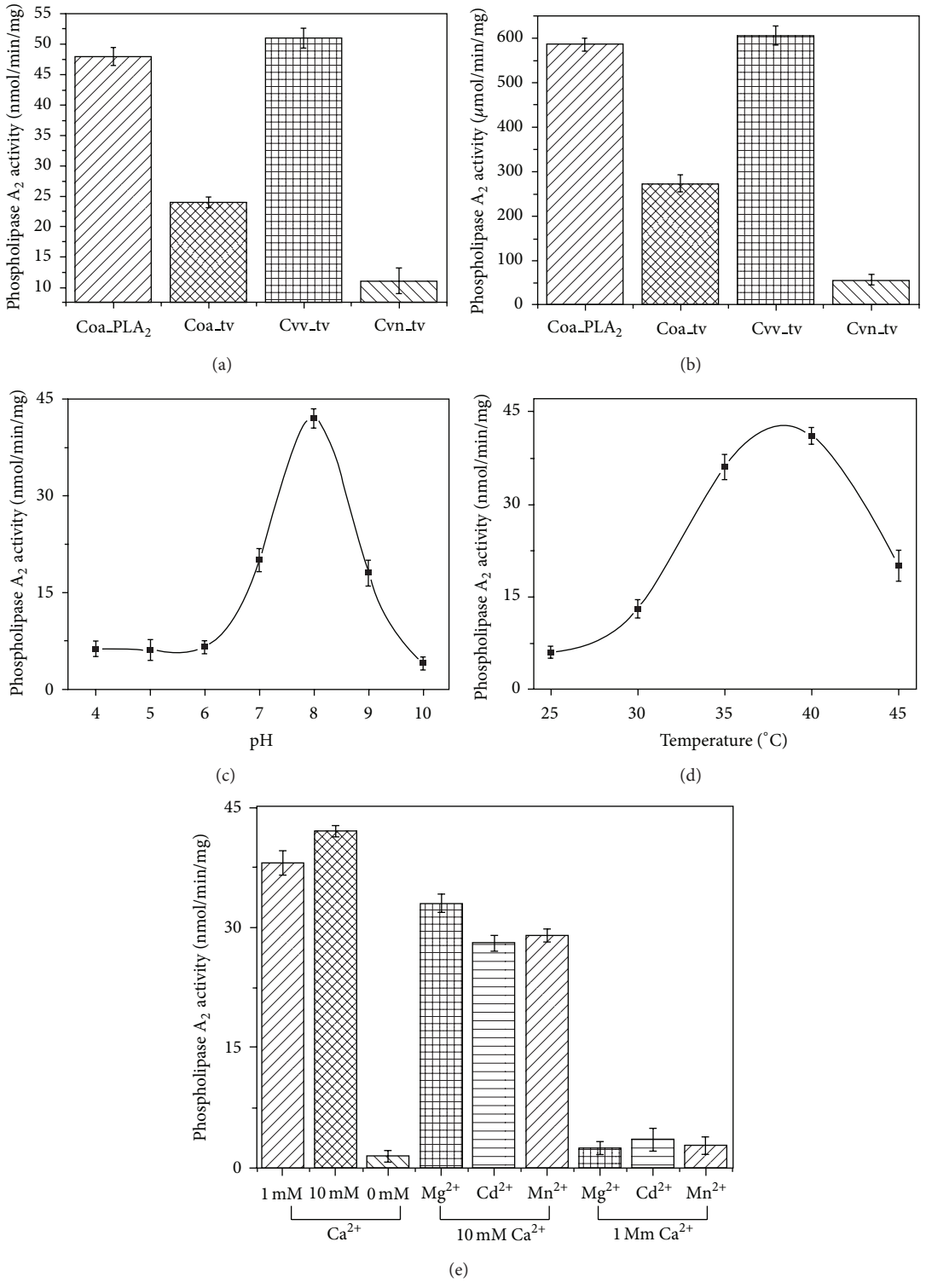


FIGURE 3: Phospholipase A_2 activities of $CoaPLA_2$ from *Crotalus Oreganus abyssus*. (a) Phospholipase A_2 activities of $CoaPLA_2$ and total venom of *Crotalus Oreganus abyssus*, *Crotalus viridis viridis,* and *Crotalus viridis nuntius* using a nonmicellar substrate (4-nitro-3-octanoyloxy benzoic acid); (b) Phospholipase A_2 activities of $CoaPLA_2$ and total venom of *Crotalus Oreganus abyssus*, *Crotalus viridis viridis,* and *Crotalus viridis nuntius* using a micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol); (c) influence of pH variations on the enzymatic activity of $CoaPLA_2$; (d) influence of temperature variations on the enzymatic activity of $CoaPLA_2$; (e) analysis of the influence of calcium ions and other divalent cations on the phospholipase A_2 activity of $CoaPLA_2$. Results are expressed as mean ± SD of three independent experiments performed in triplicate ($n = 3$).

mean ± SD of three independent experiments and each experiment was carried out in triplicate.

2.4. Biological Activity

2.4.1. Animals. Groups of 6 Swiss male mice (6–8 weeks old) were matched for body weight (18–22 g). The animals were housed for at least one week before the experiment in laminar-flow cages maintained at a temperature of 22 ± 2°C and a relative humidity of 50–60%, under a 12 : 12 h light-dark cycle. The animal experiments were carried out with the approval of the Institutional Ethics Committee, in accordance with protocols following the recommendations of the Canadian Council on Animal Care. The mice used in this study were kept under specific pathogen-free conditions.

2.4.2. 50% Lethal Dose. To evaluate the 50% lethal dose (dose that causes death in 50% of animals) of $CoaPLA_2$ and the total venoms from *C. o. abyssus*, *C. v. viridis,* and *C. v. nuntius*, groups of six Swiss male mice (18−22 g) received an intravenous injection of 100 μg of enzyme or total venom, dissolved in 100 μL of PBS. As a control, six mice were similarly injected with 100 μL of PBS alone. Animals were observed for up to 24 h after injection to record deaths. Lethal dose (LD) was expressed as mean ± SD of three independent experiments, performed in triplicate ($n = 6$) (Figure 3) [17, 18, 28, 29, 31, 33, 34].

2.4.3. Edema-Inducing Activity. Groups of six Swiss male mice (18–22 g) were injected in the subplantar region with various amounts of total venom or $CoaPLA_2$ (in a volume of 50 μL) prepared in PBS, pH 7.2. Subsequently, paw volume was measured at different time intervals (30, 60, 120, and 180 min), subtracting the initial paw volume (time 0 h). Paw edema was measured with a low-pressure pachymeter (Mitutoyo, Japan). Edema-inducing activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate ($n = 6$) (Figure 4) [17, 18, 28, 29, 31, 33, 34].

2.4.4. Myotoxic Activity. Groups of six Swiss male mice (18–22 g) were injected in the right gastrocnemius muscle with total venom or PLA_2 (50 mg/50 mL of PBS) or PBS alone (50 mL). After 3 h, blood was collected from the tail in heparinized capillary tubes and centrifuged for plasma separation. Activity of creatine kinase (CK) was then determined using 4 mL of plasma, which was incubated for 3 min at 37°C with 1.0 mL of the reagent according to the kinetic CK-UV protocol from Bioclin, Brazil. The activity was expressed in U/L, where one unit corresponds to the production of 1 mmol of NADH per minute (Figure 6). Myotoxic activity was expressed as mean ± SD of three independent experiments and each experiment was carried out in triplicate ($n = 6$) (Figure 5) [17, 18, 28, 29, 31, 33, 34].

2.5. Structural Analysis

2.5.1. MALDI-TOF Analysis of $CoaPLA_2$. The molecular mass of $CoaPLA_2$ was analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry using a MALDI-TOF/TOF—Proteomics Analyzer 4800 (Applied Biosystems). Sample treatment: $CoaPLA_2$ was analyzed at a concentration of 0.8 μL of matrix and mixed with 0.5 μL of sample on the MALDI plate. Samples were allowed to dry before analysis. The matrix consisted

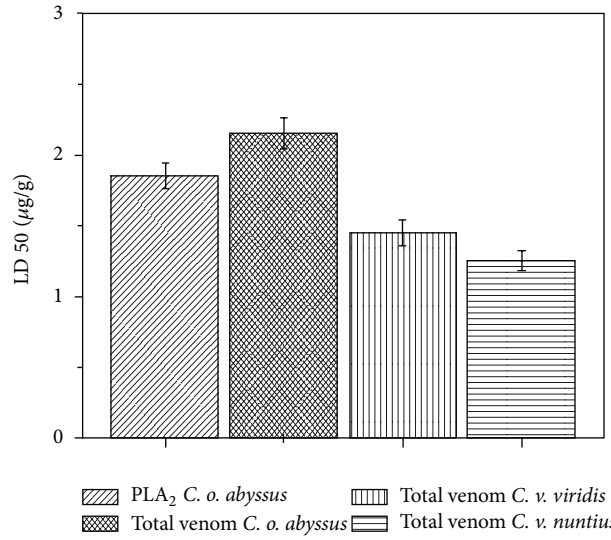


Figure 4: Lethal dose (dose that causes death in 50% of animals) of $CoaPLA_2$ and total venoms of *Crotalus oreganus abyssus*, *Crotalus viridis viridis,* and *Crotalus viridis nuntius*. Lethal dose (LD) is expressed as mean ± SD of three independent experiments performed in triplicate ($n = 6$).

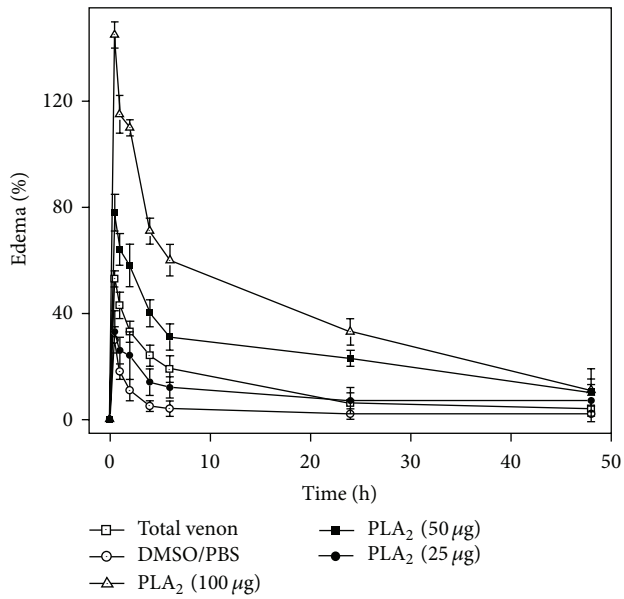


Figure 5: Mice paw edema induced by different doses of $CoaPLA_2$. Positive control was the total venom of *Crotalus Oreganus Abyssus* and negative control was DMSO/PBS solution. Edema was expressed as the percentage increase in the paw volume of the treated group, compared to that of the control group at each time interval. Edema-inducing percentage is expressed as the mean ± SD of three independent experiments performed in triplicate ($n = 6$).

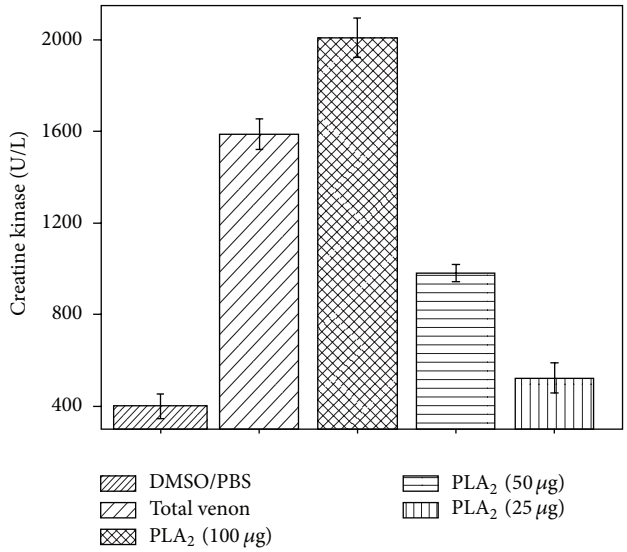


Figure 6: Myotoxic activity induced by different doses of CoaPLA$_2$ from *Crotalus Oreganus Abyssus*. Negative control was PBS solution containing 1% of DMSO. Myotoxic activity was expressed as mean ± SD of three independent experiments performed in triplicate (n = 6).

of 10 mg/mL sinapinic acid in 50% acetonitrile/miliQ water (v/v) and 0.1% trifluoroacetic acid (TFA). To calibrate the apparatus, a BSA standard solution was prepared following the same procedure, and 4 pmols were analyzed under the same conditions.

2.5.2. Sequencing Procedure

(1) In Solution Digestion. Proteins were reduced by treatment with a solution of 20 mM DTT (Dithiothreitol) in 50 mM NH_4HCO_3 for 1 h at 30°C and alkylated with a solution of 150 mM iodine acetamide in 50 mM NH_4HCO_3 for 1 h at 30°C. The sample was then digested overnight at 37°C with trypsin (sequencing grade modified, Promega). Tryptic peptides were then cleaned-up with a Proxeon Stage tip. Peptides were eluted from the tip with 70% acetonitrile/0.1% trifluoroacetic acid. The eluted peptides were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC-MS/MS analysis [45, 46].

(2) LC-MS/MS Analysis. Mass spectrometry was performed in a NanoAcquity (Waters) HPLC coupled to an OrbitrapVelos mass spectrometer (Thermo Scientific). An aliquot of the tryptic digest was injected and separated in a C18 reverse phase column (75 μm Oi, 10 cm, nano-Acquity, 1.7 μm BEH column, Waters). Bound peptides were eluted from the column with the following gradient: 1 to 40% B in 20 minutes, followed by a gradient of 40 to 60% B in 5 minutes; flow was 250 nL/min (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile). Eluted peptides were ionized in an emitter needle (PicoTipTM, New Objective). The spray voltage applied was 1900 V. Peptide masses (m/z: 300–1700) were measured in full scan in the Orbitrap at a resolution of 60,000 at m/z 400. The 5 most abundant peptides (minimum intensity of 1500 counts) were selected from each MS scan and fragmented in the HCD collision cell using a normalized collision energy (NCE) of 40% with nitrogen as the collision gas. Fragments were detected in the Orbitrap with a resolution of 7500 FWHM at 400 m/z. Raw data were collected with Thermo Xcalibur (v.2.1.0.1140). We obtained eight fragments, as shown in Table 1 [45, 46].

(3) Database Search. Raw data were analyzed using Proteome Discoverer (v.1.3.0.339) software. A search was run with the search engine MASCOT against NCBInr Serpentes database. The Percolator node was used in the Proteome Discoverer Mascot search in order to discriminate correct from incorrect peptide spectrum matches using the q-value (FDR) to improve the number of confidently identified peptides at a given false discovery rate. The results have been filtered so only high confidence peptides (FDR ≤ 0.01) have been considered for identification results [46, 47].

2.6. Tridimensional Structure Modeling

2.6.1. Threading Modeling. The initial 3D model of PLA$_2$ from *C. oreganus abyssus* venom was generated employing the threading modeling technique [39, 40, 48–57] implemented in the HHPred webserver (http://toolkit.tuebingen.mpg.de/hhpred). Initially, we generated 31 primary structure alignments using X-Ray structures available at the Protein Data Bank (PDB, http://www.rcsb.org/pdb/home/home.do) employing the global alignment option and scoring the predicted secondary structure alignment. From the 5 best ranked alignments, we evaluated the crystallographic quality (resolution and R-free value, Ramachandran distribution, and b-factor) of the target proteins. The phospholipase A$_2$ from *Crotalus durissus terrificus* (PDB ID: 3R0L chain D) [58] was chosen as a template to thread the modeling, presenting 51.1% of identity and 100% of hit probability, with an E-value of 2.1^{-41} and score of 255.95.

2.6.2. Molecular Dynamics. A molecular dynamics (MD) simulation was carried out aiming to refine the constructed PLA$_2$ model. The MD simulation was performed employing GROMACS 4.5.4 package [48, 49], running with a 8G RAM Intel Xeon processor. Explicit water molecules were used employing the Simple Point Charge (SPC) model [50]. Protonation states of charged groups were set according to pH 7.0 and counter ions were added to neutralize the system. Gromos force field [51] was chosen to perform the MD simulation. The MD simulation was performed at constant temperature and pressure in a periodic truncated cubic box with a volume that was equal to 259.14 nm^3 and at a minimum distance of 5 Å between any atom of the protein and the box wall. Sodium ions were added as counter ions to neutralize the system.

Initially, an energy minimization using the steepest descent algorithm was performed. After, 20 ps of MD simulation with position restraints applied to the protein were performed

Table 1: Peptide fragments obtained by sequencing procedure. The $CoaPLA_2$ was reduced and digested overnight with trypsin. Eluted tryptic peptides were dried in a vacuum centrifuge and resuspended in 1% formic acid for LC-MS/MS analysis.

Number	Peptide fragment	Molecular mass (MH^+-Da)
1	SLVQFEMLIMKVAKR	1793.01230
2	SGLFSYSAYGCYCGWGGHGR	2241.91338
3	PQDATDHCCFVHDCCYGK	2269.83194
4	TASYTYSEENGEIVCGGDDPCKK	2580.07798
5	QVCECDR	966.37218
6	VAAICFR	836.44066
7	DNIPTYDNK	1079.49590
8	FPPENCQEEPEPC	1632.62198

at 300 K to relax the system. Finally, an unrestrained MD simulation was performed at 300 K during 10 ns to assess the stability of the structures. During the simulation, temperature and pressure (1.0 bar) were maintained by coupling to an external heat and an isotropic pressure bath.

2.6.3. Structural Analysis and Validation. After the MD simulation, several tools of structural analysis contained in the GROMACS package were employed to evaluate the final 3D model. All figures were generated employing PyMOL 0.99c software [52]. Other validation methods were also used, such as a pseudoenergy profile, which was analyzed with Verify 3D [53, 54] and ProSA-web [55, 56], as well as the Ramachandran plot [57], ERRAT program [39], and ANOLEA web server [40].

2.7. Statistical Analysis. Results are presented as mean ± SD obtained with the indicated number of animal samples or in vitro assays. The statistical significance of differences between groups was evaluated using the Student's unpaired t-test and ANOVA analysis of variance. Significance levels were considered at a confidence interval of $0.1 > P > 0.05$.

3. Results and Discussion

3.1. Isolation and Purification of the Phospholipase A_2 from Crotalus oreganus abyssus ($CoaPLA_2$). The process used to obtain the pure protein ($CoaPLA_2$) is shown in Figure 2. Gel filtration (Figure 2(a)) demonstrated the presence of fraction II containing PLA_2 activity, which was further purified. Figure 2(b) shows the HPLC profile obtained using a reverse phase C18 column and the detachment of the peak containing $CoaPLA_2$. This peak was also further purified by rechromatography and subjected to electrophoresis (SDS-PAGE and PAGE). As shown in Figure 2(c), the purification process was efficiently purified. Nondenaturation electrophoresis showed that $CoaPLA_2$ was a dimeric protein with a molecular mass of approximately 28 kDa (lines 3 and 6), but under denaturing conditions, it was a monomer with a molecular mass of approximately 14 kDa (lines 2 and 5). This information was subsequently confirmed by MALDI-TOF mass spectrometry.

3.2. Biochemical Characterization of $CoaPLA_2$. We biochemically analyzed and characterized $CoaPLA_2$. Figure 3 shows phospholipase A_2 activity under several conditions. We measured the phospholipase A_2 activity of the isolated enzyme and total venom (using two different methods related to substrate type), as well as the optimal temperature and pH, and the influence of ions on the activity of the enzyme. Figures 3(a) and 3(b) show the PLA_2 activity of the $CoaPLA_2$ and of the total venom from *Crotalus oreganus abyssus*, *Crotalus viridis viridis,* and *Crotalus viridis nuntius*. Figure 3(a) shows that the phospholipase A_2 activity (using the nonmicellar substrate, 4-nitro-3-octanoyloxy benzoic acid) of the $CoaPLA_2$ is approximately 48 nmol/min/mg, while the total venom from *Crotalus oreganus abyssus* has a PLA_2 activity (approximately 22.5 nmol/min/mg) that is very different to the PLA_2 activity of venom from *Crotalus viridis viridis* and *Crotalus viridis nuntius* (approximately 53 and 9 nmols/min/mg, resp.). Conversely, using the micellar substrate, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol, Figure 3(b) shows that the activity of the $CoaPLA_2$ was approximately 590 μmol/min/mg, while the total venom from *Crotalus oreganus abyssus* has a PLA_2 activity (approximately 276 μmol/min/mg) that was significantly different to the PLA_2 activity of venom from *Crotalus viridis viridis* and *Crotalus viridis nuntius* (approximately 606 and 51 μmols/min/mg, resp.). Both methods clearly demonstrate that the venom from *Crotalus oreganus abyssus* that we used to isolate $CoaPLA_2$ was not derived from *Crotalus viridis viridis* or *Crotalus viridis nuntius*.

Interestingly, the enzymatic activity, obtained by Tsai et al., 2003, when cloning E6d was around 680 μmol/min/mg and when using the micellar substrate L-dipalmitoyl-glycerophosphatidyl-choline, being relatively close to the value found in the present study. We used a different, but similar, micellar substrate (1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol) and obtained a value of enzymatic activity of approximately 590 μmol/min/mg.

The optimal temperature of $CoaPLA_2$ was determined to be 37.3°C (Figure 3(d)) and optimal pH was 7.9 (Figure 3(c)). These values are in accordance with other PLA_2 measurements described in the literature [12–34]. The influence ions on the enzyme activity was determined in the presence and absence of Ca^{2+} and other divalent cations (also in the presence and absence of Ca^{2+}). Figure 3(e) shows that the PLA_2 activity of $CoaPLA_2$ is calcium-dependent. In the presence of 10 mM calcium, the PLA_2 activity was 45.8

Table 2: Multiple alignment of fragment of $CoaPLA_2$.

		|- - - Frag 1 - - -| |- - - - Frag 2 - - - - - - -| | - - Frag 3
Fragments obtained		SLVQFEMLIMKVAKR SGLFSYSAYGCYCGWGGHGR PQDATDHCC
$CoaPLA_2$	(1)	---------- SLVQFEMLIMKVAKR SGLFSYSAYGCYCGWGGHGR PQDATDHCC
Cvv_E6D	(1)	MRTLWIVAVLLLGVEG SLVQFEMLIMKVAKR SGLFSYSAYGCYCGWGGHGR PQDATDHCC
Cvv_E6E	(1)	MRTLWILAVLLLGVEG NLVQFELLIMKVAKR SGLLSYSAYGCYCGWGGHGR PQDATDRCC
Cvv_E6H	(1)	MRTLWIVAVLLLGVEG SLVQFETLIMKIAGR SGLLWYSAYGCYCGWGGHGL PQDATDRCC
Cvv_E6G	(1)	MRTLWIVAVLLLGVEG SLVQFEMMIIKVAKR SGLFWYGAYGCYCGWGGQGR PQDATDRCC
Cvv_E6A	(1)	MRTLWIVAVLLLGVEG SLVQFETLIMKIAGR SGLLWYSAYGCYCGWGGHGL PQDATDRCC
Cadam	(1)	MRTLWIVAVLLLGVEG SLVQFETLIMKVAKR SGLLWYSAYGCYCGWGGHGR PQDATDRCC
Chorrid	(1)	MRTLWIVAVLLLGVEG SLVQFEMMIMEVAKR SGLLWYSAYGCYCGWGGHGR PQDATDRCC
Chhorr	(1)	MRTLWIVAVLLLGVEG SLVQFEMMIMEVAKR SGLLWYSAYGCYCGWGGHGR PQDATDRCC
Scterg	(1)	MRTLWILAVLLLGVEG NLIQFETLILKVAKK SGMFSYSAYGCYCGWGGHGR PQDATDRCC
Catrox	(1)	---------- SLVQFETLIMKIAGR SGLLWYSAYGCYCGWGGHGL PQDATDRCC
		--------| |- - - - - - - Frag 4 - - - - - -| |Frag 5| |Frag 6| | Frag 7
Fragments obtained		FVHDCCYGK TASYTYSEENGEIVCGGDDPCKK QVCECDR VAAICFR DNIPTYD
$CoaPLA_2$	(45)	FVHDCCYGK ------- TASYTYSEENGEIVCGGDDPCKK QVCECDR VAAICFR DNIPTYD
Cvv_E6D	(61)	FVHDCCYGK VTDCNPK TASYTYSEENGEIVCGGDDPCKK QVCECDR VAAICFR DNIPTYD
Cvv_E6E	(61)	FVHDCCYGK VTDCNPK TASYTYSEENGEIVCGGDDPCKK QVCECDR VAAICFR DNIPSYD
Cvv_E6H	(61)	FVHDCCYGK ATDCNPK TVSYTYSVKNGEIICEDDDPCKR QVCECDR VAAVCFR DNIPSYN
Cvv_E6G	(61)	FVHDCCYGK ATDCNPK TVSYTYSVKNGEIICEDDDPCKK QVCECDR VAAVCFR DNIPSYN
Cvv_E6A	(61)	FVHDCCYGK ATDCNPK TVSYTYSEENGEIVCGGDNPCGT QICECDK AAAICFR DNIPSYS
Cadam	(61)	FVHDCCYGK ATNCNPK TVSYTYSEENGEIVCGGDDPCGT QICECDK AAAICFR DNIPSYD
Chorrid	(61)	FVHDCCYGK ATDCNPK RVSYTYSEENGEIVCGGDDPCGT QICECDK AAAICFR DNIPSYD
Chhorr	(61)	FVHDCCYGK ATDCNPK RVSYTYSEENGEIVCGGDDPCGT QICECDK AAAICFR DNIPSYD
Scterg	(61)	FVHDCCYGK VTDCDPK LDTYTYSEENGEIICGGDDPCKK QICECDK AAAICFR DNKNTYN
Catrox	(45)	FVHDCCYGK ATDCNPK TVSYTYSEENGEIICGGDDPCGT QICECDK AAAICFR DNIPSYD
		- - - - | |- - - Frag 8 - -|
Fragments obtained		NKYWR FPPENCQEEPEPC
$CoaPLA_2$	(98)	NKYWR FPPENCQEEPEPC
Cvv_E6D	(121)	NKYWR FPPENCQEEPEPC
Cvv_E6E	(121)	NKYIQ FPAKNCQEKPEPC
Cvv_E6H	(121)	NNYKR FPAENCREEPEPC
Cvv_E6G	(121)	NNYKR FPAENCRGDPEPC
Cvv_E6A	(121)	NKYWL FLPKNCRGDPEPC
Cadam	(121)	NKYWL FPPKNCREEPEPC
Chorrid	(121)	NKYWL FPPKNCQEEPEPC
Chhorr	(121)	NKYWL FPPKNCREEPEPC
Scterg	(121)	NKYWR LPTENCQEEPEPC
Catrox	(105)	NKYWL FPPKDCREEPEPC

$CoaPLA_2$: Crotalus oreganuis abyssus; Cvv_E6D, Cvv_E6E, Cvv_E6H, Cvv_E6G, and Cvv_E6A (cDNA from Crotalus viridis viridis [37]), Cadam: Crotalus adamanteus [39]; Chorrid and Chhorrid: Croatus horridus and Crotalus horridus horridus [40]; Scterg: Sistrurus catenatus tergeminus [41]; Catrox: Crotalus atrox [42].

nmols/min/mg. When the calcium concentration was 1 mM calcium, the phospholipase A_2 activity was slightly reduced to 38.1 nmols/min/mg. A complete absence of calcium ions drastically reduced the enzyme activity to values of approximately 3 nmols/min/mg.

When 10 mM of other divalent cations (Mg^{+2}, Cd^{+2} and Mn^{+2}) was employed, the activity of the PLA_2 was completely suppressed. However, phospholipase A_2 activity was recovered when Ca^{+2} was mixed with these divalent cations (Mg^{+2}, Cd^{+2} and Mn^{+2}), both at concentrations of 1 mM and 10 mM (Figure 3(e)).

3.3. Biological Characterization of $CoaPLA_2$. The biological characterization of $CoaPLA_2$, isolated from *Crotalus oreganus abyssus,* was carried out using measurements of lethal activity ($LA_{50\%}$—dose that causes death in 50% of animal subjects), edema-inducing and myotoxic activities. We tested the lethal activity ($LA_{50\%}$) of $CoaPLA_2$ and of the total venom of

Crotalus oreganus abyssus, Crotalus viridis viridis, and Crotalus viridis nuntius. Figure 4 shows that $LA_{50\%}$ of the venom from *Crotalus oreganus abyssus* is approximately 2.2 ± 0.4 μg of venom/g of mouse and this value is bigger in relation to *C. v. viridis* and *C. v. nuntius*. $CoaPLA_2$ has a $LA_{50\%}$ at a dose of about 1.8 μg ± 0.2 of venom/g mouse weight and was higher than that of the total venom of *C. v. viridis* and *C. v. nuntius*. The total *C. o. abyssus* venom is more lethal than $CoaPLA_2$ alone, as venom contains other enzymes that also exhibit lethality, such as serine proteases and metalloproteases [12–34].

The edema-inducing activity of $CoaPLA_2$ was measured using different dosages of the enzyme (25, 50, and 100 μg). From Figure 5, we can see that the edema-inducing activity of $CoaPLA_2$ is dose-dependent. The increase in the amount of enzyme increases the percentage of edema formed, principally in the first 24 h. After this time, this edema-inducing activity is significantly reduced and the edema is suppressed.

Similarly to the edema-inducing activity results obtained, the myotoxic activity induced by $CoaPLA_2$ was also dose-dependent. When we increased the quantity of $CoaPLA_2$ (25, 50 and 100 μg) its myotoxic effects were augmented.

The phospholipases A_2 are a group of enzymes present in most venoms or oral secretions of snakes. In addition to the digestive function of the prey, these enzymes interfere with the physiological processes and cause many pharmacological and pathophysiological effects, such as neurotoxic, cardiotoxic, anticoagulant, antiplatelet, hemolytic, hemorrhagic, and inflammatory activities [59–61].

Both the crude venom of *Crotalus oreganus abyssus* and the isolated $CoaPLA_2$, were able to induce experimental toxicity, such as myonecrosis, edema, and mortality. Due to the neurotoxic potential of this kind of snake, it was observed that the $LD_{50\%}$ of the crude venom of *C. oreganus abyssus* and its $CoaPLA_2$ showed low values of lethal doses, when compared to *Bothrops* genera venoms and its isolated PLA_2s [36].

The $CoaPLA_2$ also induced myotoxic activity, similarly to other PLA_2s isolated from snake venoms. The myotoxicity was evaluated by the activity levels of creatine kinase (CK) in the plasma of animals. Creatine kinase is an enzyme used in muscular energy metabolism and, in cases of cell damage, is released and can be detected in plasma as a marker [36, 60]. The catalytic activity of the PLA_2 on the membrane suggests an important role these enzymes in the toxicity of snake venoms ($svPLA_2$s). The breakdown of phospholipids causes severe changes in the structural and functional integrity of the plasmatic membrane with a consequent influx of calcium ions [62], release of calcium-dependent proteases [63], activation of endogenous PLA_2s [64], and mitochondrial collapse [65]. The sum of all these molecular changes could lead to cell death.

The $CoaPLA_2$ was able to induce edema in mice paws. Local inflammation is a feature of poisoning by snakes of the subfamilies of Viperidae and Crotalidae [60, 61]. The catalytically active mechanism by which PLA_2 induces edema is probably due to the release of precursors of eicosanoids due to the hydrolysis of phospholipids. Release of biogenic amines from mast cells is also proposed as a possible mechanism of induction of edema by PLA_2 [66, 67].

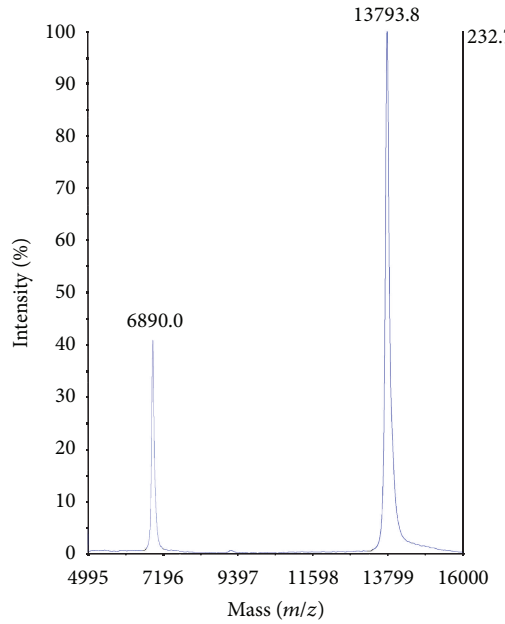


Figure 7: MALDI-TOF analysis of $CoaPLA_2$. The molecular mass of $CoaPLA_2$ (Da) was analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF mass spectrometry—Proteomics Analyzer 4800). The peak with a molecular mass of 13793.8 Da corresponds to s monoisotopic ion (m/z, where $z = +1$) and the peak with a molecular mass of 6890.0 Da corresponds to a diisotopic ion (m/z, where $z = +2$).

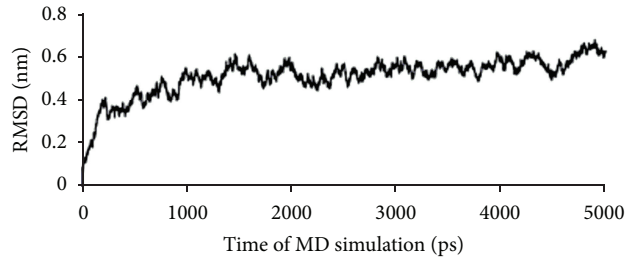


Figure 8: RMSD values of the PLA_2 backbone atoms along with the MD simulation.

3.4. Structural Characterization of $CoaPLA_2$. The molecular mass of $CoaPLA_2$ was analyzed by MALDI-TOF mass spectrometry (Figure 7) and the mass determined was 13.793.8 Da. However, interestingly, Tsai et al. [37] found the cDNA E6d product to present a molecular mass of 13.782 Da. In addition, Tsai et al. [37], based on a phylogenetic analysis, also found that all cDNA products from all specimens studied showed biological relationships among themselves, with the exception of the cDNA E6d product. Authors inferred and suggested that the specimen, initially considered as *Crotalus viridis viridis*, which produced the E6d cDNA, in reality belonged to a distinct population present in Southwestern Arizona.

To extend our structural study of $CoaPLA_2$, we determined its primary structure using LC/MS-MS, after in-solution digestion (tryptic digestion). The fragments obtained were analyzed using the Proteome Discoverer

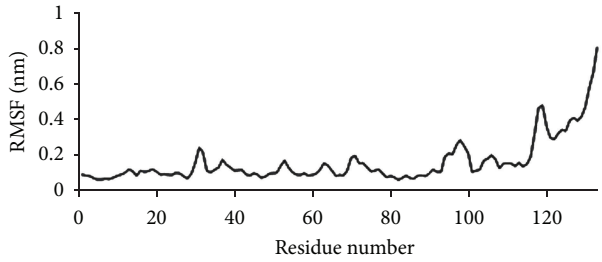


Figure 9: RMSF values per residue after protein stabilization.

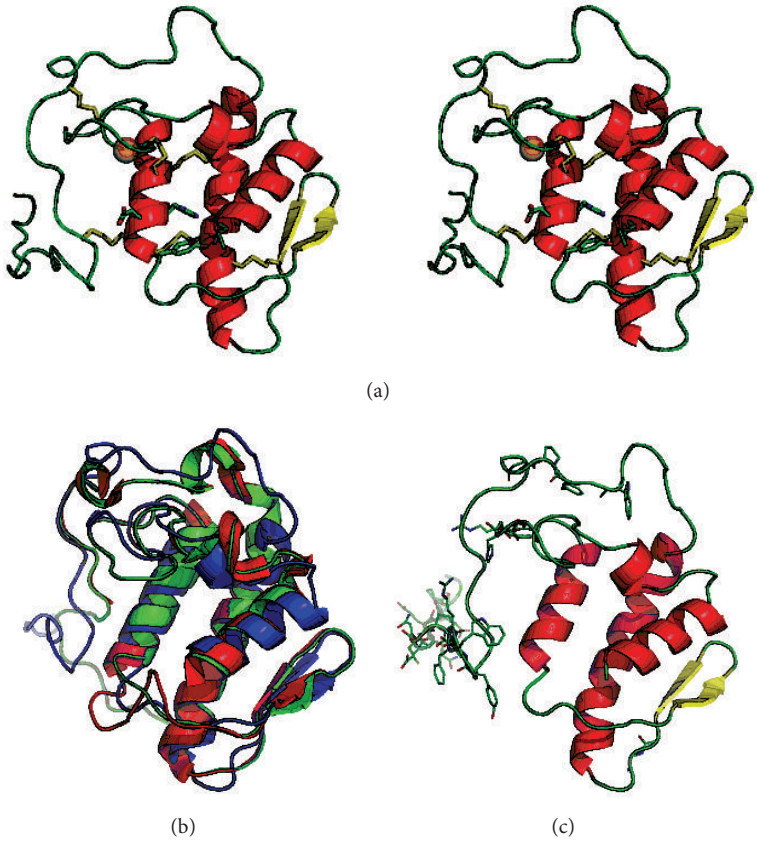
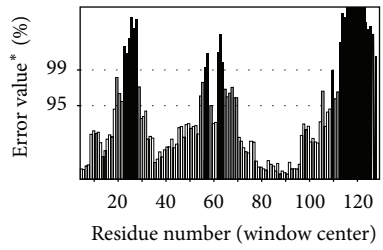
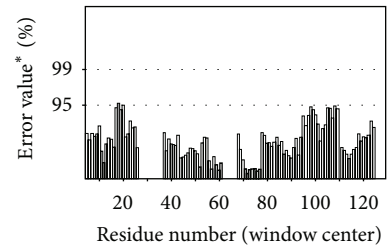
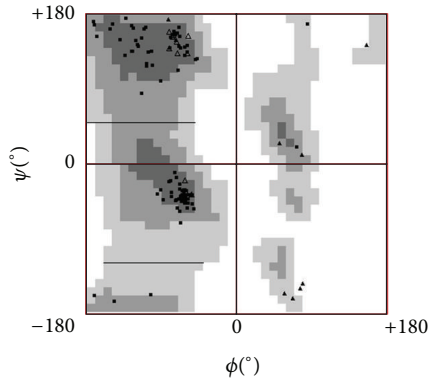
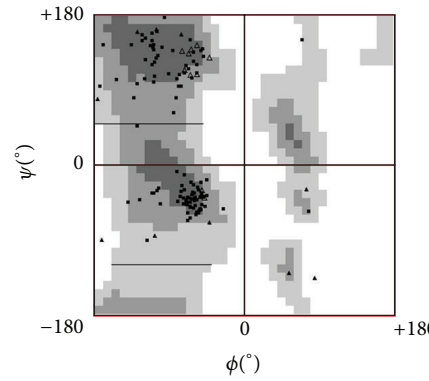


Figure 10: (a) Stereoview of the final model; (b) superimposition of the initial (green), final (blue), and template (red) models; (c) residues with high energy predicted by ANOLEA are displayed in wireframe model.

(v.1.3.0.339) software, where a search was run with the MASCOT engine against the NCBInr Serpentes database. The search found eight fragments (Table 1). The analysis using the ClustalW multiple sequence alignment showed that fragments recovered from the PLA_2 sequence produced by the cDNA E6d and described by Tsai et al. [37] displayed 94% sequential homology (Table 2), except for a unique fragment that was not found (VTDCNPK). From Table 2 we can see that this fragment is extremely conserved in all sequences analyzed and in the model proposed by us (Figure 10). We have inserted the sequence VTDCNPK in the gap of the fragment as not found, as shown in the E6D sequence.

The comparison between the E6D sequence and the $CoaPLA_2$ sequence obtained is interesting because both are exactly equal, except for the VTDCNPK fragment (not found in this study). Tsai et al., [37] suggested that the specimen, initially considered as *Crotalus viridis viridis*, may be a distinct population present in Southwestern Arizona, considered the natural habitat of *Crotalus oreganus abyssus* (Figure 1(b)). Thus, we infer that, probably, the specimen used by Tsai et al., [37] that produced the E6D cDNA was actually a *Crotalus oreganus abyssus* snake, and not a *Crotalus viridis viridis*. In addition to this information, and to enforce our conclusion, it should be remembered that the value of the enzymatic activity of $CoaPLA_2$ found in this work is very near to the value found by Tsai et al., 2003 (around 680 μmol/min/mg for the E6d clone and approximately 590 μmol/min/mg for $CoaPLA_2$).

Table 3: Results for ANOLEA, ERRAT, Verify 3D, ProSA, and Ramachandran analyses for the initial and final models.

	Initial model	Final model
ANOLEA: residues with high energy	35 (26.32%)	25 (18.80%)
ERRAT: error per residue	Overall quality factor**: 64.516 	Overall quality factor**: 99.029 
Verify 3D: residues with scores of lower than 0 have low structural quality	17 residues < 0	0 residues < 0
Ramachandran plot		
Residues in the outlier region	3 GLY (2.29%)	2 GLY (1.53%)

This fact supports the need for more studies on *Crotalus oreganus* (all subspecies) because for many years all subspecies of *Crotulus viridis* and *Crotalus oreganus* were treated as a single serpent specimen. However, as subsequent studies have shown, the old classification was incorrect and the "old" *Crotalus viridis* can in fact be divided into two subspecies of *Crotalus viridis* (*viridis* and *nuntius*) and seven subspecies of *Crotalus oreganus* (*abyssus, lutosus, concolor, oreganus, helleri, Cerberus, and caliginis*).

3.5. Molecular Modeling. To increase the understanding of the CoaPLA$_2$ structure, we conducted molecular modeling studies using Molecular Dynamics (MD) simulation. We calculated the values of root mean squared distance (RMSD) considering the protein backbone atoms, which are displayed in Figure 8. When analyzing these results, we noted that the PLA$_2$ model was stabilized after approximately 1300 ps of simulation.

From the root mean squared fluctuation (RMSF) values of the alpha carbons per residue, we can see that the fluctuation of PLA$_2$ residues from 1300 ps to 5000 ps is very low (except for the residues in the terminal loop, 119–133), indicating that there are no significant changes in the conformation of the residues (Figure 9).

Comparing the initial 3D model and the final model obtained after the MD simulation, it can be easily seen that the MD simulation is fundamental to refine the PLA$_2$ model. The ProSA energy profile indicates that both initial and final models have energy values per residue of lower than 0, indicating a good pseudoenergy profile. Table 3 displays the results obtained from ANOLEA, ERRAT, Verify 3D, ProSA, and Ramachandran analyses for the initial and final models.

The ANOLEA results indicate that the MD simulation decreased the number of high energy residues by 8%. With all high energy residues of the final model being located in the loop region (Figure 9(c)). The ERRAT results indicate that the MD simulation improved the quality of the structural model from 64% to 99%. From the Verify 3D results, the initial model had 17 residues with poor structural quality (score lower than 0) and all residues of the final model had score values of higher than 0. Finally, the Ramachandran plot analysis indicates that both initial and final models had 3 and 2 glycine residues located at an outlier region, respectively. Figure 9(a) shows the Stereoview of the final model. Figure 9(a) shows that our model displays the typical phospholipase conformation, containing three parallel α-helixes and a β-wing (one double-stranded antiparallel β-sheet) [20].

Data reinforce the necessity of rearranging and clarifying all information available regarding the two subspecies of

Crotalus oreganus and *Crotalus viridis*, considering the length of time during which these species were considered as one and the same.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Institute for Research in Biomedicine (IRB Barcelona). Authors also acknowledge Ministry of Science and Technology (MCTI), Financiadora de Estudos e Projetos (FINEP), Coordenação de Aperfeiçoamento de Nível Superior (CAPES, Projeto NanoBiotec), Rede de Biodiversidade e Biotecnologia da Amazônia Legal (BIONORTE/CNPq/MCTI), Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT- INPeTAm/CNPq/MCTI), Instituto Nacional para Pesquisa em Toxinas (INCT-Tox), and Secretary of Development of Rondonia State (SEPLAN/PRONEX/CNPq) for financial support and to Conselho de Gestão do Patrimônio Genético (CGEN/MMA) for the authorization number 010627/2011-1.

References

[1] L. M. Klauber, *Their Habits, Life Histories and Influences on Mankind*, vol. 2, University of California Press, Berkeley, Calif, USA, 1956.

[2] S. P. Mackessy, "Evolutionary trends in venom composition in the Western Rattlesnakes (*Crotalus viridis sensu lato*): toxicity versus tenderizers," *Toxicon*, vol. 55, no. 8, pp. 1463–1474, 2010.

[3] K. G. Ashton, "Body size variation among mainland populations of the western rattlesnake (*Crotalus viridis*)," *Evolution*, vol. 55, no. 12, pp. 2523–2533, 2001.

[4] K. G. Ashton and A. de Queiroz, "Molecular systematics of the western rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogenetic studies of snakes," *Molecular Phylogenetics and Evolution*, vol. 21, no. 2, pp. 176–189, 2001.

[5] C. E. Pook, W. Wüster, and R. S. Thorpe, "Historical biogeography of the Western Rattlesnake (Serpentes: *Viperidae*: *Crotalus viridis*), inferred from mitochondrial DNA sequence information," *Molecular Phylogenetics and Evolution*, vol. 15, no. 2, pp. 269–282, 2000.

[6] D. C. I. Koh, A. Armugam, and K. Jeyaseelan, "Snake venom components and their applications in biomedicine," *Cellular and Molecular Life Sciences*, vol. 63, no. 24, pp. 3030–3041, 2006.

[7] R. K. Arni and R. J. Ward, "Phospholipase A_2: a structural review," *Toxicon*, vol. 34, no. 8, pp. 827–841, 1996.

[8] C. D. Funk, "Prostaglandins and leukotrienes: advances in eicosanoid biology," *Science*, vol. 294, no. 5548, pp. 1871–1875, 2001.

[9] F. S. Markland Jr., "Snake venoms and the hemostatic system," *Toxicon*, vol. 36, no. 12, pp. 1749–1800, 1998.

[10] S. L. da Silva, M. Comar Jr., K. M. T. Oliveira et al., "Molecular modeling of the inhibition of enzyme PLA_2 from snake venom by dipyrone and 1-phenyl-3-methyl-5-pyrazolone," *International Journal of Quantum Chemistry*, vol. 108, no. 13, pp. 2576–2585, 2008.

[11] Z. Mallat, G. Lambeau, and A. Tedgui, "Lipoprotein-associated and secreted phospholipases A_2 in cardiovascular disease: roles as biological effectors and biomarkers," *Circulation*, vol. 122, no. 21, pp. 2183–2200, 2010.

[12] D. de Luca, A. Minucci, P. Cogo et al., "Secretory phospholipase A_2 pathway during pediatric acute respiratory distress syndrome: a preliminary study," *Pediatric Critical Care Medicine*, vol. 12, no. 1, pp. e20–e24, 2011.

[13] R. Ezzeddini, M. Darabi, B. Ghasemi et al., "Circulating phospholipase-A_2 activity in obstructive sleep apnea and recurrent tonsillitis," *International Journal of Pediatric Otorhinolaryngology*, vol. 76, no. 4, pp. 471–474, 2012.

[14] S. Chalbot, H. Zetterberg, K. Blennow et al., "Blood-cerebrospinal fluid barrier permeability in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 25, no. 3, pp. 505–515, 2011.

[15] A. A. Farooqui, M. L. Litsky, T. Farooqui, and L. A. Horrocks, "Inhibitors of intracellular phospholipase A_2 activity: their neurochemical effects and therapeutical importance for neurological disorders," *Brain Research Bulletin*, vol. 49, no. 3, pp. 139–153, 1999.

[16] M. E. S. Martins-Santos, R. R. Resende, F. C. H. Pinto et al., "Effect of a pool of peptides isolated from crotalus durissus terrificus (South American Rattlesnake) venom on glucose levels of mice fed on a high-fat diet," *International Journal of Peptide Research and Therapeutics*, vol. 17, no. 3, pp. 225–230, 2011.

[17] S. L. da Silva, J. R. Almeida, L. M. Resende et al., "Isolation and characterization of a natriuretic peptide from *Crotalus oreganus abyssus* (grand canyon rattlesnake) and its effects on systemic blood pressure and nitrite levels," *International Journal of Peptide Research and Therapeutics*, vol. 17, no. 3, pp. 165–173, 2011.

[18] S. L. da Silva, C. A. Dias-Júnior, P. Baldasso et al., "Vascular effects and electrolyte homeostasis of the natriuretic peptide isolated from *Crotalus oreganus abyssus* (North American Grand Canyon rattlesnake) venom," *Peptides*, vol. 36, pp. 206–212, 2012.

[19] L. B. Silveira, D. P. Marchi-Salvador, N. A. Santos-Filho et al., "Isolation and expression of a hypotensive and anti-platelet acidic phospholipase A_2 from Bothrops moojeni snake venom," *Journal of Pharmaceutical and Biomedical Analysis*, vol. 73, pp. 35–43, 2013.

[20] D. C. S. Damico, T. Vassequi-Silva, F. D. Torres-Huaco et al., "LmrTX, a basic PLA_2 (D49) purified from *Lachesis muta rhombeata* snake venom with enzymatic-related antithrombotic and anticoagulant activity," *Toxicon*, vol. 60, pp. 773–781, 2012.

[21] S. Marcussi, R. G. Stábeli, N. A. Santos-Filho et al., "Genotoxic effect of Bothrops snake venoms and isolated toxins on human lymphocyte DNA," *Toxicon*, vol. 65, pp. 9–14, 2013.

[22] S. Marcussi, P. R. S. Santos, D. L. Menaldo et al., "Evaluation of the genotoxicity of *Crotalus durissus terrificus* snake venom and its isolated toxins on human lymphocytes," *Mutation Research*, vol. 724, no. 1-2, pp. 59–63, 2011.

[23] D. C. S. Damico, M. A. C. Höfling, M. Cintra et al., "Pharmacological study of edema and myonecrosis in mice induced by venom of the bushmaster snake (*Lachesis muta muta*) and its basic Asp49 phospholipase A_2 (LmTX-I)," *Protein Journal*, vol. 27, no. 6, pp. 384–391, 2008.

[24] L. Romero, S. Marcussi, D. P. Marchi-Salvador et al., "Enzymatic and structural characterization of a basic phospholipase A_2

from the sea anemone Condylactis gigantea," *Biochimie*, vol. 92, no. 8, pp. 1063–1071, 2010.

[25] S. S. Teixeira, L. B. Silveira, F. M. N. da Silva et al., "Molecular characterization of an acidic phospholipase A_2 from Bothrops pirajai snake venom: synthetic C-terminal peptide identifies its antiplatelet region," *Archives of Toxicology*, vol. 85, no. 10, pp. 1219–1233, 2011.

[26] C. Z. Oliveira, N. A. Santos-Filho, D. L. Menaldo et al., "Structural and functional characterization of a γ-type phospholipase A_2 inhibitor from *Bothrops jararacussu* Snake Plasma," *Current Topics in Medicinal Chemistry*, vol. 11, no. 20, pp. 2509–2519, 2011.

[27] J. A. F. P. Villar, F. T. D. Lima, C. L. Veber et al., "Synthesis and evaluation of nitrostyrene derivative compounds, new snake venom phospholipase A_2 inhibitors," *Toxicon*, vol. 51, no. 8, pp. 1467–1478, 2008.

[28] R. C. S. Nunomura, V. G. Oliveira, S. L. da Silva, and S. M. Nunomura, "Characterization of bergenin in Endopleura uchi bark and its anti-inflammatory activity," *Journal of the Brazilian Chemical Society*, vol. 20, no. 6, pp. 1060–1064, 2009.

[29] M. L. da Silva, S. Marcussi, R. S. Fernandes et al., "Anti-snake venom activities of extracts and fractions from callus cultures of *Sapindus saponaria*," *Pharmaceutical Biology*, vol. 50, no. 3, pp. 366–375, 2012.

[30] E. S. de Alvarenga, S. A. Silva, L. C. A. Barosa et al., "Synthesis and evaluation of sesquiterpene lactone inhibitors of phospholipase A_2 from *Bothrops jararacussu*," *Toxicon*, vol. 57, no. 1, pp. 100–108, 2011.

[31] S. L. da Silva, A. K. Calgarotto, V. Maso et al., "Molecular modeling and inhibition of phospholipase A_2 by polyhydroxy phenolic compounds," *European Journal of Medicinal Chemistry*, vol. 44, no. 1, pp. 312–321, 2009.

[32] A. D. L. Souza, E. Rodrigues-Filho, A. Q. L. Souza et al., "Koninginins, phospholipase A_2 inhibitors from endophytic fungus Trichoderma koningii," *Toxicon*, vol. 51, no. 2, pp. 240–250, 2008.

[33] S. L. da Silva, A. K. Calgarotto, J. S. Chaar, and S. Marangoni, "Isolation and characterization of ellagic acid derivatives isolated from *Casearia sylvestris* SW aqueous extract with anti-PLA_2 activity," *Toxicon*, vol. 52, no. 6, pp. 655–666, 2008.

[34] M. Murakami, Y. Taketomi, C. Girard, K. Yamamoto, and G. Lambeau, "Emerging roles of secreted phospholipase A_2 enzymes: lessons from transgenic and knockout mice," *Biochimie*, vol. 92, no. 6, pp. 561–582, 2010.

[35] J. I. dos Santos, M. Cintra-Francischinelli, R. J. Borges et al., "Structural, functional, and bioinformatics studies reveal a new snake venom homologue phospholipase A_2 class," *Proteins*, vol. 79, no. 1, pp. 61–78, 2011.

[36] A. M. Soares, M. R. M. Fontes, and J. R. Giglio, "Phospholipase A_2 myotoxins from Bothrops snake venoms: structure-function relationship," *Current Organic Chemistry*, vol. 8, no. 17, pp. 1677–1690, 2004.

[37] I. Tsai, Y. Wang, Y. Chen, and A. T. Tu, "Geographic variations, cloning, and functional analyses of the venom acidic phospholipases A_2 of *Crotalus viridis viridis*," *Archives of Biochemistry and Biophysics*, vol. 411, no. 2, pp. 289–296, 2003.

[38] W. Cho, M. A. Markowitz, and F. J. Kézdy, "A new class of phospholipase A_2 substrates: kinetics of the phospholipase A_2 catalyzed hydrolysis of 3-(Acyloxy)-4-nitrobenzoic acids," *Journal of the American Chemical Society*, vol. 110, no. 15, pp. 5166–5171, 1988.

[39] C. Colovos and T. O. Yeates, "Verification of protein structures: patterns of nonbonded atomic interactions," *Protein Science*, vol. 2, no. 9, pp. 1511–1519, 1993.

[40] F. Melo, D. Devos, E. Depiereux, and E. Feytmans, "ANOLEA: a www server to assess protein structures," *Intelligent Systems for Molecular Biology*, vol. 97, pp. 110–113, 1997.

[41] J. Jasti, M. Paramasivam, A. Srinivasan, and T. P. Singh, "Structure of an acidic phospholipase A_2 from Indian saw-scaled viper (Echis carinatus) at 2.6 A resolution reveals a novel intermolecular interaction," *Acta Crystallographica D*, vol. 60, no. 1, pp. 66–72, 2004.

[42] M. Pasek, C. Keith, D. Feldman, and P. B. Sigler, "Characterization of crystals of two venom phospholipases A_2," *Journal of Molecular Biology*, vol. 97, no. 3, pp. 395–397, 1975.

[43] M. Holzer and S. P. Mackessy, "An aqueous endpoint assay of snake venom phospholipase A_2," *Toxicon*, vol. 34, no. 10, pp. 1149–1155, 1996.

[44] B. P. Smart, Y. H. Pan, A. K. Weeks, J. G. Bollinger, B. J. Bahnson, and M. H. Gelb, "Inhibition of the complete set of mammalian secreted phospholipases A_2 by indole analogues: a structure-guided study," *Bioorganic and Medicinal Chemistry*, vol. 12, no. 7, pp. 1737–1749, 2004.

[45] Y. Han, B. Ma, and K. Zhang, "Spider: software for protein identification from sequence tags with de novo sequencing error," *Journal of Bioinformatics and Computational Biology*, vol. 3, no. 3, pp. 697–716, 2005.

[46] B. Ma and R. Johnson, "De Novo sequencing and homology searching," *Molecular and Cellular Proteomics*, vol. 11, no. 2, Article ID O111.014902, 2011.

[47] J. U. Bowie, R. Luthy, and D. Eisenberg, "A method to identify protein sequences that fold into a known three-dimensional structure," *Science*, vol. 253, no. 5016, pp. 164–170, 1991.

[48] H. J. C. Berendsen, D. van der Spoel, and R. van Drunen, "GROMACS: a message-passing parallel molecular dynamics implementation," *Computer Physics Communications*, vol. 91, no. 1–3, pp. 43–56, 1995.

[49] E. Lindahl, B. Hess, and D. van der Spoel, "GROMACS 3.0: a package for molecular simulation and trajectory analysis," *Journal of Molecular Modeling*, vol. 7, no. 8, pp. 306–317, 2001.

[50] H. J. C. Berendsen, J. P. M. Postma, W. F. V. Gunsteren, and J. Hermans, "Interaction models for water in relation to protein hydration," in *Intermolecular Forces*, B. Pullman, Ed., pp. 331–342, Reidel, Dordrecht, The Netherlands, 1981.

[51] W. F. V. Gunsteren, S. R. Billeter, A. A. Eising et al., *Biomolecular Simulation: The GROMOS96 Manual and User Guide*, VdF Hochschulverlag ETHZ Zurich, 1996.

[52] W. L. de Lano, *The PyMOL Molecular Graphics System, Volume 0. 99*, DeLano Scientific, San Francisco, Calif, USA, 2006.

[53] R. Luthy, J. U. Bowie, and D. Eisenberg, "Assesment of protein models with three-dimensional profiles," *Nature*, vol. 356, no. 6364, pp. 83–85, 1992.

[54] J. U. Bowie, R. Luthy, and D. Eisenberg, "A method to identify protein sequences that fold into a known three-dimensional structure," *Science*, vol. 253, no. 5016, pp. 164–170, 1991.

[55] M. Wiederstein and M. J. Sippl, "ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins," *Nucleic Acids Research*, vol. 35, pp. W407–410, 2007.

[56] M. J. Sippl, "Recognition of errors in three-dimensional structures of proteins," *Proteins*, vol. 17, no. 4, pp. 355–362, 1993.

[57] K. Gopalakrishnan, G. Sowmiya, S. S. Sheik, and K. Sekar, "Ramachandran plot on the web (2.0)," *Protein and Peptide Letters*, vol. 14, no. 7, pp. 669–671, 2007.

[58] G. Faure, H. Xu, and F. A. Saul, "Crystal structure of crotoxin reveals key residues involved in the stability and toxicity of this potent heterodimeric β-neurotoxin," *Journal of Molecular Biology*, vol. 412, no. 2, pp. 176–191, 2011.

[59] J. B. Harris, *Snake Toxins*, Pergamont Press, New York, NY, USA, 1991.

[60] J. Gutiérrez and B. Lomonte, "Phospholipase A_2 myotoxins from Bothrops snake venoms," *Toxicon*, vol. 33, no. 11, pp. 1405–1424, 1995.

[61] E. A. Dennis, J. Cao, Y. Hsu, V. Magrioti, and G. Kokotos, "Phospholipase A_2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention," *Chemical Reviews*, vol. 111, no. 10, pp. 6130–6185, 2011.

[62] J. M. Gutierrez, F. Chaves, J. A. Gene, B. Lomonte, Z. Camacho, and K. Schosinsky, "Myonecrosis induced in mice by a basic myotoxin isolated from the venom of the snake *Bothrops nummifer* (jumping viper) from Costa Rica," *Toxicon*, vol. 27, no. 7, pp. 735–745, 1989.

[63] C. J. Duncan, "Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition," *Experientia*, vol. 34, no. 12, pp. 1531–1535, 1978.

[64] B. F. Trump, I. K. Berezesky, and A. R. Vargas, "Cell death the disease process. The role of calcium," in *Cell Death in Biology and Pathology*, Chapman and Hall, 1981.

[65] P. Gopalakrishnakone, D. W. Dempster, B. J. Hawgood, and H. Y. Elder, "Cellular and mitochondrial changes induced in the structure of murine skeletal muscle by crotoxin, a neurotoxic phospholipase A_2 complex," *Toxicon*, vol. 22, no. 1, pp. 85–98, 1984.

[66] E. C. T. Landucci, R. C. Castro, M. F. Pereira et al., "Mast cell degranulation induced by two phospholipase A_2 homologues: dissociation between enzymatic and biological activities," *European Journal of Pharmacology*, vol. 343, no. 2-3, pp. 257–263, 1998.

[67] E. C. T. Landucci, R. C. de Castro, M. Toyama et al., "Inflammatory oedema induced by the Lys-49 phospholipase A_2 homologue piratoxin-I in the rat and rabbit. Effect of polyanions and p-bromophenacyl bromide," *Biochemical Pharmacology*, vol. 59, no. 10, pp. 1289–1294, 2000.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 203639, 19 pages
http://dx.doi.org/10.1155/2014/203639

Review Article

Antitumoral Activity of Snake Venom Proteins: New Trends in Cancer Therapy

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Received 20 September 2013; Revised 7 December 2013; Accepted 8 December 2013; Published 13 February 2014

Academic Editor: Fernando Albericio

For more than half a century, cytotoxic agents have been investigated as a possible treatment for cancer. Research on animal venoms has revealed their high toxicity on tissues and cell cultures, both normal and tumoral. Snake venoms show the highest cytotoxic potential, since ophidian accidents cause a large amount of tissue damage, suggesting a promising utilization of these venoms or their components as antitumoral agents. Over the last few years, we have studied the effects of snake venoms and their isolated enzymes on tumor cell cultures. Some *in vivo* assays showed antineoplastic activity against induced tumors in mice. In human beings, both the crude venom and isolated enzymes revealed antitumor activities in preliminary assays, with measurable clinical responses in the advanced treatment phase. These enzymes include metalloproteases (MP), disintegrins, L-amino acid oxidases (LAAOs), C-type lectins, and phospholipases A_2 (PLA_2s). Their mechanisms of action include direct toxic action (PLA_2s), free radical generation (LAAOs), apoptosis induction (PLA_2s, MP, and LAAOs), and antiangiogenesis (disintegrins and lectins). Higher cytotoxic and cytostatic activities upon tumor cells than normal cells suggest the possibility for clinical applications. Further studies should be conducted to ensure the efficacy and safety of different snake venom compounds for cancer drug development.

1. Introduction

Cancer is a chronic degenerative disease considered to be the second most common cause of death in economically developing countries [1, 2]. According to a recent report by the International Agency for Research on Cancer (IARC), there are currently more than 10 million cases of cancer per year worldwide. In 2008 alone there were 12.7 million new cases of cancer worldwide and the WHO estimates that the disease will cause about 13.1 million deaths by 2030 [3].

Cancer is characterized by an accelerated and uncontrolled multiplication of a set of aberrant cells which lose their apoptotic ability. Research has been undertaken in order to find out the factors which promote uncontrolled multiplication of cells and how cancer genes affect cell signaling, chromatin, and epigenomic regulation and RNA splicing, protein homeostasis, metabolism, and lineage maturation [4–6].

Understanding the events that transform a normal cell into a cancer cell has caused new therapies to develop that are

more precisely designed to treat a critical gene or biological pathway [7]. Based on their mechanism of action, antitumor drugs that target the cell cycle can be divided generally into three categories, namely, blocking DNA synthesis, causing DNA damage, and stopping mitosis [8]. However, cancer therapy continues involving invasive procedures, including catheter application of chemotherapy, surgery to remove the tumor(s), the use of radiation, and even nonselective cytotoxic drugs [9, 10]. Therefore, the search for new active drugs for cancer therapy is one of the goals of biotechnological research. The expansion of new drugs in oncology represents one of the most promising objectives of the pharmaceutical industry. Many of these compounds are derived from the extraction and purification of toxins and secondary metabolites originating from microorganisms, plants, and animals [11, 12].

Several compounds from venomous animals, such as snakes, spiders, scorpions, caterpillars, bees, insects, wasps, centipedes, ants, toads, and frogs, have largely shown biotechnological or pharmacological applications [13–17]. Numerous examples may be mentioned. Compound TM-601, a modified form of the peptide Chlorotoxin (CTX), isolated from *Leiurus quinquestriatus* scorpion venom, has been shown to bind specifically to glioma cell surfaces as a specific chloride channel blocker and is currently in phase II of human trials [18, 19]. Another example is the venom-derived drug Prialt (ziconotide) generated from the venom peptide of the marine snail *Conus magus* [20] and the drug Byetta (exenatide), a synthetic version of exendin-4 utilized in the treatment of Type 2 diabetes, from the saliva of the Gila monster lizard [21, 22].

The ability of some snake venom toxins to cause toxicity is associated with their high specificity and affinity for cell and tissues. In spite of their toxicological effects, several isolated snake venom proteins and peptides have practical applications as pharmaceutical agents [23]. For example, thrombolytic agents have been used in several cases of vascular disorder [24], antimicrobial activity against gram-positive and gram-negative bacteria [25, 26], antiviral activity against several types of viruses including the herpes simplex virus [27], yellow fever and dengue [28], antiparasitic activity against *Leishmania* [29] and *Plasmodium falciparum* [30], and antifungal activity [31], among other examples.

For cancer treatment, there is great interest in drug design, providing structural templates for the study of new molecules or cellular mechanisms. The use of snake venom in the treatment of some diseases began about sixty years ago in folk medicine. Thus, the biological and toxicological mechanisms involved in snakebites led physicians to study new methods on the isolation of venom constituents, as well as to understand how these compounds could help in medicine.

2. Antitumoral Activity of Snake Venoms

Snake venom is a complex mixture of different components that include peptides, proteins, enzymes, carbohydrates, and minerals. Inside a group of enzymes may be found acetylcholinesterases, L-amino acid oxidases, serineproteases, metalloproteases, and phospholipases A_2 [32] (Figure 1). The cytotoxicity of snake venoms is related to cellular metabolism alterations with a major effect on tumor cells when compared with normal cells. This observation stimulated the development of most chemotherapeutic drugs based on toxins produced in animals, which have the capacity to be highly cytotoxic.

The ability of snake venoms to act upon tumor cells has been known for a long time. The first reported studies on using snake venom against tumor cells were related to the defibrination process. It was suggested that Ancrod, a polypeptide from *Agkistrodon rhodostoma*, administered with cyclophosphamide, could produce defibrination, thus decreasing the tumor weight by fibrinolysis and contributing to both detachment and decreased spread of some tumors [33]. However, their results showed that, besides defibrination, other complex mechanisms including platelet aggregation could be involved in the process.

Braganca et al. [34, 35] assayed a small fraction of *Naja naja* venom on cell cultures of Yoshida sarcoma, calling it cobra venom factor (CVF). Kaneda et al. [36] studied the antitumoral potential of purified peptides (cardiotoxin and cytotoxin) from the *Naja naja atra* snake. Then, Chiam-Matyas and Ovadia [37] showed the cytotoxic properties of several crude venoms from terrestrial snakes with lytic effects on cultures of malignant melanoma tumor cells.

In the past, snake venoms were used to understand the molecular mechanism of some receptors, such as acetylcholine (ACh), and their involvement with some diseases. Two groups of toxins (α-BuTX and Erabutoxin and b—ETXa and ETXb) isolated from *Bungarus multicinctus* and *Laticauda semifasciata*, respectively, showed high affinity toward normal and tumor cells, displaying both cytolytic and cytotoxic effects. Interactions of such toxins with ACh led to the application of these compounds as probes not only to elucidate neurophysiology but also to study some tumor cells. Although α-BuTX inhibited neuroblastomas, it was too toxic for *in vivo* assays [38]. Moreover, no relationship was observed between a cytotoxic effect and ACh receptors [39].

Experiments have shown that cytotoxic effects displayed by snake venoms are specifically related to the species, genus, and tissue targets. Thus, snake venoms were grouped according to their pathophysiological activities as follows: (i) venoms which cause irreversible alterations on the cell, totally destroying it (this group includes Elapidae venoms); (ii) Crotalidae venoms which cause loss of the cell process viability; and (iii) Viperidae venoms, which cause alterations of cell aggregation. *In vivo*, it was demonstrated that the venom of *Naja nigricollis* inhibited the growth of melanoma through one of these mechanisms. Thus, these findings gave new direction and probable application of snake venom as well as isolated toxins for cancer treatment [40].

Snake venom toxins were also investigated as blockers of metastasis. Metastasis is one of the major causes of death in patients with cancer, being dependent on steps such as adhesion, migration, invasion of blood or lymph vessels, exiting the vessel (with the help of matrix metalloproteinases—MMPs), and finally interaction with the tissue target [41]. Yang et al. [42] studied an inhibitor of integrins that is an important class of cell surface receptors, critically involved

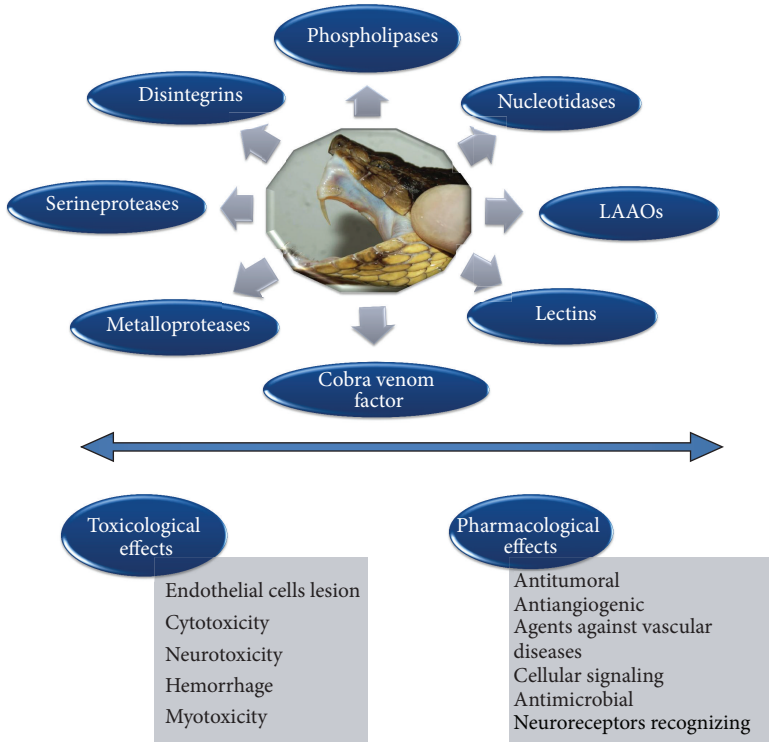

Figure 1: The wide spectrum of action and recent applications of snake venom toxins. The figure depicts the paradigms between toxicological and pharmacological effects of isolated toxins. Different cellular targets are related to different kinds of mechanisms.

in cell-cell and cell-matrix interactions. Particularly, the subfamilies $\beta 1$ and $\beta 3$ play a key role in tumor invasion and dissemination. The group isolated contortrostatin, a disintegrin from *Agkistrodon contortrix contortrix* venom, which is a potent inhibitor of $\beta 1$-integrin-mediated adhesion in human metastatic melanoma cells. Cardiotoxin III (CTX-III) isolated from *Naja naja atra* in the study by Jokhio and Ansari [43] also demonstrated antimetastatic potential by decreasing the expression and activity of matrix metalloproteinase MMP-9, caused by the inactivation of p38 MAPK and PI3K/Akt signaling pathways and NF-κB activity. This suppressive effect assists in inhibiting the migration and invasion of cells causing breast cancer.

As of the last decade, a new strategy has been applied to research on snake venoms with antitumor action, with the focus not only on identifying components with this feature but also on understanding the mechanism of action of toxins that reduce cancer. Several mechanisms of action have been related, as in the study of a cardiotoxin that induces apoptosis in K562 cells through an ROS-independent mitochondrial dysfunction pathway and the caspase-dependent mechanism of Bax/Bcl-2 ratio in human colorectal Colo205 cancer [44]. Juhl et al. [45] described the feasibility of using snake venom in suppressing breast cancer tissue through the inhibition of nucleic acid synthesis. This study shows that snake venom strongly inhibited the formation of nucleic acids in breast cancer tissues. It may cause a decrease in cell proliferation.

The ability of snake venom toxins to destroy malignant cells or to share cytotoxic activity was interesting in areas such as immunology. The use of monoclonal antibodies as antitumor therapeutic agents has not been very promising. However, in coupling a nontoxic CVF isolated from *Naja naja siamensis* to monoclonal antitumor antibodies, several nontoxic antibodies were activated and converted into cytotoxic compounds [46, 47]. Thus, these antibody-CVF conjugates might be a promising therapeutic approach, mediating selective complement-dependent agents of human melanoma, leukemia, and neuroblastomas. Later, it was confirmed that CVF is an important factor for the synthesis of immunoconjugates, which are more specific towards carcinoma cells [48]. In another study using cytotoxin P4, isolated from the same snake, primary conclusions showed for the first time, *in vitro* and *in vivo*, that this peptide caused histopathological changes in leukemia cells and specifically in organelles such as mitochondria [47, 48]. Cytotoxins CT1 and CT2 from *Naja oxiana*, CT3 from *Naja kaouthia,* and CT1 from *Naja haje* were demonstrated to possess this property against human lung adenocarcinoma A549 and promyelocytic leukemia HL60 cells [49].

There are studies showing that *Bothrops jararaca* venom (BjV) induces inhibition of Ehrlich ascites tumor (EAT) growth, accompanied by an increase of mononuclear (MN) leukocytes in all groups inoculated with EAT and/or venom [50]. Different effects were reported with *Crotalus durissus terrificus* venom, one of which was analgesic activity. Zhang et

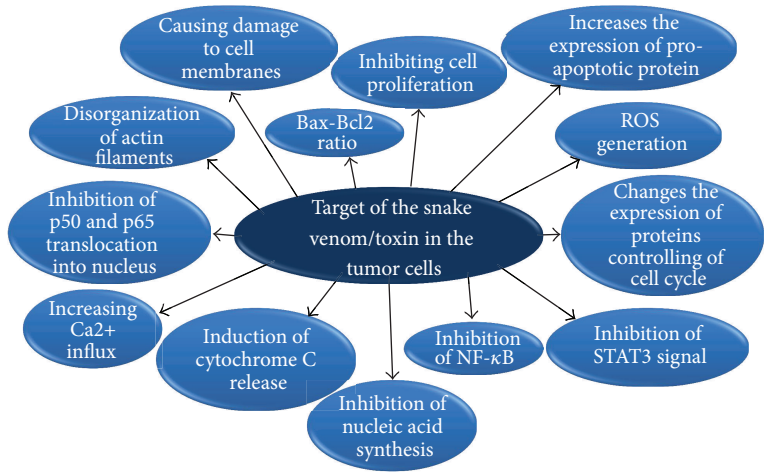

Figure 2: Actions triggered by venoms and/or snake toxins which cause an antitumor effect.

al. [51] showed that the administration of crotoxin to cancer patients reduced the consumption of analgesics.

Several studies suggest the application of snake venom toxins for the treatment of animal tumors. Despite several findings and much evidence, there is much controversy regarding this subject. New advances in cellular and molecular biology, as well as biotechnology, focus on the need to understand new mechanisms displayed by snake venom toxins (Figure 2).

3. "Targets" in Tumor Cells

Understanding snake venom toxins not only helps relieve the healthcare burden of snakebites but also contributes significantly to the treatment of many other medical conditions. In the early 20th century, the idea of utilizing purified toxins as a source of therapeutics emerged [21]. Anticancer drug developments from natural biological resources are ventured throughout the world. The biodiversity of venoms or toxins makes them a tool from which new therapeutic agents may be developed. Snake venom has been shown to possess a wide spectrum of biological activities. Anticarcinogenic activities of snake crude venoms have been recognized, and their components, including cytotoxins, have been isolated and characterized. These components exhibit various physiological effects such as cytotoxicity, inhibition of platelet aggregation, cardiac arrest, and hemolysis [20].

One of the targets investigated is integrins. They are cell surface adhesion molecules coupling the extracellular environment to the cytoskeleton and are also receptors for transmitting important signals for cell migration, invasion, proliferation, and survival. At least six integrin inhibitors are being evaluated in clinical trials for cancer. The parallel development of integrin antagonists as imaging tools for patient selection may accelerate the discovery of new ways for their use [52].

Integrins play multiple important roles in cancer pathology including tumor cell proliferation, angiogenesis, invasion, and metastasis. The inhibition of angiogenesis is one of the most heavily explored treatment options for cancer, and snake venom disintegrins represent a library of molecules with different structures, potencies, and specificities and are good starting points for developing antiangiogenesis therapeutics [21, 53–55].

Recently, Bazaa et al. [56] characterized MVL-PLA_2, a novel phospholipase A_2 from *Macrovipera lebetina* venom, reporting that it exhibited anti-integrin activity. Chwetzoff [57] studied the cytotoxic activity of a basic phospholipase A_2 from *Naja nigricollis* venom on different cell types and its esterase activity. The cytotoxicity observed was not due to a contaminant, since that would have been eliminated after immunoprecipitation of the basic phospholipase A_2 by specific monoclonal antibodies. All eukaryotic cells tested were sensitive to the cytotoxic action of the basic phospholipase A_2. In contrast, the *Escherichia coli* K-12 wild strain was resistant. Thus, the participation of cell membranes in whether the cell is sensitive or resistant to phospholipase A_2's attack was investigated using *E. coli* K-12 membrane mutants, and some of them were sensitive. Whether or not esterase activity was required for phospholipase A_2's cytotoxic attack was dependent on the cell line tested. Indeed, when the esterase activity of the basic PLA_2 was eliminated by treatment with p-bromophenacyl bromide, the enzyme retained cytotoxic potency inducing necrosis of certain tumor cells grown *in vitro*, but not of other cells, such as erythrocytes, for which concomitant esterase activity was also necessary. *In vivo* toxicity studies showed that the loss of either cytotoxic potency or esterase activity eliminated the lethal character of the basic PLA_2. This leads to the proposal that the *in vivo* phospholipase A_2 toxicity depends on the simultaneous expression of esterase activity and a nonenzymatic property, manifested by the *in vitro* cytotoxic potency.

da Silva et al. [58] demonstrated that *Bothrops jararaca* venom (BjV) has an antitumoral effect on Ehrlich ascites tumor (EAT) cells and induces an increase of polymorphonuclear leukocytes in early stages of tumor growth. The study reported that this venom presents an important inflammatory effect when inoculated in animal models and in human snakebites, and that cytokine levels have been

detected in these cases. To evaluate whether the cytokines are involved with the suppression of the tumor's growth, the authors evaluated the cytokine profile in the peritoneal cavity of mice inoculated with EAT cells and treated with BjV. It was observed that EAT implantation induces IL-6, IL-10, and tumor necrosis factor-alpha (TNF-α) production and that the treatment with BjV suppresses production of these cytokines. Furthermore, it was suggested that the IL-6 detected in the present study was produced by the EAT cells and the suppression of its production could be associated with the antitumoral effect of BjV.

Pituitary adenomas are neuroendocrine tumors that produce different endocrine and metabolic alterations, including hyperprolactinemia, acromegaly, and Cushing's disease. These different clinical features of pituitary tumors are the result of the overproduction of hormones by the different pituitary cell types. Recent advances in the understanding of the signaling pathways that control hormone production in pituitary cells provide a source of potential therapeutic targets. Therefore, the study of signaling pathways that control hormone production and proliferation is a good source of candidate targets in pituitary tumors [59].

Structural and functional investigations of these proteins and enzymes from snake venoms may contribute to the advancement of toxinology and to the elaboration of novel therapeutic agents [60].

4. Snake Venom L-Amino Acid Oxidases (svLAAOs)

L-amino acid oxidases (LAAO) are enzymes diffusely distributed in several organisms, such as bacteria, fungi, algae, and snakes. They are dimeric flavoenzymes, which catalyse the oxidative deamination of L-amino acids into ammonia, alpha-keto acids, and H_2O_2 through an intermediary amino acid. These glycoproteins are typically found in the homodimeric form accompanied by a cofactor, which can be flavin adenine dinucleotide or flavin mononucleotide. LAAOs are also found in venoms of several snake species [61]. They are purified generally in either acidic or basic form, with an isoelectric point between 4.4 and 8.5, having FMN and FAD as cofactors (approximately 2 mol/mol), with a relative molecular mass of 120.000–150,000 in native form and 55,000–66,000 in its monomeric form [61–68].

Until the nineties, researchers were restricted to the study of structural and functional characteristics of these enzymes [61]. From this decade, the correlation between the production of L-amino acid oxidases and their use in the metabolic pathways that involve nitrogen, as well as the production of hydrogen peroxide and ammonia, started to represent a horizon in the development of new biotechnological applications.

The high toxicity presented by this class of enzymes is not yet completely understood. Hypotheses have been studied through interaction with cell membrane receptors, which have the potential to produce high hydrogen peroxide concentrations [69]. The first function of LAAO is probably to promote hypotension in the victim by activating soluble guanylate cyclase in the presence of superoxide dismutase [70]. It has been demonstrated that the isolated enzymes of *C. adamanteus* and *C. atrox* can interact specifically with mammal endothelial cells possibly by increasing hydrogen peroxide production [61, 71]. Snake venom LAAOs and their studies in structural and molecular biology have been very important for pharmacology [61, 72]. They have been characterized through their different functions, such as substrate preference, apoptosis induction, cytotoxicity, hemolysis, activation or inhibition of platelet aggregation, hemorrhage induction, edema, and bactericidal activity [61, 68, 73, 74]. Hemorrhage is a common phenomenon caused by snake venom LAAOs, which unchains complex processes, such as apoptosis of endothelial or vascular cells [61].

Many research groups describe LAAOs as apoptosis inducers, in human embryonic cells (293T) [75], human promyelocytic leukemia cells (HL-60) [65, 76, 77], human monocytic cells (MM6) [68], rat lymphocytic leukemia cells (L1210), and human leukemia T cells [71].

In this review, we will focus on the results obtained through several assays regarding their cytotoxic effect upon cell cultures and animal models, as well as the mechanisms involved and reactions able to explain these effects.

Araki et al. [78] showed that cytotoxic substances in the venoms of *C. atrox*, *T. flavoviridis*, *G. h. blomhoffii*, *V. ammodytes,* and *B. arietans* cause apoptosis in cell lines in a selective way: being active on vascular endothelial cells of the human lung and inactive on the same cell line of rats, smooth muscle cells of bovines, and embryonic fibroblasts of human beings [70].

Interestingly, the assays that evaluated the cytotoxic activity of LAAOs attributed the apoptotic effect to H_2O_2 produced by the oxidative reaction, but other studies showed that different mechanisms might exist [71]. Several mice tumor cell lines were assayed for LAAO from *Agkistrodon halys* with high apoptosis induction, even at low concentrations. In the presence of the enzyme, cultured L1210 cell nuclei were split and showed the characteristic ladder-like pattern of DNA fragmentation. The enzyme binds directly to the cell surface, thereby increasing the local concentration of H_2O_2. However, experimental evidence suggests that the apoptotic mechanism induced by LAAO is distinguished from the one caused by exogenous H_2O_2 [71].

In 1997, the Korean group of Ahn et al. [79] published an interesting study investigating the LAAO from *Ophiophagus hannah*, starting from its purification, biochemical characterization up to its cytotoxic activity upon several tumor cell cultures, both human and murine, achieving around 74% of inhibition of tumor proliferation at a concentration of 2 μg/mL. A different mechanism for H_2O_2 production was also postulated, by inhibition of thymidine incorporation, with a consequent interaction with DNA. Markland [80] suggested that this enzyme probably prevents the adhesion of tumor cells and the formation of metastasis in the host by inhibition of platelet aggregation and activation of phagocytic cells from the immunological system.

In 1999, Souza et al. [67] showed the cytotoxicity level of an LAAO from *Agkistrodon contortrix laticinctus* through the fragmentation of DNA on HL-60 cultures hybrid

cells. Results showed signs of induction of apoptosis after extraction of DNA. Apoptosis related to svLAAO activity is a polemic subject since some authors postulate that this activity derives from H_2O_2 action from the enzymatic reaction. Suhr and Kim [71] already showed that apoptosis induction was not related to H_2O_2 alone. Dipietrantonio et al. [81] detected an increase of caspase 3 activity in HL-60 cells exposed to H_2O_2. Caspases are proteases of the cysteine family that are commonly apoptosis markers.

Suhara et al. [82] found that H_2O_2 induces the regulation of the TNF receptor superfamily (FAS) in human endothelial cells and that the activation of the protein tyrosine kinase may be involved in the expression of FAS induced by H_2O_2. Thus, apoptosis mediated by FAS in human endothelial cells can contribute to the mechanism of H_2O_2 in inducing cellular damage.

Stábeli et al. [83] showed that inhibition of the toxic effect of LAAO from *Bothrops moojeni* was retained when catalase, an H_2O_2 scavenger, was added. This same enzyme showed a cytotoxic effect upon Ehrlich ascite tumor cells and showed efficiency as a bactericidal, trypanocidal, leishmanicidal and apoptotic agent through DNA fragmentation. The same observation was made regarding other isoforms of this enzyme, isolated from the same species, on leishmanicidal activity [84].

BjarLAAO-I, an L-amino acid oxidase from *Bothrops jararaca* snake venom, was purified by de Vieira Santos et al. [85]. This LAAO inhibited Ehrlich ascites tumor growth and induced an influx of polymorphonuclear cells, as well as spontaneous liberation of H_2O_2 from peritoneal macrophages. Later, BjarLAAO-I induced mononuclear influx and peritoneal macrophage spreading but the mechanisms that inhibit tumor growth have not been clarified. Animals treated with BjarLAAO-I showed higher survival time.

Zhang et al. [76] evaluated the activity of an LAAO from *Trimeresurus stejnegeri* as an antiviral agent, as well as the cytotoxic effect of this enzyme upon lymphocytic leukemia C8166 cells, discussing the role of H_2O_2 in cytotoxic activity. Using catalase, an H_2O_2 scavenger, these authors observed that LAAO greatly lost its activity, but even in the absence of H_2O_2 cytotoxicity was still significant, supporting the hypothesis that other mechanisms of action are probably involved. This was already postulated by the assay that evaluated the bactericidal activity of the mouse milk enzyme as a protecting agent of mastitis [86].

In investigating apoxin, an LAAO from *Crotalus atrox*, Torii et al. [75] concluded that H_2O_2 indeed played an important role in apoptosis induction. Thus Ali et al. [68] showed that the studies with LAAO from *Eristicophis macmahoni* reinforce the already proposed theory of the participation of H_2O_2, produced during the enzyme's activity, in biological and pharmacological effects, such as apoptosis, cytotoxicity, bactericidal activity, and platelet aggregation induction.

Sun et al. [87] showed the antitumoral effect on an LAAO from *Trimeresurus flavoviridis* at several concentrations upon human glioma cell cultures. They evaluated apoptosis using flow cytometry, showing the inhibitory effect of the enzyme in the presence of catalase. Once more it was shown that concentrations as low as 10 μg/mL were able to destroy 90% of the cells. However, in addition to inhibition by catalase, there was still apoptosis, probably related to the binding of the enzyme to the cell membrane.

Bp-LAAO, an L-amino acid oxidase from *Bothrops pauloensis* snake venom purified by Rodrigues et al. [60], showed dose-dependent leishmanicidal, bactericidal, and antitumoral activities. This antitumor activity was observed in human breast cancer cells (SKBR-3), acute T leukemia cell (JURKAT), and Ehrlich ascitic tumor (EAT) cell lines. Moreover Bp-LAAO induced platelet aggregation in platelet-rich plasma by inhibiting catalase.

In 2012, LAAOs isolated from *Ophiophagus hannah* venom decreased thymidine uptake in murine melanoma, fibrosarcoma, colorectal cancer, and Chinese hamster ovary cell line and also showed reduction in cellular proliferation [79]. In addition, an LAAO isolated from *Agkistrodon acutus* snake venom showed an accumulation of tumor cells at the sub-G1 phase of the cell cycle. It also induced apoptosis via the Fas pathway in A549 cells (human alveolar epithelial cell line) [88–90].

5. Snake Venom Phospholipases A_2 (svPLA$_2$s)

Phospholipases A_2 (PLA$_2$s) are enzymes of high medical-scientific interest due to their involvement in several inflammatory human diseases and in envenomation by snake and bee venoms. PLA$_2$s also play an important role in diet lipid catabolism and in the general metabolism of lipid membranes. In addition, arachidonic acid, one of their hydrolysis products, is the precursor of important eicosanoids displaying prominent biological activities, namely, prostaglandins, prostacyclins, thromboxanes, and leucotrienes. PLA$_2$s constitute a super-family of different enzymes belonging to four groups based on their source, amino acid sequences, and biochemical characteristics [91–93].

Altered lipid biosynthesis and deregulated lipogenesis are typical features of cancer. Consequently, these pathways have been investigated as novel therapeutic targets. Lipolytic phospholipase A_2 (PLA$_2$) enzymes have been explored as novel anticancer agents [94–96].

Different types of phospholipases have been shown to possess antitumor and antiangiogenic properties, such as acidic and basic PLA$_2$s, and synthetic peptides derived from PLA$_2$ homologues [97–100]. Recently, two phospholipases A_2 from *Cerastes cerastes* venom, CC-PLA$_2$-1 and CC-PLA$_2$-2, were purified and characterized. They were able to inhibit cancerous cell adhesion and migration, along with angiogenesis, both *in vitro* and *in vivo* [101, 102]. Phospholipase A_2 from *Macrovipera lebetina transmediterranea* venom (MVL-PLA$_2$) inhibited tumor cell adhesion and migration, as well as angiogenesis. This process occurs through an increase in microtubule dynamics and disorganization of focal adhesions [56, 103].

Some PLA$_2$s isolated from Viperidae venoms are capable of inducing antitumoral activity, suggesting that these molecules may be a new class of anticancer agents and provide new molecular and biological insights into cancer drug development [60, 102].

PLA_2 activity is related to the metabolism of cell membranes. In 1989, Chwetzoff et al. reported that a *Naja nigricollis* PLA_2, called nigexin, displays important cytotoxicity upon cell cultures of several tumors, such as epithelial, neuroblastoma, and leukemia tumors [104]. Most PLA_2s do not show this profile and the authors suggest that the enzymatic activity is not responsible for the cytotoxic effect and other mechanisms must be involved.

VRCTC-310-Onco is a pharmaceutical product under development, composed of crotoxin (from *Crotalus durissus terrificus*) and cardiotoxin (from *Naja naja atra*) at an equimolar ratio. Crotoxin B is the main component, a 14 kDa neurotoxic secretory phospholipase A_2, that, in addition to its classic enzymatic activity, binds and activates cell receptors located in the plasma membrane [105]. By these or other mechanisms, crotoxin interferes with the signaling of the epidermal growth factor receptor [106]. Addition of cardiotoxin dissociates cytotoxicity (required for antitumoral activity) and neurotoxicity (otherwise, its main side effect) and allows a useful concentration to be achieved *in vivo* [107]. Injection of crotoxin into mice has been reported to increase the *in vivo* production of tumor necrosis factor α (TNF- α) together with the stimulation of the hypothalamic-pituitary axis [108].

Preliminary data showed that a protein fraction of the venom could be a useful tool for cancer therapy. Costa et al. [108] evaluated the probable mechanism of action of this union and concluded that PLA_2s act on the receptors of the epidermal growth factor. Another route of action might be a decreased production of tumor necrosis factor. In 2002, the same group suggested intravenous (i.v) administration of the drug, and that route did not show toxicity for the kidneys, heart, or lungs [109].

Roberto et al. [97] showed that the *Bothrops jararacussu* acidic PLA_2, BthA-I-PLA_2, displays antitumoral effects upon Ehrlich ascites tumor, leukemia (Jurkat), and breast cell lines, using several enzyme concentrations. At 100 μg/mL, the toxicity was close to that of the control drug (methotrexate). This activity seems to be related to apoptosis. It is postulated that PLA_2s probably speed up the turnover of phospholipids, what may generate typical changes to apoptosis.

Gebrim et al. [110] evaluated both *in vitro* and *in vivo* antitumor activity of BPB-modified BthTX-I (PLA_2 Lys49) and its cationic synthetic peptide derived from the 115–129 C-terminal region. BPB-BthTX-I presented 70 and 90% cytotoxicity upon Jurkat, B16F10, and S180 tumor cell lines, which were also susceptible to the lytic action of the synthetic peptide. BPB-BthTX-I showed dose-dependent cytotoxicity and this effect was shown to be inferior to that of the native toxin on all tumor cell lines and macrophages.

Several articles state the affinity of secretory PLA_2s for different membrane receptors and lipids, but since those related to *Bothrops* myotoxins are still unknown, two mechanisms have been proposed to explain Lys49 myotoxin cytotoxicity: a fatty acid-dependent lysis by means of an interaction with a receptor able to activate the myotoxin and the activation of intracellular lipase unleashed by the binding of the myotoxin to the receptor [110–112].

A phospholipase B purified from *Pseudechis colletti* was assayed on rabdomiosarcoma A673 tumor cells. A cytotoxic effect on sarcoma cells was observed, but no lytic activity against fibroblasts was found. This effect was related to destructive action of the enzyme upon the striated muscles [113]. Another study with recombinant sea snake PLA_2 (rSSBPLA_2) from *Lapemis hardwickii* venom showed its *in vivo* and *in vitro* enzymatic activity on different tumor cell lines [114].

The synthetic peptides p-AppK and pEM-2 derived from Lys49 phospholipase A_2 homologues from *Agkistrodon piscivorus piscivorus* snake venom were evaluated against different tumor cell lines (B16 melanoma, EMT6 mammary carcinoma, S-180 sarcoma, P3X myeloma, and tEnd endothelial cells) and showed a rapid cytotoxic effect. In general, peptide p-AppK was slightly more potent than pEM-2 against various tumor cell lines, except for the P3X myeloma cells, which were slightly more susceptible to pEM-2 [98].

Ferguson et al. [96] have proposed dextrin-PLA_2 as a bioresponsive anticancer therapeutic polymer and a new example of polymer-enzyme liposome therapy (PELT). Cytotoxicity was assessed in MCF-7, B16F10, and HT29 tumor cell lines using an MTT assay. Therefore, prior to designing protocols for *in vivo* studies it was considered important to further investigate the dextrin-PLA_2 action mechanism, particularly since this could potentially involve multiple cellular targets. Preliminary experiments show that the conjugate is internalized by endocytosis more readily than PLA_2 alone. The resulting redistribution of intracellular vesicles suggests a multimodal mechanism involving both plasma and intracellular-vesicle membrane interactions.

Documented literature reported that the PLA_2 from *Macrovipera lebetina* venom exhibits anti-integrin activity. In their study with HMEC-1 (human microvascular endothelial cells), MVL-PLA_2 has shown inhibition of cell adhesion and migration, as well as disturbed the actin cytoskeleton and the distribution of $\alpha v\beta 3$ integrin [56, 90].

Khunsap et al. [100] purified a PLA_2 (Drs-PLA_2) from *Daboia russelii siamensis* venom which exhibited indirect hemolytic, anticoagulant, and cytotoxic activities. Moreover, this PLA_2 decreased human skin melanoma (SK-MEL-28) cell viability in a dose-dependent manner, as well as migration with an IC_{50} of 25.6 nM. Moreover, Drs-PLA_2 inhibited the colonization of skin melanoma cells (B16F10) in BALB/c mice lungs by 65%.

6. Snake Venom Lectins

Lectins, proteins that bind to carbohydrates, are found in several animal and vegetal species. They interfere with tumor cell proliferation and the studies about their relationship with cancer are based on the characteristics of endogenous lectins from tumor cells. Some plant lectins showed an inhibitory effect on human prostatic tumor cells [115].

Lectins are polyvalent carbohydrate-binding proteins of nonimmune origin and have been used extensively as histochemical probes to describe changes in tumor cell surface. These glycoproteins are known to influence the

growth of cancer cells. BJcuL, a lectin isolated from *Bothrops jararacussu* snake venom, was purified and functionally characterized, and its effect on the proliferation of a number of established human cancer cell lines was determined. The growth of eight cancer cell lines was inhibited in a dose-related manner in the presence of BJcuL. This lectin was a potent growth inhibitor in renal (Caki-1 and A-498) and pancreatic (CFPAC-1) cancer cell lines, with an inhibitory concentration of 50%. These results suggest that BJcuL lectin is an effective inhibitor of cell growth in some cancer cell lines [116].

In 2001, de Carvalho et al. [117] observed that human metastatic breast cancer (MDA-MB-435) and human ovarian carcinoma (OVCAR-5) cell lines adhere, although weakly, to BJcuL. However, the lectin did not inhibit the adhesion of cells to the extracellular matrix proteins fibronectin, laminin, and type I collagen. Importantly, the viability of these tumor cells, other human tumor cell lines, and bovine brain endothelial cell lines were suppressed by BJcuL. These findings suggest that the lectin BJcuL may serve as an interesting tool for combating tumor progression by inhibiting the growth of tumor and endothelial cells. The integrin family of adhesion receptors play an essential role during tumor progression and thus represent interesting potential targets for the development of new therapeutic agents. More recently, Sarray et al. [118] reinforced the cytotoxicity of BJcuL on tumor cells mainly by altering cell adhesion and inducing apoptosis in gastric carcinoma cells MKN45 and AGS. The authors suggested that BJcuL may compete for binding to the cell surface with extracellular matrix glycoproteins and promote actin disassembly and possibly accelerate cellular detachment from the extracellular matrix. After, it was demonstrated that lebecetin, a C-type lectin isolated from *Macrovipera lebetina* venom, displays anti-integrin activity. Lebecetin inhibited integrin-mediated attachment of various tumor cell lines to different adhesion substrates. This protein was able to inhibit adhesion, migration, and invasion of tumor cells. Apparently, the fact that lectin acts on the integrin domains and its antiproliferative activity was significant. At 10 μg/mL, melanoma and sarcoma cell lines had their proliferative profile strongly inhibited [118].

Sarray et al. (2001) demonstrated that lebecetin also interferes with the adhesion of IGR39 melanoma and HT29D4 adenocarcinoma cells. In fact, these two cancer cell lines tightly adhere to immobilized lebecetin. Lebecetin is also able to strongly reduce IGR39 and HT29D4 cell adhesion to fibrinogen and laminin but not to fibronectin and collagen types I and IV, respectively. Adhesion properties of lebecetin may thus involve integrin receptors [118]. Six years later, the same group [119] presented a second C-type lectin, isolated from the same venom which showed potent inhibition of platelet aggregation and seemingly affected cell adhesion, migration, invasion, and proliferation by inhibiting $\alpha5\beta1$ and αv-containing integrins. Moreover, the inhibition of $\alpha5\beta1$ and αv integrins is likely due to the binding of venom peptides, as both lebectin and lebecetin coimmunoprecipitate with these integrins. Lebectin and lebecetin were the first examples of venom C-type lectins inhibiting an integrin other than the collagen receptor $\alpha2\beta1$.

In a short communication, Nunes et al. [120] showed the cytotoxic activity on tumor cells and apoptosis in K562 cells of BlL, a galactoside-binding lectin isolated from *Bothrops leucurus* venom. Antitumor activity was verified by phosphatidylserine externalization analysis and mitochondrial membrane potential determination.

Therefore, lectins have been proved to be prospects for potential use in cancer therapy.

7. Snake Venom Metalloproteases

Metalloproteases are important compounds of most viperid and crotalid venoms. They can trigger hemorrhage by causing changes in blood coagulation or interaction with the main components of the extracellular matrix such as collagen, laminin, and fibronectin [121–123]. Also known as zinc-proteases, snake venom metalloproteases (SVMPs) are multidomain proteins that, through autoproteolysis, can generate biologically active products [124, 125]. According to their structure, these proteins are classified as either part of the mature P-I class, which has only a metalloprotease domain, P-II, which contains a metalloprotease domain followed by a disintegrin domain, P-III, a metalloprotease, with disintegrin-like and cysteine-rich domains, or P-IV, the heterotrimeric class of SVMPs, which possesses an additional snake C-type lectin-like (snaclec) domain, found close to the carboxyl end of the protease which is now included in the P-III group as a subclass (P-IIId) [125–131].

High molecular weight SVMPs are classified as metalloprotease/disintegrin-like/cysteine-rich (MDC) proteins. The complex hemorrhage mechanism induced by these enzymes has led to the investigation of the relationship between the disintegrin domain and the main components of blood coagulation, especially platelets and integrins α, β [132]. In addition, the application of these enzymes in platelet physiology research contributed to elucidating other probable mechanisms involved in cellular adhesion, which was widely studied with jararhagin, a high molecular weight hemorrhagic metalloprotease isolated from *Bothrops jararaca* venom [128, 133–136].

Various groups of matrix metalloproteases/ADAMs have been shown to be involved in the formation of new vessels during tumor growth [137]. These multidomain proteins are involved in both cancerous cell proliferation and indeed in cell-cell/cell-ECM adhesion [138–144].

Molecular approaches have been performed with high molecular weight metalloproteases from a number of Viperidae species in order to elucidate the complex integrin-disintegrin interactions. SVMPs containing disintegrin-like domains (PIII/PIIIb class) may play a role in targeting the protein to a particular site in cells such as platelets, and endothelial cells, as well as in integrins, extracellular matrix and other substrates [122, 134, 145–148]. The structural similarity between mammalian MMPs (ADAM) and SVMPs (low and high MMPs), including disintegrins, reinforces the idea that snake venom components captivate medical interest

as potential molecules for the treatment of animal tumors [149].

8. Disintegrins

Along with metalloproteases, disintegrins are important compounds in most viperid and crotalid venoms. Disintegrins represent a family of nontoxic and nonenzymatic low molecular weight (5–10 kDa) RGD-containing peptides naturally presented in snake venoms or synthetics. Originally, these compounds are characterized by their ability to interact with integrins αIIbβ3, α5β1, and αvβ3lls expressed by a number of cells including those involved in tumor development and proliferation [150, 151].

Based on binding experiments, $\alpha\beta$ integrins and their subtypes have been identified as major functional adhesion receptors on tumor cells. Indeed, disintegrins from several snake venoms have revealed new possibilities of uses not only in cardiovascular diseases but also as potent inhibitors of tumor cells [128, 149, 152]. Thus, a number of toxins containing RGD-peptides or RGD-containing disintegrins isolated from snake venoms have also been used to elucidate target receptors in a wide variety of primary cultured tumor cells (Table 1).

Studies with metalloproteases and respective peptides that contain the disintegrin domain called RGD have focused on their antitumoral effects. These peptides can act in the extracellular matrix and play an important role in the evolution of angiogenesis and metastatic dissemination of cancer [152–154].

Venoms of *Trimeresurus* and *Agkistrodon* genera contain peptides that potentially inhibit platelet aggregation [150, 155–161], frequently induced by tumor cells. In order to discover new natural compounds to inhibit tumor cells, Kang et al. [88] showed strong *in vivo* evidence that a disintegrin containing the RGD sequence from *Agkistrodon halys brevicaudus* could suppress tumor angiogenesis through αvβ3 integrin interactions. A number of isolated disintegrins demonstrated potential inhibition of tumor cells: Contortrostatin [162], Eritostatin [163], Rhodostomin [164, 165], Obtustatin [166], Trigramin [167], Salmosin [168], Triflavin [169], Albolabrin [170], and Echistatin [171].

Contortrostatin (CN), a disintegrin containing Arg-Gly-Asp, isolated from *Agkistrodon contortrix contortrix* venom, interacts with different epithelial carcinoma and endothelial cell surface receptors. The anticancer potential of CN, a 13.5 kDa protein, was demonstrated because CN recognizes integrins αIIbβ3; α5β1; α5β3; α5β5. Schmitmeier et al. [172] demonstrated that CN exerted antitumor activity along with tumor necrosis factor (TNF-α) on human glioblastoma cell lines. This activity may be related to the fact that CN induced the disruption and prevented the binding of integrins to the extracellular matrix [173]. It has been demonstrated that this peptide inhibits *in vitro* and *in vivo* ovarian cancer dissemination and the recruitment of blood vessels to tumors [162, 174, 175]. This first study reinforces that CN is a potent and important molecule not only for therapeutic use in cancer but also to elucidate the several steps involved in tumor progression and metastasis. In 2004, the pharmacological effectiveness of CN was investigated using a mouse model of human mammary cancer. Clinically, the peptide is nonimmunogenic and stable when submitted to a relevant method of drug delivery, such as the liposomal delivery system. This study also shows the effectiveness of the inhibitory effect of contortrostatin on breast cancer progression in orthotopic and xenographic models, as well as the importance of integrins in cellular migration, invasion, matrix degradation, proliferation, and angiogenesis. This protein binds and affects the function of some integrins expressed in cancer, platelet, and endothelial cell surfaces. Encapsulated liposomes were used to release the active protein at the tumor site, thus having a chemotherapeutic application [176].

Another study using the human metastatic breast cancer cell line MDA-MB-435 in mice revealed that contortrostatin from *Agkistrodon contortrix contortrix* has potent antitumoral and antiangiogenic activities and demonstrated that contortrostatin may have potential as a therapeutic agent for the treatment of malignant gliomas [177].

The exact mechanisms that cause tumor regression in experimental animals after treatment with disintegrins are not well established. However, in addition to cell detachment, antiangiogenic activity is an important characteristic of these groups of peptides. Obtustatin, a disintegrin isolated from the venom of *Vipera lebetina obtusa*, showed significant *in vivo* inhibition of Lewis lung carcinoma growth and interacted selectively with the integrin α1β1, promoting inhibition of angiogenesis [166]. Rhodostomin, a disintegrin isolated from *Calloselasma rhodostoma* venom, affected tumor formation and the angiogenic process in different ways. It was first demonstrated that rhodostomin caused inhibition of Saos-2 (human osteosarcoma cells), TCIPA (tumor cell-induced platelet aggregation), and adhesion of tumor cells to the ECM, suggesting that host platelets may act as causative agents in tumor formation and metastasis [164, 165]. Yang and Huang [178] also showed that rhodostomin strongly inhibits the adhesion activity of ROS 17/2.8 cells (osteosarcoma cells) by TGF-β1 (transforming growth factor-β1). In 2001, Huang et al. [179] reported that rhodostomin showed antimetastatic activity due to the fact that it is an antiangiogenic substance, which selectively inhibits basic fibroblast growth factor (bFGF-treated) and the viability of human umbilical vein endothelial cells (HUVEC). The interaction between tumor cells and microvasculature including cell-cell adhesion as well as their migratory activity and angiogenesis was also reported using albolabrin, a disintegrin isolated from *Trimeresurus albolabris* venom. The peptide inhibited the adhesion of melanoma cells to extracellular matrix proteins. Inhibition exerted by albolabrin was dose-dependent and reached 70% for fibronectin and 60% for laminin at 20 μg/mL [170]. At 30 μg/mouse, it inhibits the tumor alone in a dose-dependent manner, showing no damage to animal health. The effect of albolabrin on metastasis was evaluated using a B16-BL6 melanoma line and compared with data from the synthetic RGD peptides. Albolabrin was 2000-fold more effective on the mouse melanoma cells. In addition to its inhibitory effect on platelets, salmosin, a disintegrin isolated

Table 1: Antitumor activity of snake venoms and isolated compounds.

	Protein name	Snakes	Cellular target/mechanism	Reference
Phospholipase A_2 (PLA_2)	Nigexine	*Naja nigricollis nigricollis*	Cytotoxic, altered cell viability and prevented cell proliferation.	[104]
	BthA-I-PLA_2	*Bothrops jararacussu*	Effect against breast adenocarcinoma, human leukemia T, and Ehrlich ascitic tumor	[97]
	rSSBPLA_2	*Lapemis hardwickii* (sea snake)	Antitumor effect	[114]
	PLB	*Pseudechis colletti*	Cytotoxicity	[113]
	PLA_2	*Agkistrodon piscivorus piscivorus*	Synergistic effects with antineoplastic drugs against S49 lymphoma cells	[98]
	BPB-BthTX-I	*B. jararacussu*	Cytotoxicity on S180 tumor cells	[189]
	CC-PLA2-1 and 2	*Cerastes cerastes*	Antitumor and antiangiogenic activities	[101]
	Drs-PLA_2	*Daboia russelii siamensis*	Inhibition of SK-MEL-28 cell migration and inhibition of the colonization of B16F10 cells in lungs	[100]
	MVL-PLA2	*Macrovipera lebetina*	Inhibits angiogenesis and induces changes in actin cytoskeleton	[56, 102]
L-Aminoacid oxidases (LAAOs)	LAAO	*Ophiophagus hannah*	Cytotoxicity in stomach cancer, murine melanoma, fibrosarcoma, and colorectal and ovary cell lines	[79]
	LAAO	*Agkistrodon halys*	Apoptosis	[71]
	Apoxin-I	*Crotalus atrox*	Apoptosis	[75]
	LAAO	*Eristicophis macmahoni*	Apoptosis	[68]
	BmooLAAO-I	*Bothrops moojeni*	Cytotoxicity and apoptosis	[83]
	LAAO	*Trimeresurus stejnegeri*	Cytotoxicity	[76]
	LAAO	*Trimeresurus flavoviridis*	Antitumor activity	[87]
	AHP-LAAO	*Agkistrodon halys pallas*	Apoptosis	[190]
	LAAO	*Vipera berus berus*	Apoptosis	[77]
	ACTX-6	*Agkistrodon acutus*	Induces apoptosis in human cervical cancer Hela cell line	[191]
	Bl-LAAO	*Bothrops leucurus*	Cytotoxicity in the stomach cancer MKN-45, adenocarcinoma HUTU, colorectal RKO, and human fibroblast LL-24 cell lines.	[192]
Metalloproteases	Crovidisin	*Crotalus viridis*	Detachment of ROS 17/2.8 osteosarcoma cells.	[147]
	Jararhagin	*Bothrops jararaca*	Inhibition of melanoma cells and proapoptotic effect selective, interfering with the adhesion mechanisms	[136]
	leucurolysin-B	*Bothrops leucurus*	Potent cytotoxic effect in a micromolar range against T98, U87 and RT2, MCF7, EAC, and UACC cancer cell lines	[193]
	TSV-DM	*Trimeresurus stejnegeri*	Apoptosis, inhibitor of cell proliferation and inducer cell morphologic changes.	[194]
Disintegrin	Contortrostatin	*Agkistrodon contortrix contortrix*	Anti-invasive and antiadhesive activities on tumor cells and endothelial cells. Antitumor, antiangiogenic activities and inhibitor of tumor growth.	[171–175]
	Leucurogin	*Bothrops leucurus*	Anti-angiogenesis effect	[195]
	Saxatilin	*Gloydius saxatilis*	Inhibitor of tumor growth.	[181]
	Obtustatin	*Vipera lebetina*	Anti-angiogenesis effect	[165]
	Adinbitor	*Agkistrodon halys stejneger*	Inhibits angiogenesis (*in vitro* and *in vivo*)	[196]
	Albolabrin	*Trimeresurus albolabris*	Inhibits RGD-dependent integrins and metastasis	[169]
	Rhodocetin	*Calloselasma rhodostoma*	Inhibits the cell adhesion, migration, and collagen contraction	[197]
	Salmosin	*Agkistrodon halys brevicaudus*	Antiangiogenic and antitumorigenic	[88, 179, 180, 185]
	Trigramin	*Trimeresurus gramineus*	Inhibits the adhesion melanoma cells to fibronectin and fibrinogen.	[164, 166]
	Triflavin	*Trimeresurus flavoviridis*	Inhibits adhesion and migration cell and angiogenesis.	[168]
	Rhodostomin	*Agkistrodon rhodostoma*	Inhibits angiogenesis and grow tumor cell adhesion.	[163, 164, 177]
	Echistatin	*Echis carinatus*	Inhibits the adhesion of melanoma cells to extracellular matrix components	[170]

Table 1: Continued.

	Protein name	Snakes	Cellular target/mechanism	Reference
Serineproteases	Crotalase	*Crotalus adamanteus*	Inhibition of tumor growth	[153]
	Batroxobin	*B. moojeni*	Antimetastatic effect	[183]
Lectins	BjcuL	*B. jararacussu*	Cytotoxic effects and inhibits cell adhesion	[117]
	Lebectin and Lebecetin	*Macrovipera lebetina*	Inhibits adhesion, migration, and invasion of tumor cells; inhibits angiogenesis	[119]
	EM16	*Echis multisquamatus*	Cytoskeleton disassembly; inhibits adhesion and migration of HUVEC cells	[198]
Peptides	Cardiotoxin III (CTX III)	*Naja naja atra*	Blocks migration and invasion of MDA-MB-231 breast cancer cells	[42, 108]
	Cytotoxin P4	*Naja n. nigricollis*	Cytotoxicity	[40, 47, 48]
	Cathelicidin-BF	*Bungarus fasciatus*	Inhibits B16F10 and B16 proliferation	[199]
Inhibitors	BJ46a	*B. jararaca*	Inhibits the invasion and metastasis of tumor cells B16F10, a melanoma cell line, and MHCC97H, a human hepatocellular carcinoma cell line	[200]
	PIVL	*Macrovipera lebetina transmediterranea*	Serine protease inhibitor; exhibits an anti-tumor effect and displays integrin inhibitory activity without being cytotoxic. Inhibit the adhesion, migration, and invasion of human glioblastoma U87 cells.	[201]
Crude	WEV—venom extracted	*Montivipera xanthina*	Cytotoxic effect	[202]
		Walterinnesia aegyptia	Induction of apoptosis	[203]
		Vipera lebetina turanica	Inhibits the growth of human ovarian cancer through induction of apoptosis	[204]

from *Agkistrodon halys brevicaudus* venom, acts on tumor growth through an antiangiogenic mechanism [88]. Some studies have reported salmosin activity on melanoma cell metastasis and proliferation. Kang et al. [180] and Chung et al. [181] investigated the action of salmosin on integrin receptors, which are mediators of human tumor cell proliferation. Salmosin significantly inhibited the proliferation of human melanoma cells (SK-Mel-2 and B16 cells) in a dose-dependent manner. These authors also observed that salmosin inhibits the adhesion process via two possible mechanisms: (i) it specifically blocks the adhesion of cells to the ECM, via $\alpha v\beta 3$ or $\beta 1$ or (ii) via induction of apoptosis, also blocking the αv receptor. A novel disintegrin purified from *Gloydius saxatilis* snake venom, named saxatilin, was able to strongly inhibit tumor growth and may be an alternative procedure for antiangiogenic cancer therapy [182].

In 2009, Colombistatin, a disintegrin that inhibited ADP-induced platelet aggregation, human urinary (T24) and skin melanoma (SK-Mel-28) cancer cell adhesion to fibronectin, and cell migration was isolated from the venom of *Bothrops colombiensis* [183].

Thus, along with metalloproteinases, disintegrins from snake venoms have shown a high potential for treatment against cancer.

9. Snake Venom Serineproteases

Serineproteases are enzymes found in microorganisms, plants, and many animals. These enzymes display several biological functions and may be involved with digestion, activation of the complement system, cell differentiation, hemostasis, and others. Serineproteases affect several steps of blood clotting, often not specifically, inactivating blood clotting factors involved with platelet aggregation, clotting, and fibrinolysis [184–186]. The antitumoral potential of these proteins is still not well explored, and there are few studies published on their effects and mechanisms of action.

Markland [153] evaluated the *in vitro* and *in vivo* effects of crotalase, a serine protease from *Crotalus adamanteus*, on the growth of B16 melanoma cells in C57BL/6 mice and concluded that the enzyme was able to inhibit the growth of B16 melanoma cells *in vitro*, did not show cytotoxic or cytostatic effects on cells *in vivo*, and did not significantly increase the survival time of the animals.

The effect of defibrinogenation of batroxobin, a thrombin-like enzyme found in *Bothrops atrox moojeni*, presently *Bothrops moojeni* snake venoms, was studied on artificial lung metastasis in mice. The role of natural killer (NK) cells in the inhibitory effects of defibrinogenation on metastasis was also investigated. The administration of batroxobin had no effect at all on spleen lymphocyte NK activity. These results indicated that defibrinogenation due to batroxobin inhibits lung metastasis, and these effects depend on the NK activity of the host [187].

SVSPs have shown great potential for therapeutic and diagnostic use of coagulant disorders, which has generated a positive outlook regarding their study, even though few studies have been published to date.

10. Concluding Remarks

An uncontrolled rush toward the development of cytotoxic agents, selective for tumor cells, has already started. Several

classes of drugs were produced, as happened in the seventies with platinum derivatives able to inhibit the growth of bacterium cultures. In the sixties, we find the first reports of cytotoxic activity of snake venom PLA_2s on Yoshida sarcoma cell cultures [188]. So far, the association of animal venoms with important tissue damages in ophidian accidents is known and the investigation of active fractions for clinical use started to stand out in the scientific literature.

Nowadays, some works have been published with emphasis on the evaluation of specific points of tumor metabolism, like its immunological aspects and the induction of apoptosis. Cellular proliferation is not the only event to be fought in cancer treatment; the capacity of the tumor to invade adjacent tissues and to create new blood vessels can also be used as targets for new treatments. These relationships between cells and components of the extracellular matrix are fundamental in the events that occur in cancer for the invasion of tumor cells and also for the mechanisms of angiogenesis.

In this review, we focused on the new paradigms of both cytotoxic and pharmacological effects of snake venom toxins. A number of *in vivo* and *in vitro* experiments have demonstrated that snake venoms can contribute to the development of new drugs to combat a number of diseases including cancer. Moreover, from 1980 to the 1990s, snake venom peptides isolated from Viperidae and Crotalidae species and containing RGD/ECD sequences in their structures proved to be invaluable tools to recognize specific structures/receptors of platelets and some kinds of cells, as well as to promote physiological and biochemical changes on a cellular level. To date, these compounds are also complements for new therapeutic strategies in mutagenesis-related diseases.

Disintegrins interact with integrins via glycoprotein receptors located on cellular surfaces, which are related to cell-cell and cell-matrix interactions. These complex mechanisms have led to several studies on the elucidation of events or factors that may affect focal adhesion in cancer. Enthusiastic studies using these promising "Leads" or templates have demonstrated that peptides containing the RGD sequence from several snake venom species have the ability either to inhibit angiogenic activity via subtypes of $\alpha\beta$ receptors or to exhibit selective apoptotic effects. These observations were essential according to a wide variety of pharmacological targets that make snake venom toxins invaluable sources of binders for studying new drugs and a considerable number of inhibitors [140, 189].

Abbreviations

svMP: Snake venom metalloproteases
svSP: Snake venom serineproteases
$svPLA_2s$: Snake venom phospholipases A_2
svLAAO: Snake venom L-amino acid oxidases
svCTL: Snake venom C-type lectin-like
CVF: Cobra venom factor.

Conflict of Interests

The authors state that there is no conflict of interests.

Acknowledgments

The authors are grateful to the Ministry of Science and Technology (MCTI), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), Fundação de Tecnologia do Acre (FUNTAC/FDCT), Coordenação de Aperfeiçoamento de Nível Superior (CAPES)-Projeto NanoBiotec, Rede de Biodiversidade e Biotecnologia da Amazônia Legal (BIONORTE/CNPq/MCTI), Instituto Nacional para Pesquisa Translacional em Saúde e Ambiente na Região Amazônica (INCT-INPeTAm/CNPq/MCTI) e Instituto Nacional para Pesquisa em Toxinas (INCT-Tox), Secretary of Development of Rondonia State (SEPLAN/PRONEX/CNPq) for financial support.

References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] N. Hidetomo, J. R. David, G. L. D. David et al., "Can anesthetic techniques or drugs affect cancer recurrence in patients undergoing cancer surgery?" *Journal of Anesthesia*, vol. 27, no. 5, pp. 731–741, 2013.

[3] F. Bray, J. S. Ren, E. Masuyer, and J. Ferlay, "Global estimates of cancer prevalence for 27 sites in the adult population in 2008," *Journal of Cancer*, vol. 132, pp. 1133–1145, 2013.

[4] F. de Vita, M. Orditura, E. Martinelli, L. Moraca, A. de Chiara, and G. Catalano, "The new european gold standard treatment for rectum cancer," *Tumori*, vol. 86, pp. S26–S29, 2000.

[5] T. Boveri, "Concerning the origin of malignant tumours by Theodor Boveri. Translated and annotated by Henry Harris," *Journal of Cell Science*, vol. 121, pp. 1–84, 2008.

[6] L. A. Garraway and E. S. Lander, "Lessons from the cancer genome," *Cell*, vol. 153, pp. 117–137, 2013.

[7] H. L. Mcleod, "Cancer pharmacogenomics: early promise, but concerted effort needed," *Science*, vol. 339, no. 6127, pp. 1563–1566, 2013.

[8] N. Jiang, X. Wang, Y. Yang, and W. Dai, "Advances in mitotic inhibitors for cancer treatment," *Mini-Reviews in Medicinal Chemistry*, vol. 6, no. 8, pp. 885–895, 2006.

[9] L. Brannon-Peppas and J. O. Blanchette, "Nanoparticles and systems, target for cancer therapy," *Advanced Drug Delivery Reviews*, vol. 64, pp. 206–212, 2012.

[10] S. Kamath and J. K. Buolamwini, "Targeting EGFR and HER-2 receptor tyrosine kinases for cancer drug discovery and development," *Medicinal Research Reviews*, vol. 26, no. 5, pp. 569–594, 2006.

[11] M. C. Perry, C. M. Anderson, and R. C. Donebwer, "Chemotherapy," in *Clinical Oncology*, M. D. Abeloff, J. O. Armitage, A. S. Lichter, and J. E. Niederbuber, Eds., pp. 379–422, Churclhill-Livingstone, Philadelphia, Pa, USA, 3rd edition, 2000.

[12] I. Adkins, H. Jana, M. Kosova, and L. Sadilkova, "Bacteria and their toxins tamed is immunotherapy," *Current Pharmaceutical Biotechnologyp*, vol. 13, pp. 1446–1473, 2012.

[13] R. J. Lewis and M. L. Garcia, "Therapeutic potential of venom peptides," *Nature Reviews Drug Discovery*, vol. 2, no. 10, pp. 790–802, 2003.

[14] M. F. Fernandes-Pedrosa, J. Félix-Silva, and Y. A. S. Menezes, "Toxins from venomous animals: gene cloning, protein expression and biotechnological applications," in *An Integrated View of the Molecular Recognition and Toxinology: From Analytical Procedures to Biomedical Applications*, InTech, 2013.

[15] R. L. Warner, S. D. McClintock, A. G. Barron, and F. de la Iglesia, "Hemostatic properties of a venomic protein in rodent dermal injuries," *Experimental and Molecular Pathology*, vol. 83, no. 2, pp. 241–248, 2007.

[16] R. L. Warner, S. D. McClintock, A. G. Barron, and F. A. de la Iglesia, "Hemostatic properties of a venomic protein in rat organ trauma," *Experimental and Molecular Pathology*, vol. 87, no. 3, pp. 204–211, 2009.

[17] D. Fenard, G. Lambeau, E. Valentin, J. Lefebvre, M. Lazdunski, and A. Doglio, "Secreted phospholipases A_2, a new class of HIV inhibitors that block virus entry into host cells," *The Journal of Clinical Investigation*, vol. 104, no. 5, pp. 611–618, 1999.

[18] P. Escoubas and G. F. King, "Venomics as a drug discovery platform," *Expert Review of Proteomics*, vol. 6, no. 3, pp. 221–224, 2009.

[19] Y. Fu, N. Na, K. Li et al., "Chlorotoxin-conjugated nanoparticles as potential glioma-targeted drugs," *Journal of Neuro-Oncology*, vol. 107, pp. 457–462, 2012.

[20] I. Vetter and R. J. Lewis, "Therapeutic potential of cone snail venom peptides (conopeptides)," *Current Topics in Medicinal Chemistry*, vol. 12, pp. 1546–1552, 2012.

[21] A. Bond, "Exenatide (Byetta) as a novel treatment option for type 2 diabetes mellitus," *Proceedings (Baylor University. Medical Center)*, vol. 19, no. 3, pp. 281–284, 2006.

[22] L. R. Aramadhaka, A. Prorock, B. Dragulev et al., "Connectivity maps for biosimilar drug discovery in venoms: the case of Gila Monster Venom and the anti-diabetes drug Byetta," *Toxicon*, vol. 69, pp. 160–167, 2013.

[23] V. K. Vyas, K. Brahmbhatt, H. Bhatt et al., "Therapeutic potential of snake venom in cancer therapy: current perspectives," *Asian Pacific Journal Tropical of Biomedicine*, vol. 3, no. 2, pp. 156–162, 2013.

[24] C. Y. Koh and R. M. Kini, "From snake venom toxins to therapeutics: cardiovascular examples," *Toxicon*, vol. 59, no. 4, pp. 497–506, 2012.

[25] R. P. Samy, P. Gopalakrishnakone, B. G. Stiles et al., "Snake venom phospholipases A_2: a novel tool against bacterial diseases," *Current Medicinal Chemistry*, vol. 19, no. 36, pp. 6150–6162, 2012.

[26] Y. Wen, W. U. Bao-Jueng, K. Pei-Hsiu et al., "Antibacterial and membrane-damaging activities of b-bungarotoxin B chain," *Journal of Peptide Science*, vol. 19, pp. 1–8, 2013.

[27] S. Hubbard, S. Choudhary, E. Maus et al., "Contortrostatin, a homodimeric disintegrin isolated from snake venom inhibits herpes simplex virus entry and cell fusion," *Antiviral Therapy*, vol. 17, no. 7, pp. 1319–1326, 2012.

[28] V. D. M. Muller, R. R. Russo, A. C. Cintra et al., "Crotoxin and phospholipases A_2 from *Crotalus durissus* terrificus showed antiviral activity against dengue and yellow fever viruses," *Toxicon*, vol. 59, no. 4, pp. 507–515, 2012.

[29] L. F. D. Passero, T. Y. Tomokane, C. E. P. Corbett, M. D. Laurenti, and M. H. Toyama, "Comparative studies of the anti-leishmanial activity of three Crotalus durissus ssp. venoms," *Parasitology Research*, vol. 101, no. 5, pp. 1365–1371, 2007.

[30] J. C. Castillo, L. J. Vargas, C. Segura et al., "*In vitro* antiplasmodial activity of phospholipases A_2 and a phospholipase homologue isolated from the venom of the snake *Bothrops asper*," *Toxins*, vol. 4, no. 12, pp. 1500–1516, 2012.

[31] E. S. Yamane, C. B. A. Fernando, E. B. Oliveira et al., "Unraveling the antifungal activity of a South American rattlesnake toxin crotamine," *Biochimie*, vol. 95, pp. 231–240, 2013.

[32] J. J. Calvete, P. Juárez, and L. Sanz, "Snake venomics. Strategy and applications," *Journal of Mass Spectrometry*, vol. 42, no. 11, pp. 1405–1414, 2007.

[33] W. D. DeWys, H. C. Kwaan, and S. Bathina, "Effect of defibrination on tumor growth and response to chemotherapy," *Cancer Research*, vol. 36, no. 10, pp. 3584–3587, 1976.

[34] B. M. Braganca, "Biologically active components of cobra venom in relation to cancer research," *Indian Journal of Medical Research*, vol. 64, no. 8, pp. 1197–1207, 1976.

[35] B. M. Braganca and A. V. Hospattankar, "Potentiating action of cobra venom cytotoxin on the antitumour effects of an alkylating agent (melphalan)," *European Journal of Cancer and Clinical Oncology*, vol. 14, no. 6, pp. 707–712, 1978.

[36] N. Kaneda, M. Hamaguchi, and K. Kojima, "Action of cobra venom cardiotoxin on chick embryonal fibroblasts transformed with a temperature-sensitive mutant of Rous sarcoma virus," *FEBS Letters*, vol. 192, no. 2, pp. 313–316, 1985.

[37] A. Chiam-Matyas and M. Ovadia, "Cytotoxic activity of various snake venoms on melanoma, B16F10 and chondrosarcoma," *Life Sciences*, vol. 40, no. 16, pp. 1601–1607, 1987.

[38] G. C. Y. Chiou and M. K. Martin, "Chemotherapy of neuroblastoma in mice with anticancer agents," *Journal of Pharmaceutical Sciences*, vol. 69, no. 5, pp. 592–594, 1980.

[39] E. Kato and T. Narahashi, "Low sensitivity of the neuroblastoma cell cholinergic receptors to erabutoxins and α-bungarotoxin," *Brain Research*, vol. 245, no. 1, pp. 159–162, 1982.

[40] G. Borkow, A. Chaim-Matyas, and M. Ovadia, "Binding of cytotoxin P4 from Naja nigricollis nigricollis to B16F10 melanoma and WEHI-3B leukemia cells," *FEMS Microbiology Immunology*, vol. 5, no. 1–3, pp. 139–146, 1992.

[41] M. Trikha, Y. A. de Clerck, and F. S. Markland, "Contortrostatin, a snake venom disintegrin, inhibits β1 integrin- mediated human metastatic melanoma cell adhesion and blocks experimental metastasis," *Cancer Research*, vol. 54, no. 18, pp. 4993–4998, 1994.

[42] S. Yang, M. Lu, C. Chien et al., "Induction of apoptosis in human leukemia K562 cells by cardiotoxin III," *Life Sciences*, vol. 76, no. 21, pp. 2513–2522, 2005.

[43] R. Jokhio and A. F. Ansari, "Cobra snake venom reduces significantly tissue nucleic acid levels in human breast cancer," *Journal of the Pakistan Medical Association*, vol. 55, no. 2, pp. 71–73, 2005.

[44] H. Juhl, E. C. Petrella, N.-K. V. Cheung, R. Bredehorst, and C.-W. Vogel, "Complement killing of human neuroblastoma cells: a cytotoxic monoclonal antibody and its F(ab)'2-cobra venom factor conjugate are equally cytotoxic," *Molecular Immunology*, vol. 27, no. 10, pp. 957–964, 1990.

[45] H. Juhl, E. C. Petrella, N. V. Cheung, R. Bredehorst, and C. Vogel, "Additive cytotoxicity of different monoclonal antibody-cobra venom factor conjugates for human neuroblastoma cells," *Immunobiology*, vol. 197, no. 5, pp. 444–459, 1997.

[46] X. Wang and S. Huang, "The selective cytotoxicity of cobra venom factor immunoconjugate on cultured human nasopharyngeal carcinoma cell line," *Human and Experimental Toxicology*, vol. 18, no. 2, pp. 71–76, 1999.

[47] A. Chaim-Matyas, G. Borkow, and M. Ovadia, "Isolation and characterization of a cytotoxin P4 from the venom of Naja nigricollis nigricollis preferentially active on tumor cells," *Biochemistry International*, vol. 24, no. 3, pp. 415–421, 1991.

[48] U. Oron, A. Chaim-Matyas, and M. Ovadia, "Histopathological changes in WEHI-3B leukemia cells following intoxication by cytotoxin P4 from *Naja nigricollis nigricollis* venom," *Toxicon*, vol. 30, no. 9, pp. 1122–1126, 1992.

[49] A. V. Feofanov, G. V. Sharonov, M. V. Astapova, D. I. Rodionov, Y. N. Utkin, and A. S. Arseniev, "Cancer cell injury by cytotoxins from cobra venom is mediated through lysosomal damage," *Biochemical Journal*, vol. 390, no. 1, pp. 11–18, 2005.

[50] M. M. de Vieira Santos, R. J. da Silva, M. G. da Silva, and D. Fecchio, "Subpopulations of mononuclear leukocytes associated with inhibition of Ehrlich ascites tumor growth by treatment with Bothrops jararaca venom," *Mediators of Inflammation*, vol. 13, no. 1, pp. 29–32, 2004.

[51] H. Zhang, R. Han, Z. Chen et al., "Opiate and acetylcholine-independent analgesic actions of crotoxin isolated from *Crotalus durissus* terrificus venom," *Toxicon*, vol. 48, no. 2, pp. 175–182, 2006.

[52] R. J. Gould, M. A. Polokoff, P. A. Friedman et al., "Disintegrins: a family of integrin inhibitory proteins from viper venoms (43129B)," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 195, no. 2, pp. 168–171, 1990.

[53] S. Niewiarowski, M. A. McLane, M. Kloczewiak, and G. J. Stewart, "Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors," *Seminars in Hematology*, vol. 31, no. 4, pp. 289–300, 1994.

[54] R.-S. Yang, C.-H. Tang, W. J. Chuang et al., "Inhibition of tumor formation by snake venom disintegrin," *Toxicon*, vol. 45, pp. 661–669, 2005.

[55] M. S. Dennis, W. J. Henzel, R. M. Pitti et al., "Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: evidence for a family of platelet-aggregation inhibitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 7, pp. 2471–2475, 1990.

[56] A. Bazaa, E. Pasquier, C. Defilles et al., "MVL-PLA$_2$, a snake venom phospholipase A$_2$, inhibits angiogenesis through an increase in microtubule dynamics and disorganization of focal adhesions," *PLoS ONE*, vol. 5, no. 4, Article ID e10124, 2010.

[57] S. Chwetzoff, "On the mode of action of basic phospholipase A$_2$ from *Naja nigricollis* venom," *Biochimica et Biophysica Acta*, vol. 1045, no. 3, pp. 285–290, 1990.

[58] R. J. da Silva, M. G. da Silva, L. C. Vilela, and D. Fecchio, "Cytokine profile of Ehrlich ascites tumor treated with *Bothrops jararaca* venom," *Mediators of Inflammation*, vol. 11, no. 4, pp. 197–201, 2002.

[59] M. Paez-Pereda, F. Kuchenbauer, E. Arzt, and G. K. Stalla, "Regulation of pituitary hormones and cell proliferation by components of the extracellular matrix," *Brazilian Journal of Medical and Biological Research*, vol. 38, no. 10, pp. 1487–1494, 2005.

[60] R. S. Rodrigues, J. F. da Silva, J. B. França et al., "Structural and functional properties of Bp-LAAO, a new l-amino acid oxidase isolated from Bothrops pauloensis snake venom," *Biochimie*, vol. 91, no. 4, pp. 490–501, 2008.

[61] X. Du and K. J. Clemetson, "Snake venom L-amino acid oxidases," *Toxicon*, vol. 40, no. 6, pp. 659–665, 2002.

[62] N. H. Tan and M. N. Saifuddin, "Isolation and characterization of an unusual form of L-amino acid oxidase from King cobra (*Ophiophagus hannah)* venom," *Biochemistry International*, vol. 19, no. 4, pp. 937–944, 1989.

[63] N.-H. Tan and S. Swaminathan, "Purification and properties of the L-amino acid oxidase from monocellate cobra (*Naja naja kaouthia*) venom," *International Journal of Biochemistry*, vol. 24, no. 6, pp. 967–973, 1992.

[64] G. Ponnudurai, M. C. M. Chung, and N.-H. Tan, "Purification and properties of the L-amino acid oxidase from Malayan pit viper (*Calloselasma rhodostoma*) venom," *Archives of Biochemistry and Biophysics*, vol. 313, no. 2, pp. 373–378, 1994.

[65] S. Torii, M. Naito, and T. Tsuruo, "Apoxin I, a novel apoptosis-inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom," *The Journal of Biological Chemistry*, vol. 272, no. 14, pp. 9539–9542, 1997.

[66] Y. Abe, Y. Shimoyama, H. Munakata, J. Ito, N. Nagata, and K. Ohtsuki, "Characterization of an apoptosis-inducing factor in Habu snake venom as a glycyrrhizin (GL)-binding protein potently inhibited by GL *in vitro*," *Biological and Pharmaceutical Bulletin*, vol. 21, no. 9, pp. 924–927, 1998.

[67] D. H. F. Souza, L. M. Eugenio, J. E. Fletcher et al., "Isolation and structural characterization of a cytotoxic L-amino acid oxidase from *Agkistrodon contortrix laticinctus* snake venom: preliminary crystallographic data," *Archives of Biochemistry and Biophysics*, vol. 368, no. 2, pp. 285–290, 1999.

[68] S. A. Ali, S. Stoeva, A. Abbasi et al., "Isolation, structural, and functional characterization of an apoptosis-inducing L-amino acid oxidase from leaf-nosed viper (*Eristocophis macmahoni*) snake venom," *Archives of Biochemistry and Biophysics*, vol. 384, no. 2, pp. 216–226, 2000.

[69] R. Iijima, J. Kisugi, and M. Yamazaki, "L-amino acid oxidase activity of an antineoplastic factor of a marine mollusk and its relationship to cytotoxicity," *Developmental and Comparative Immunology*, vol. 27, no. 6-7, pp. 505–512, 2003.

[70] S. D. Aird, "Ophidian envenomation strategies and the role of purines," *Toxicon*, vol. 40, no. 4, pp. 335–393, 2002.

[71] S. Suhr and D. Kim, "Identification of the snake venom substance that induces apoptosis," *Biochemical and Biophysical Research Communications*, vol. 224, no. 1, pp. 134–139, 1996.

[72] R. G. Stábeli, S. Marcussi, G. B. Carlos et al., "Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom," *Bioorganic and Medicinal Chemistry*, vol. 12, no. 11, pp. 2881–2886, 2004.

[73] L. F. M. Izidoro, M. C. Ribeiro, G. R. L. Souza et al., "Biochemical and functional characterization of an l-amino acid oxidase isolated from *Bothrops pirajai* snake venom," *Bioorganic and Medicinal Chemistry*, vol. 14, no. 20, pp. 7034–7043, 2006.

[74] L. D. Campos, M. D. Pucca, E. C. Roncolato et al., "Analysis of phospholipase A$_2$, L-amino acid oxidase, and proteinase enzymatic activities of the *Lachesis muta rhombeata* venom," *Journal of Biochemical and Molecular Toxicology*, vol. 26, no. 8, pp. 308–314, 2012.

[75] S. Torii, M. Naito, and T. Tsuruo, "Apoxin I, a novel apoptosis-inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom," *Biochemistry*, vol. 39, pp. 3197–3205, 2000.

[76] Y. Zhang, J. Wang, W. Lee et al., "Molecular characterization of *Trimeresurus stejnegeri* venom L-amino acid oxidase with potential anti-HIV activity," *Biochemical and Biophysical Research Communications*, vol. 309, no. 3, pp. 598–604, 2003.

[77] M. Samel, H. Vija, G. Rönnholm, J. Siigur, N. Kalkkinen, and E. Siigur, "Isolation and characterization of an apoptotic

and platelet aggregation inhibiting l-amino acid oxidase from *Vipera berus berus* (common viper) venom," *Biochimica et Biophysica Acta*, vol. 1764, no. 4, pp. 707–714, 2006.

[78] S. Araki, T. Ishida, T. Yamamoto, K. Kaji, and H. Hayashi, "Induction of apoptosis by hemorrhagic snake venom in vascular endothelial cells," *Biochemical and Biophysical Research Communications*, vol. 190, no. 1, pp. 148–153, 1993.

[79] M. Y. Ahn, B. M. Lee, and Y. S. Kim, "Characterization and cytotoxicity of L-amino acid oxidase from the venom of king cobra (*Ophiophagus hannah*)," *International Journal of Biochemistry and Cell Biology*, vol. 29, no. 6, pp. 911–919, 1997.

[80] F. S. Markland, "Snake venoms and the hemostatic system," *Toxicon*, vol. 36, no. 12, pp. 1749–1800, 1998.

[81] A. M. Dipietrantonio, T. Hsieh, and J. M. Wu, "Activation of caspase 3 in HL-60 cells exposed to hydrogen peroxide," *Biochemical and Biophysical Research Communications*, vol. 255, no. 2, pp. 477–482, 1999.

[82] T. Suhara, K. Fukuo, T. Sugimoto et al., "Hydrogen peroxide induces up-regulation of Fas in human endothelial cells," *Journal of Immunology*, vol. 160, no. 8, pp. 4042–4047, 1998.

[83] R. G. Stábeli, L. M. Pimenta Magalhães, H. S. Selistre-de-Araujo, and E. B. Oliveira, "Antibodies to a fragment of the *Bothrops moojeni* L-amino acid oxidase cross-react with snake venom components unrelated to the parent protein," *Toxicon*, vol. 46, no. 3, pp. 308–317, 2005.

[84] A. G. Tempone, H. F. Andrade Jr., P. J. Spencer, C. O. Lourenço, J. R. Rogero, and N. Nascimento, "*Bothrops moojeni* venom kills *Leishmania* spp. with hydrogen peroxide generated by its L-amino acid oxidase," *Biochemical and Biophysical Research Communications*, vol. 280, no. 3, pp. 620–624, 2001.

[85] M. M. de Vieira Santos, C. D. Sant'Ana, J. R. Giglio et al., "Antitumoural effect of an L-amino acid oxidase isolated from *Bothrops jararaca* snake venom," *Basic and Clinical Pharmacology and Toxicology*, vol. 102, no. 6, pp. 533–542, 2008.

[86] Y. Sun, E. Nonobe, Y. Kobayashi et al., "Characterization and expression of L-amino acid oxidase of mouse milk," *The Journal of Biological Chemistry*, vol. 277, no. 21, pp. 19080–19086, 2002.

[87] L. Sun, Y. Yoshii, A. Hyodo et al., "Apoptotic effect in the glioma cells induced by specific protein extracted from *Okinawa Habu* (*Trimeresurus flavoviridis*) venom in relation to oxidative stress," *Toxicology in Vitro*, vol. 17, no. 2, pp. 169–177, 2003.

[88] I. Kang, Y. Lee, and D. Kim, "A novel disintegrin salmosin inhibits tumor angiogenesis," *Cancer Research*, vol. 59, no. 15, pp. 3754–3760, 1999.

[89] J. E. Cura, D. P. Blanzaco, C. Brisson et al., "Phase I and pharmacokinetics study of crotoxin (cytotoxic PLA_2, NSC-624244) in patients with advanced cancer," *Clinical Cancer Research*, vol. 8, no. 4, pp. 1033–1041, 2002.

[90] D. Jain and S. Kumar, "Snake venom: a potentanticancer agent," *Asian Pacific Journal of Cancer Prevention*, vol. 13, no. 10, pp. 4855–4860, 2012.

[91] J. Gutiérrez and B. Lomonte, "Phospholipase A_2 myotoxins from *Bothrops* snake venoms," *Toxicon*, vol. 33, no. 11, pp. 1405–1424, 1995.

[92] A. M. Soares and J. R. Giglio, "Chemical modifications of phospholipases A_2 from snake venoms: effects on catalytic and pharmacological properties," *Toxicon*, vol. 42, no. 8, pp. 855–868, 2003.

[93] C. F. P. Teixeira, E. C. T. Landucci, E. Antunes, M. Chacur, and Y. Cury, "Inflammatory effects of snake venom myotoxic phospholipases A_2," *Toxicon*, vol. 42, no. 8, pp. 947–962, 2003.

[94] B. S. Cummings, "Phospholipase A_2 as targets for anti-cancer drugs," *Biochemical Pharmacology*, vol. 74, no. 7, pp. 949–959, 2007.

[95] T. Mashima, H. Seimiya, and T. Tsuruo, "De novo fatty-acid synthesis and related pathways as molecular targets for cancer therapy," *British Journal of Cancer*, vol. 100, no. 9, pp. 1369–1372, 2009.

[96] E. L. Ferguson, S. C. W. Richardson, and R. Duncan, "Studies on the mechanism of action of dextrin-phospholipase A_2 and its suitability for use in combination therapy," *Molecular Pharmaceutics*, vol. 7, no. 2, pp. 510–521, 2010.

[97] P. G. Roberto, S. Kashima, S. Marcussi et al., "Cloning and identification of a complete cDNA coding for a bactericidal and antitumoral acidic phospholipase A_2 from *Bothrops jararacussu* venom," *Protein Journal*, vol. 23, no. 4, pp. 273–285, 2004.

[98] C. Araya and B. Lomonte, "Antitumor effects of cationic synthetic peptides derived from Lys49 phospholipase A_2 homologues of snake venoms," *Cell Biology International*, vol. 31, no. 3, pp. 263–268, 2007.

[99] G. Maity, S. Mandal, A. Chatterjee, and D. Bhattacharyya, "Purification and characterization of a low molecular weight multifunctional cytotoxic phospholipase A_2 from Russell's viper venom," *Journal of Chromatography B*, vol. 845, no. 2, pp. 232–243, 2007.

[100] S. Khunsap, N. Pakmanee, O. Khow et al., "Purification of a phospholipase A_2 from *Daboia russelii siamensis* venom with anticancer effects," *Journal of Venom Research*, vol. 2, pp. 42–51, 2011.

[101] R. Zouari-Kessentini, J. Luis, A. Karray et al., "Two purified and characterized phospholipases A_2 from Cerastes cerastes venom, that inhibit cancerous cell adhesion and migration," *Toxicon*, vol. 53, no. 4, pp. 444–453, 2009.

[102] R. Kessentini-Zouari, J. Jebali, S. Taboubi et al., "CC-PLA2-1 and CC-PLA2-2, two Cerastes cerastes venom-derived phospholipases A_2, inhibit angiogenesis both *in vitro* and *in vivo*," *Laboratory Investigation*, vol. 90, no. 4, pp. 510–519, 2010.

[103] A. Bazaa, J. Luis, N. Srairi-Abid et al., "MVL-PLA2, a phospholipase A_2 from *Macrovipera lebetina transmediterranea* venom, inhibits tumor cells adhesion and migration," *Matrix Biology*, vol. 28, no. 4, pp. 188–193, 2009.

[104] S. Chwetzoff, S. Tsunasawa, F. Sakiyama, and A. Menez, "Nigexine, a phospholipase A_2 from cobra venom with cytotoxic properties not related to esterase activity. Purification, amino acid sequence, and biological properties," *The Journal of Biological Chemistry*, vol. 264, no. 22, pp. 13289–13297, 1989.

[105] L. A. Costa, H. Miles, C. E. Araujo, S. González, and V. G. Villarrubia, "Tumor regression of advanced carcinomas following intra-and/or peri-tumoral inoculation with VRCTC-310 in humans: preliminary report of two cases," *Immunopharmacology and Immunotoxicology*, vol. 20, no. 1, pp. 15–25, 1998.

[106] N. J. Donato, C. A. Martin, M. Perez, R. A. Newman, J. C. Vidal, and M. Etcheverry, "Regulation of epidermal growth factor receptor activity by crotoxin, a snake venom phospholipase A_2 toxin: a novel growth inhibitory mechanism," *Biochemical Pharmacology*, vol. 51, no. 11, pp. 1535–1543, 1996.

[107] R. E. Corin, L. J. Viskatis, J. C. Vidal, and M. A. Etcheverry, "Cytotoxicity of crotoxin on murine erythroleukemia cells *in vitro*," *Investigational New Drugs*, vol. 11, no. 1, pp. 11–15, 1993.

[108] L. A. Costa, M. C. Fornari, V. E. Berardi, H. A. Miles, and R. A. Diez, "*In vivo* effect of snake phospholipase A_2 (crotoxin + cardiotoxin) on serum IL-1α, TNF-α and IL-1ra level in humans," *Immunology Letters*, vol. 75, no. 2, pp. 137–141, 2001.

[109] N. O. Stanchi, D. Arias, P. E. Martino, R. A. Diez, and L. A. Costa, "30-day intravenous administration of VRCTC-310-ONCO in rabbits," *Farmaco*, vol. 57, no. 2, pp. 167–170, 2002.

[110] L. C. Gebrim, S. Marcussi, D. L. Menaldo et al., "Antitumor effects of snake venom chemically modified Lys49 phospholipase A_2-like BthTX-I and a synthetic peptide derived from its C-terminal region," *Biologicals*, vol. 37, no. 4, pp. 222–229, 2009.

[111] C. L. Ownby, H. S. Selistre-de-Araujo, S. P. White, and J. E. Fletcher, "Lysine 49 phospholipase A_2 proteins," *Toxicon*, vol. 37, no. 3, pp. 411–445, 1999.

[112] K. Hanasaki and H. Arita, "Biological and pathological functions of phospholipase A_2 receptor," *Archives of Biochemistry and Biophysics*, vol. 372, no. 2, pp. 215–223, 1999.

[113] A. W. Bernheimer, R. Linder, S. A. Weinstein, and K.-S. Kim, "Isolation and characterization of a phospholipase B from venom of collett's snake, *Pseudechis colletti*," *Toxicon*, vol. 25, no. 5, pp. 547–554, 1987.

[114] Y. Liang, X. Yang, J. Wei et al., "Correlation of antitumor effect of recombinant sea snake basic phospholipase A_2 to its enzymatic activity," *Ai Zheng*, vol. 24, no. 12, pp. 1474–1478, 2005.

[115] I. Camby, I. Salmon, K. Rombaut, J. Pasteels, R. Kiss, and A. Danguy, "Influence of culture media and multidrug resistance on the wheat germ agglutinin (WGA) glycocytochemical expression of two human glioblastoma cell lines," *Anticancer Research*, vol. 16, no. 4 A, pp. 1719–1725, 1996.

[116] M. Pereira-Bittencourt, D. D. Carvalho, A. R. Gagliardi, and D. C. Collins, "The effect of a lectin from the venom of the snake, *Bothrops jararacussu*, on tumor cell proliferation," *Anticancer Research B*, vol. 19, pp. 4023–4025, 1999.

[117] D. D. de Carvalho, S. Schmitmeier, J. C. Novello, and F. S. Markland, "Effect of BJcuL (a lectin from the venom of the snake *Bothrops jararacussu*) on adhesion and growth of tumor and endothelial cells," *Toxicon*, vol. 39, no. 10, pp. 1471–1476, 2001.

[118] S. Sarray, N. Srairi, J. Luis, J. Marvaldi, M. El Ayeb, and N. Marrakchi, "Lebecetin, a C-lectin protein from the venom of *Macrovipera lebetina* that inhibits platelet aggregation and adhesion of cancerous cells," *Haemostasis*, vol. 31, no. 3–6, pp. 177–182, 2001.

[119] S. Sarray, E. Delamarre, J. Marvaldi, M. E. Ayeb, N. Marrakchi, and J. Luis, "Lebectin and lebecetin, two C-type lectins from snake venom, inhibit $\alpha 5\beta 1$ and αv-containing integrins," *Matrix Biology*, vol. 26, no. 4, pp. 306–313, 2007.

[120] E. S. Nunes, M. A. A. Souza, A. F. M. Vaz et al., "Cytotoxic effect and apoptosis induction by *Bothrops leucurus* venom lectin on tumor cell lines," *Toxicon*, vol. 59, no. 7-8, pp. 667–671, 2012.

[121] A. S. Kamiguti, C. R. M. Hay, R. D. G. Theakston, and M. Zuzel, "Insights into the mechanism of haemorrhage caused by snake venom metalloproteinases," *Toxicon*, vol. 34, no. 6, pp. 627–642, 1996.

[122] M. I. Estêvão-Costa, C. R. Diniz, A. Magalhães, F. S. Markland, and E. F. Sanchez, "Action of metalloproteinases mutalysin I and II on several components of the hemostatic and fibrinolytic systems," *Thrombosis Research*, vol. 99, no. 4, pp. 363–376, 2000.

[123] G. D. Loría, A. Rucavado, A. S. Kamiguti et al., "Characterization of "basparin A", a prothrombin-activating metalloproteinase, from the venom of the snake *Bothrops asper* that inhibits platelet aggregation and induces defibrination and thrombosis," *Archives of Biochemistry and Biophysics*, vol. 418, no. 1, pp. 13–24, 2003.

[124] L. Jia, K. Shimokawa, J. B. Bjarnason, and J. W. Fox, "Snake venom metalloproteinaes: structure, function and relationship to the adams family of proteins," *Toxicon*, vol. 34, no. 11-12, pp. 1269–1276, 1996.

[125] T. Matsui, Y. Fujimura, and K. Titani, "Snake venom proteases affecting hemostasis and thrombosis," *Biochimica et Biophysica Acta*, vol. 1477, no. 1-2, pp. 146–156, 2000.

[126] J. M. Gutiérrez and A. Rucavado, "Snake venom metalloproteinases: their role in the pathogenesis of local tissue damage," *Biochimie*, vol. 82, no. 9-10, pp. 841–850, 2000.

[127] J. M. Gutiérrez, "Comprendiendo los venenos de serpientes: 50 Años de investigaciones en América Latina," *Revista de Biologia Tropical*, vol. 50, no. 2, pp. 377–394, 2002.

[128] A. S. Kamiguti, M. Zuzel, and R. D. G. Theakston, "Snake venom metalloproteinases and disintegrins: interactions with cells," *Brazilian Journal of Medical and Biological Research*, vol. 31, no. 7, pp. 853–862, 1998.

[129] J. B. Bjarnason and J. W. Fox, "Hemorrhagic metalloproteinases from snake venoms," *Pharmacology and Therapeutics*, vol. 62, no. 3, pp. 325–372, 1994.

[130] K. J. Clemetson, T. Morita, and K. R. Manjunatha, "Scientific and standardization committee communications: classification and nomenclature of snake venom C-type lectins and related proteins," *Journal of Thrombosis and Haemostasis*, vol. 7, no. 2, p. 360, 2009.

[131] S. Sarray, E. L. Luis, M. Ayeb, and N. Marrakchi, "Snake venom peptides: promising molecules with anti-tumor effects," in *Bioactive Food Peptides in Health and Disease*, B. Hernández-Ledesma and C. C. Hsieh, Eds., InTech, 2013.

[132] R. K. Andrews and M. C. Berndt, "Snake venom modulators of platelet adhesion receptors and their ligands," *Toxicon*, vol. 38, no. 6, pp. 775–791, 2000.

[133] A. S. Kamiguti, A. M. Moura-da-Silva, G. D. Laing et al., "Collagen-induced secretion-dependent phase of platelet aggregation is inhibited by the snake venom metalloproteinase jararhagin," *Biochimica et Biophysica Acta*, vol. 1335, no. 1-2, pp. 209–217, 1997.

[134] A. S. Kamiguti, F. S. Markland, Q. Zhou, G. D. Laing, R. D. G. Theakston, and M. Zuzel, "Proteolytic cleavage of the $\beta 1$ subunit of platelet $\alpha 2\beta 1$ integrin by the metalloproteinase jararhagin compromises collagen-stimulated phosphorylation of pp72(syk)," *The Journal of Biological Chemistry*, vol. 272, no. 51, pp. 32599–32605, 1997.

[135] A. M. Moura-da-Silva, C. Marcinkiewicz, M. Marcinkiewicz, and S. Niewiarowski, "Selective recognition of $\alpha 2\beta 1$ integrin by jararhagin, a metalloproteinase/disintegrin from *Bothrops jararaca* venom," *Thrombosis Research*, vol. 102, no. 2, pp. 153–169, 2001.

[136] M. C. Corrêa, D. A. Maria, A. M. Moura-da-Silva et al., "Inhibition of melanoma cells tumorigenicity by the snake venom toxin jararhagin," *Toxicon*, vol. 40, pp. 739–748, 2002.

[137] E. H. Kraut, J. L. Rojko, R. G. Olsen, and D. L. Tuomari, "Effects of treatment with cobra venom factor on experimentally induced feline leukemia," *American Journal of Veterinary Research*, vol. 48, no. 7, pp. 1063–1066, 1987.

[138] Y. A. DeClerck, "Interactions between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer," *European Journal of Cancer*, vol. 36, no. 10, pp. 1258–1268, 2000.

[139] L. Howard, Y. Zheng, M. Horrocks, R. A. Maciewicz, and C. Blobel, "Catalytic activity of ADAM28," *FEBS Letters*, vol. 498, no. 1, pp. 82–86, 2001.

[140] J. T. Peterson, "Matrix metalloproteinase inhibitor development and the remodeling of drug discovery," *Heart Failure Reviews*, vol. 9, no. 1, pp. 63–79, 2004.

[141] K. Poindexter, N. Nelson, R. F. Dubose, R. A. Black, and D. P. Cerretti, "The identification of seven metalloproteinase-disintegrin (ADAM) genes from genomic libraries," *Gene*, vol. 237, no. 1, pp. 61–70, 1999.

[142] P. Primakoff and D. G. Myles, "The ADAM gene family: surface proteins with adhesion and protease activity," *Trends in Genetics*, vol. 16, no. 2, pp. 83–87, 2000.

[143] B. L. Tang, "ADAMTS: a novel family of extracellular matrix proteases," *International Journal of Biochemistry and Cell Biology*, vol. 33, no. 1, pp. 33–44, 2001.

[144] K. Eto, C. Huet, T. Tarui et al., "Functional classification of ADAMs based on a conserved motif for binding to integrin $\alpha 9\beta 1$. Implications for sperm-egg binding and other cell interactions," *The Journal of Biological Chemistry*, vol. 277, no. 20, pp. 17804–17810, 2002.

[145] J. B. Smith, C. Dangelmaier, and M. Selak, "Identification of 50 kDa snake venom proteins which specifically inhibit platelet adhesion to collagen," *FEBS Letters*, vol. 283, no. 2, pp. 307–310, 1991.

[146] E. F. Sanchez, L. R. Bush, S. Swenson, and F. S. Markland, "Chimeric fibrolase: covalent attachment of an RGD-like peptide to create a potentially more effective thrombolytic agent," *Thrombosis Research*, vol. 87, no. 3, pp. 289–302, 1997.

[147] C. Liu and T. Huang, "Crovidisin, a collagen-binding protein isolated from snake venom of *Crotalus viridis*, prevents platelet-collagen interaction," *Archives of Biochemistry and Biophysics*, vol. 337, no. 2, pp. 291–299, 1997.

[148] S. Swenson, L. R. Bush, and F. S. Markland Jr., "Chimeric derivative of fibrolase, a fibrinolytic enzyme from southern copperhead venom, possesses inhibitory activity on platelet aggregation," *Archives of Biochemistry and Biophysics*, vol. 384, no. 2, pp. 227–237, 2000.

[149] C. Tang, R. Yang, C. Liu, T. Huang, and W. Fu, "Differential susceptibility of osteosarcoma cells and primary osteoblasts to cell detachment caused by snake venom metalloproteinase protein," *Toxicon*, vol. 43, no. 1, pp. 11–20, 2004.

[150] R. J. Gould, M. A. Polokoff, P. A. Friedman et al., "Disintegrins: a family of integrin inhibitory proteins from viper venoms (43129B)," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 195, no. 2, pp. 168–171, 1990.

[151] S. Niewiarowski, M. A. McLane, M. Kloczewiak, and G. J. Stewart, "Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors," *Seminars in Hematology*, vol. 31, no. 4, pp. 289–300, 1994.

[152] S. A. Brooks, H. J. Lomax-Browne, T. M. Carter, C. E. Kinch, and D. M. S. Hall, "Molecular interactions in cancer cell metastasis," *Acta Histochemica*, vol. 112, no. 1, pp. 3–25, 2010.

[153] F. S. Markland Jr., "Antitumor action of crotalase, a defibrinogenating snake venom enzyme," *Seminars in Thrombosis and Hemostasis*, vol. 12, no. 4, pp. 284–290, 1986.

[154] H. S. Selistre-de-Araujo, C. L. S. Pontes, C. F. Montenegro, and A. C. B. M. Martin, "Snake venom disintegrins and cell migration," *Toxins*, vol. 2, no. 11, pp. 2606–2621, 2010.

[155] M. S. Dennis, W. J. Henzel, R. M. Pitti et al., "Platelet glycoprotein IIb-IIIa protein antagonists from snake venoms: evidence for a family of platelet-aggregation inhibitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 7, pp. 2471–2475, 1990.

[156] Z.-R. Gan, R. J. Gould, J. W. Jacobs, P. A. Friedman, and M. A. Polokoff, "Echistatin. A potent platelet aggregation inhibitor from the venom of the viper, *Echis carinatus*," *The Journal of Biological Chemistry*, vol. 263, no. 36, pp. 19827–19832, 1988.

[157] T. F. Huang and C. Ouyang, "Action mechanism of the potent platelet aggregation inhibitor from *Trimeresurus gramineus* snake venom," *Thrombosis Research*, vol. 33, no. 2, pp. 125–138, 1984.

[158] T.-F. Huang, J. C. Holt, H. Lukasiewicz, and S. Niewiarowski, "Trigramin: a low molecular weight peptide inhibiting fibrinogen interaction with platelet receptors expressed on glycoprotein IIb-IIIa complex," *The Journal of Biological Chemistry*, vol. 262, no. 33, pp. 16157–16163, 1987.

[159] M. P. Neeper and M. A. Jacobson, "Sequence of a cDNA encoding the platelet aggregation inhibitor trigramin," *Nucleic Acids Research*, vol. 18, no. 14, p. 4255, 1990.

[160] C. Ouyang and T. F. Huang, "Potent platelet aggregation inhibitor from *Trimeresurus gramineus* snake venom," *Biochimica et Biophysica Acta*, vol. 757, no. 3, pp. 332–341, 1983.

[161] C. Ouyang, H. Yeh, and T. F. Haung, "A potent platelet aggregation inhibitor purified from *Agkistrodon halys* (mamushi) snake venom," *Toxicon*, vol. 21, no. 6, pp. 797–804, 1983.

[162] F. S. Markland Jr., K. Shieh, Q. Zhou et al., "A novel snake venom disintegrin that inhibits human ovarian cancer dissemination and angiogenesis in an orthotopic nude mouse model," *Haemostasis*, vol. 31, no. 3–6, pp. 183–191, 2001.

[163] E. H. J. Danen, C. Marcinkiewicz, I. M. H. A. Cornelissen et al., "The disintegrin eristostatin interferes with integrin $\alpha 4\beta 1$ function and with experimental metastasis of human melanoma cells," *Experimental Cell Research*, vol. 238, no. 1, pp. 188–196, 1998.

[164] H.-S. Chiang, R.-S. Yang, and T.-F. Huang, "The Arg-Gly-Asp-containing peptide, rhodostomin, inhibits *in vitro* cell adhesion to extracellular matrices and platelet aggregation caused by Saos-2 human osteosarcoma cells," *British Journal of Cancer*, vol. 71, no. 2, pp. 265–270, 1995.

[165] M. W. Swaim, H.-S. Chiang, and T.-F. Huang, "Characterization of platelet aggregation induced by PC-3 human prostate adenocarcinoma cells and inhibited by venom peptides, trigramin and rhodostomin," *European Journal of Cancer*, vol. 32, no. 4, pp. 715–721, 1996.

[166] C. Marcinkiewicz, P. H. Weinreb, J. J. Calvete et al., "Obtustatin: a potent selective inhibitor of $\alpha 1\beta 1$ integrin *in vitro* and angiogenesis *in vivo*," *Cancer Research*, vol. 63, no. 9, pp. 2020–2023, 2003.

[167] K. A. Knudsen, G. P. Tuszynski, T.-F. Huang, and S. Niewiarowski, "Trigramin, an RGD-containing peptide from snake venom, inhibits cell-substratum adhesion of human melanoma cells," *Experimental Cell Research*, vol. 179, no. 1, pp. 42–49, 1988.

[168] S. I. Kim, K. S. Kim, H. S. Kim et al., "Inhibition of angiogenesis by salmosin expressed *in vitro*," *Oncology Research*, vol. 14, no. 4-5, pp. 227–233, 2003.

[169] J. R. Sheu, C. H. Lin, and T. F. Huang, "Triflavin, an antiplatelet peptide, inhibits tumor cell-extracellular matrix adhesion through an arginine-glycine-aspartic acid-dependent mechanism," *Journal of Laboratory and Clinical Medicine*, vol. 123, no. 2, pp. 256–263, 1994.

[170] T. Soszka, K. A. Knudsen, L. Beviglia, C. Rossi, A. Poggi, and S. Niewiarowski, "Inhibition of murine melanoma cell-matrix adhesion and experimental metastasis by albolabrin, an RGD-containing peptide isolated from the venom of *Trimeresurus*

albolabris," *Experimental Cell Research*, vol. 196, no. 1, pp. 6–12, 1991.

[171] N. Staiano, G. R. D. Villani, E. Di Martino, C. Squillacioti, P. Vuotto, and P. Di Natale, "Echistatin inhibits the adhesion of murine melanoma cells to extracellular matrix components," *Biochemistry and Molecular Biology International*, vol. 35, no. 1, pp. 11–19, 1995.

[172] S. Schmitmeier, F. S. Markland Jr., and T. C. Chen, "Anti-invasive effect of contortrostatin, a snake venom disintegrin, and TNF-α on malignant glioma cells," *Anticancer Research*, vol. 20, no. 6 B, pp. 4227–4233, 2000.

[173] S. Schmitmeier, F. S. Markland, A. H. Schönthal, and T. C. Chen, "Potent mimicry of fibronectin-induced intracellular signaling in glioma cells by the homodimeric snake venom disintegrin contortrostatin," *Neurosurgery*, vol. 57, no. 1, pp. 141–153, 2005.

[174] V. Golubkov, D. Hawes, and F. S. Markland Jr., "Anti-angiogenic activity of contortrostatin, a disintegrin from *Agkistrodon contortrix contortrix* snake venom," *Angiogenesis*, vol. 6, no. 3, pp. 213–224, 2003.

[175] Q. Zhou, M. T. Nakada, C. Arnold, K. Y. Shieh, and F. S. Markland Jr., "Contortrostatin, a dimeric disintegrin from *Agkistrodon contortrix contortrix*, inhibits angiogenesis," *Angiogenesis*, vol. 3, no. 3, pp. 259–269, 1999.

[176] S. Swenson, F. Costa, R. Minea et al., "Intravenous liposomal delivery of the snake venom disintegrin contortrostatin limits breast cancer progression," *Molecular Cancer Therapeutics*, vol. 3, no. 4, pp. 499–511, 2004.

[177] I. Radu, A. Nicola, D. Micu, and D. Minea, "A rare case of glioblastoma multiforme," *Romanian Journal of Morphology and Embryology*, vol. 46, no. 2, pp. 109–112, 2005.

[178] R. Yang and T. Huang, "Rhodostomin inhibits the transforming growth factor-β1-enhanced adhesion activity of ROS 17/2.8 osteosarcoma cells," *Tohoku Journal of Experimental Medicine*, vol. 191, no. 3, pp. 145–155, 2000.

[179] T. F. Huang, C. H. Yeh, and W. B. Wu, "Viper venom components affecting angiogenesis," *Haemostasis*, vol. 31, no. 3–6, pp. 192–206, 2001.

[180] I. Kang, D. Kim, Y. Jang, and K. Chung, "Suppressive mechanism of salmosin, a novel disintegrin in B16 melanoma cell metastasis," *Biochemical and Biophysical Research Communications*, vol. 275, no. 1, pp. 169–173, 2000.

[181] K. Chung, S. Kim, K. Han et al., "Inhibitory effect of salmosin, a Korean snake venom-derived disintegrin, on the integrin αv-mediated proliferation of SK-Mel-2 human melanoma cells," *Journal of Pharmacy and Pharmacology*, vol. 55, no. 11, pp. 1577–1582, 2003.

[182] K. S. Kim, D. S. Kim, K. H. Chung, and Y. S. Park, "Inhibition of angiogenesis and tumor progression by hydrodynamic cotransfection of angiostatin K1-3, endostatin, and saxatilin genes," *Cancer Gene Therapy*, vol. 13, no. 6, pp. 563–571, 2006.

[183] E. E. Sánchez, A. Rodríguez-Acosta, R. Palomar et al., "Colombistatin: a disintegrin isolated from the venom of the South American snake (*Bothrops colombiensis*) that effectively inhibits platelet aggregation and SK-Mel-28 cell adhesion," *Archives of Toxicology*, vol. 83, no. 3, pp. 271–279, 2009.

[184] S. Braud, C. Bon, and A. Wisner, "Snake venom proteins acting on hemostasis," *Biochimie*, vol. 82, no. 9-10, pp. 851–859, 2000.

[185] C. M. White, "Thrombin-directed inhibitors: pharmacology and clinical use," *American Heart Journal*, vol. 149, pp. S54–S60, 2005.

[186] J. D. O. Costa, K. C. Fonseca, M. S. Garrote-Filho et al., "Structural and functional comparison of proteolytic enzymes from plant latex and snake venoms," *Biochimie*, vol. 92, no. 12, pp. 1760–1765, 2010.

[187] M. Shibuya, H. Niitani, A. Aoyama et al., "Antimetastatic effect of defibrinogenation with batroxobin depends on the natural killer activity of host in mice," *Journal of Cancer Research and Clinical Oncology*, vol. 116, no. 2, pp. 168–172, 1990.

[188] B. M. Yoshida, N. T. Patel, and P. G. Badrinath, "Isolation and properties of a cobravenom factor selectively cytotoxic to yoshida sarcoma cells," *Biochimica et Biophysica Acta*, vol. 136, no. 3, pp. 508–520, 1967.

[189] A. R. Kennedy, "Chemopreventive agents: protease inhibitors," *Pharmacology and Therapeutics*, vol. 78, no. 3, pp. 167–209, 1998.

[190] L. C. Gebrim, S. Marcussi, D. L. Menaldo et al., "Antitumor effects of snake venom chemically modified Lys49 phospholipase A_2-like BthTX-I and a synthetic peptide derived from its C-terminal region," *Biologicals*, vol. 37, no. 4, pp. 222–229, 2009.

[191] L. Zhang and W. T. Wu, "Isolation and characterization of ACTX-6: a cytotoxic L-amino acid oxidase from *Agkistrodon acutus* snake venom," *Natural Product Research*, vol. 22, no. 6, pp. 554–563, 2008.

[192] L. Zhang and L. J. Wei, "ACTX-8, a cytotoxic L-amino acid oxidase isolated from *Agkistrodon acutus* snake venom, induces apoptosis in Hela cervical cancer cells," *Life Sciences*, vol. 80, no. 13, pp. 1189–1197, 2007.

[193] G. B. Naumann, L. F. Silva, L. Silva et al., "Cytotoxicity and inhibition of platelet aggregation caused by an L-amino acid oxidase from *Bothrops leucurus* venom," *Biochimica et Biophysica Acta*, vol. 1810, no. 7, pp. 683–694, 2011.

[194] L. M. Gabriel, E. F. Sanchez, S. G. Silva et al., "Tumor cytotoxicity of leucurolysin-B, a P-III snake venom metalloproteinase from *Bothrops leucurus*," *Journal of Venomous Animals and Toxins including Tropical Diseases*, vol. 18, no. 1, pp. 24–33, 2012.

[195] S. G. Wan, Y. Jin, W. H. Lee et al., "A snake venom metalloproteinase that inhibited cell proliferation and induced morphological changes of ECV304 cells," *Toxicon*, vol. 47, no. 4, pp. 480–489, 2006.

[196] D. A. Higuchi, M. C. Almeida, C. C. Barros et al., "Leucurogin, a new recombinant disintegrin cloned from *Bothrops leucurus* (white-tailed-jararaca) with potent activity upon platelet aggregation and tumor growth," *Toxicon*, vol. 58, no. 1, pp. 123–129, 2011.

[197] J. H. Wang, Y. Wu, F. Ren et al., "Cloning and characterization of Adinbitor, a novel disintegrin from the snake venom of Agkistrodon halys brevicaudus stejneger," *Acta Biochimica et Biophysica Sinica*, vol. 36, no. 6, pp. 425–429, 2004.

[198] J. A. Eble, S. Niland, A. Dennes et al., "Rhodocetin antagonizes stromal tumor invasion *in vitro* and other alpha 2 beta 1 integrin-mediated cell functions," *Matrix Biology*, vol. 21, no. 7, pp. 547–558, 2002.

[199] C. Marcinkiewicz, R. R. Lobb, M. M. Marcinkiewicz et al., "Isolation and characterization of EMS16, a C-lectin type protein from *Echis multisquamatus* venom, a potent and selective inhibitor of the alpha 2 beta 1 integrin," *Biochemistry*, vol. 39, no. 32, pp. 9859–9867, 2000.

[200] Y. Tian, H. Wang, B. Li et al., "The cathelicidin-BF Lys16 mutant Cbf-K16 selectively inhibits non-small cell lung cancer proliferation *in vitro*," *Oncology Reports*, vol. 30, no. 5, pp. 2502–2510, 2013.

[201] M. K. Ji, Y. Shi, J. W. Xu et al., "Recombinant snake venom metalloproteinase inhibitor BJ46A inhibits invasion and metastasis

of B16F10 andMHCC97H cells through reductions of matrix metalloproteinases 2 and 9 activities," *Anticancer Drugs*, vol. 24, no. 5, pp. 461–472, 2013.

[202] M. Morjen, O. Kallech-Ziri, A. Bazaa et al., "Matrix PIVL, a new serine protease inhibitor from *Macrovipera lebetina transmediterranea* venom, impairs motility of human glioblastoma cells," *Biology*, vol. 32, no. 1, pp. 52–62, 2013.

[203] H. T. Yalcın, M. O. Ozen, B. Gocmen et al., "Effect of ottoman viper (*Montivipera xanthina* (Gray, 1849)) venom on various cancer cells and on microorganisms," *Cytotechnology*, vol. 66, no. 1, pp. 87–94, 2014.

[204] M. K. Al-Sadoon, M. A. Abdel-Maksoud, D. M. Rabah et al., "Induction of apoptosis and growth arrest in human breast carcinoma cells by a snake (*Walterinnesia aegyptia*) venom combined with silica nanoparticles: crosstalk between Bcl2 and caspase 3," *Cellular Physiology and Biochemistry*, vol. 30, no. 3, pp. 653–665, 2012.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 683123, 13 pages
http://dx.doi.org/10.1155/2014/683123

Research Article

Activation of J77A.1 Macrophages by Three Phospholipases A_2 Isolated from *Bothrops atrox* Snake Venom

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Received 4 September 2013; Revised 18 November 2013; Accepted 18 November 2013; Published 27 January 2014

Academic Editor: Saulo Luís da Silva

In the present study, we investigated the *in vitro* effects of two basic myotoxic phospholipases A_2 (PLA_2), BaTX-I, a catalytically inactive Lys-49 variant, and BaTX-II, a catalytically active Asp-49, and of one acidic myotoxic PLA_2, $BaPLA_2$, a catalytically active Asp-49, isolated from Bothrops atrox snake venom, on the activation of J774A.1 macrophages. At noncytotoxic concentrations, the toxins did not affect the adhesion of the macrophages, nor their ability to detach. The data obtained showed that only BaTX-I stimulated complement receptor-mediated phagocytosis. However, BaTX-I, BaTX-II, and $BaPLA_2$ induced the release of the superoxide anion by J774A.1 macrophages. Additionally, only BaTX-I raised the lysosomal volume of macrophages after 15 min of incubation. After 30 min, all the phospholipases increased this parameter, which was not observed within 60 min. Moreover, BaTX-I, BaTX-II, and $BaPLA_2$ increased the number of lipid bodies on macrophages submitted to phagocytosis and not submitted to phagocytosis. However, BaTX-II and $BaPLA_2$ induced the release of TNF-α by J774A.1 macrophages. Taken together, the data show that, despite differences in enzymatic activity, the three toxins induced inflammatory events and whether the enzyme is acidic or basic does not seem to contribute to these effects.

1. Introduction

Bothrops atrox is responsible for most cases of envenoming in the Northern region of Brazil [1]. Its venom contains, among others, proteins serine proteinases, metalloproteinases, L-amino acid oxidases, C-type lectin-like enzymes, and phospholipases A_2. Phospholipases A_2 (PLA_2; EC 3.1.1.4) belong to a diverse super family of lipolytic enzymes that catalyse the hydrolysis of the sn-2 ester bond of membrane glycerophospholipids to produce free fatty acids and lysophospholipids. These enzymes can differ in structure, molecular weight, substrate specificity, requirement for Ca^{2+}, cell localization, and mechanism of action [2]. PLA_2s are classified into five major families: extracellular or secretory PLA_2s ($sPLA_2$), intracellular cytosolic PLA_2s ($cPLA_2$), Ca^{2+}-independent PLA_2s ($iPLA_2$), PAF acetylhydrolases, and lysossomal PLAs (lPLAs) [3–6].

Snake venom PLA_2s of the Viperidae family, belong to the subgroup IIA, which share structural features with PLA_2s present in inflammatory exudates in mammals [7, 8]. These snake venom PLA_2s display a variety of pharmacological activities, such as myotoxic, neurotoxic, anticoagulant, hypotensive, hemolytic, platelet aggregation inhibiting, bactericidal, and proinflammatory activities [9–12]. Proteins of this subgroup can be further subdivided into two types: (i) catalytically active PLA_2s, which have conserved residues at

the catalytic network and at the calcium-binding loop, including Asp49, and (ii) catalytically inactive variants having a Lys instead of Asp at position 49 [10, 13, 14].

In addition, among the pro inflammatory activities, it was shown that various venom PLA_2s are able to stimulate neutrophil chemotaxis, degranulate mast cells *in vitro*, induce prostaglandin production in peritoneal leukocytes, and activate macrophage functions [12, 15–19].

Macrophages play a central role in a wide variety of processes associated with tissue maintenance, antigen presentation, inflammation, and tissue repair [20]. Resident macrophages constitute one of the first lines of host defense and are present in many tissues. Upon stimulation, these quiescent cells are activated and display diverse cellular functions such as phagocytosis. This process consists of the uptake and destruction of invading microorganisms, an essential part of the host response against infections. It is initiated by engaging receptors on the surface of the macrophages which express various receptors that participate in target cell recognition and internalization of ligands [21]. There are four major classes of receptor-mediated phagocytosis: (a) Fcγ receptors, which recognize the Fcγ domain of IgG-coated particles; (b) complement receptor type 3 (CR3, also referred to as CD11b/CD18, Mac-1, and integrin $\alpha M\beta 2$) which recognizes complement-coated particles; (c) mannose receptors which recognize mannose and fucose on the surface of pathogens; and (d) β-glucan receptors or Dectin-1 which recognize β-glucans-bearing ligands [20, 21].

Subsequent to phagocytosis, an abrupt production of oxygen free radicals such as the superoxide anion occurs, referred to as an oxidative burst. The enzyme complex responsible for the production of this reactive oxygen species is the NADPH oxidase complex. When macrophages are stimulated, the membrane and cytosolic components of this complex associate on the phagosomal membranes to form the active oxidase complex [22–25]. The reactive oxygen species (ROS) formed, such as superoxide anion and hydrogen peroxide, are used by macrophages to kill ingested microorganisms [22–25]. Parallel to this reaction occurs lysossomal membrane destabilization involving increased fusion of lysosomes and phagosomes deriving from cellular stress. Lysossomal activities mediate several processes in cell feeding, homeostasis, and antimicrobial defense, which involve lysosome fusion with endosomes and phagosomes [26]. Furthermore, activated macrophages express a large amount of inclusions called lipid bodies (LBs). These LBs are neutral lipid storage organelles with key roles in cell signaling, intracellular trafficking, cell activation, and eicosanoid formation [27]. LBs were shown to characteristically increase both the size and number of cells associated with the inflammatory process. Additionally, macrophages, upon stimulation, release cytokines such as TNF-α, an important inflammatory mediator involved in many inflammatory reactions [28].

The present study was therefore designed to evaluate the effects of *Bothrops atrox* Lys-49 and Asp-49 PLA_2s, acidic and basic proteins, on J774A.1 macrophage function. Our results indicate that these PLA_2s, at noncytotoxic concentrations, are able to directly stimulate macrophages. Despite differences in enzymatic activity, the toxins induced inflammatory events and whether the enzyme is acidic or basic does not seem to contribute to these effects.

2. Materials and Methods

2.1. Chemicals and Reagents. Trypan blue, RPMI-1640, L-glutamine, gentamicin, zymosan, thiocarbohydrazide (TCH), nitroblue tetrazolium (NBT), mouse INF-γ and neutral red were purchased from Sigma (MO, USA). Fetal bovine serum was obtained from Cultilab (Brazil). Formaldehyde and OsO_4^- (osmium tetroxide) were purchased from Electron Microscopy Sciences (PA, USA). DuoSet Elisa Mouse TNF-alpha was purchased from R&D Systems (Oxon, United Kingdom). All salts and reagents used were obtained from Merck (Darmstadt, Germany) with low endotoxin or endotoxin-free grades.

2.2. Venom. *Bothrops atrox* specimens collected around the city of Porto Velho, State of Rondônia, Brazil, were kept at the Fiocruz Rondônia bioterium in order to be used for venom production under authorization from IBAMA (license number 27131-1) and CGEN (license number 010627/2011-1). The crude venom was dehydrated and stored at a temperature of −20°C in the Amazon Venom Bank at CEBio.

2.3. Cell Line and Culture. The murine macrophage cell line J774A.1 was obtained from the Rio de Janeiro Cell Bank Collection (Brazil). Briefly, monolayers of J774A.1 macrophages were maintained in flasks in a RPMI-1640 medium supplemented with 100 μg/mL gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS), in humidified air with 5% CO_2 at 37°C.

2.4. Isolation and Biochemical Characterization of Phospholipases A_2. Lys49 (BaTX-I) and Asp49 (BaTX-II) basic PLA_2s as well as Asp49 ($BaPLA_2$) acidic PLA_2 from *Bothrops atrox* snake venom were purified according to the method previously described [29, 30]. *B. atrox* crude venom (100 mg) was dissolved in 1.0 mL of 0.05 M Tris-HCl buffer, pH 7.4, centrifuged at 755 ×g for 10 min at room temperature and the clear supernatant applied on a CM-Sepharose FF column (1 × 40 cm), which was previously equilibrated with the same buffer. Elution was carried out with a continuous gradient up to a concentration of 1.0 M of NaCl at a flow rate of 1.0 mL/min (HPLC, Akta Purifier, GE). Absorbance of the effluent solution was recorded at 280 nm. The last fraction with phospholipase and myotoxic activity was collected and freeze-dried. The acidic fraction (Peak 1) was freeze-dried and then diluted with 0.05 M Tris-HCl buffer, pH 7.4 containing 4 M NaCl prior to the next chromatographic step in a butyl-HP (Hitrap, 5 mL) column, previously equilibrated with the same buffer. The chromatography was carried out at a flow rate of 2.5 mL/min, with a decreasing gradient of NaCl (4 to 0 M), followed by Milli-Q H_2O. The active fractions showing PLA_2 activity were desalinized, lyophilized, and applied on a C18 reverse-phase column (Supelco, 250 mm × 4.6 mm), previously equilibrated with 0.1% TFA and then eluted with

an acetonitrile-TFA gradient (0 to 70%), at a flow rate of 1.0 mL/min and monitored at $\lambda = 280$ nm.

Homogeneity was demonstrated using SDS-polyacrylamide gel electrophoresis under reducing conditions [31]. PLA_2 activity of the proteins was evaluated *in vitro* by indirect erythrocyte lysis in agar containing human erythrocytes and egg yolk, as previously described [32]. A prior agreement from all participants involved was made in order to be included in the study, and the Center of Tropical Medicine Research (Rondonia, Brazil) Research Ethics Committees (number CAAE: 14204413.5.0000.0011) approved this study. The assay for myotoxicity was carried out using the creatine kinase (CK)-UV kinetic kit from Bioclin, Brazil [33].

2.5. Cytotoxic Assay. Cell viability was measured by trypan blue exclusion. In brief, J774A.1 macrophages were centrifuged at 200 ×g for 5 min at 4°C and the cell pellets were resuspended in 1 mL of RPMI-1640 medium supplemented with 100 μg/mL gentamicin, 2 mM L-glutamine, and 10% fetal bovine serum (FBS). After counting, 2×10^5 macrophages/80 μL were added to plastic vials and incubated with 20 μL of different concentrations of BaTX-I or BaTX-II or $BaPLA_2$ (1.5; 3 and 6 μg/mL) diluted in assay medium or RPMI (control), for 1 h and 4 h at 37°C in a humidified atmosphere (5% CO_2). Then, 20 μL 0.1% trypan blue was added to 100 μL of macrophage suspension. The viable cell index was determined in a Neubauer's chamber by counting a total of 100 cells. Results were expressed as percentage of viable cells.

2.6. Adhesion Assay. Macrophage adhesion was assayed according to the procedure described by [34]. The cells (2×10^5 cells/well) were cultured for 24 h in a supplemented medium. After incubation, the plates were washed three times with PBS and the adherent cells were fixed with methanol. After staining with 10% Giemsa solution for 10 min, the plates were washed with water, and the remaining dye was solubilized with methanol. Adherence of control cells was considered to be 100%. Absorbance was determined spectrophotometrically at 550 nm.

2.7. Detachment Assay. Macrophage detachment was assayed according to the procedure described by [34]. In this test, J774A.1 macrophages were plated in 96-well plates at a density of 2×10^5 cells/well and allowed to attach for 24 h at 37°C in a 5% CO_2 atmosphere. Nonadherent cells were removed by washing with PBS. After 24 h the cells were incubated with 200 μL of supplemented RPMI (control) or BaTX-I or BaTX-II or $BaPLA_2$ (6 μg/mL) diluted in RPMI at 37°C in a humidified atmosphere (5% CO_2) for 1 h. After another washing with PBS, the cells were stained with 50 μL of 0.1% Giemsa for 40 min. Finally, the cells were washed with PBS and 100 μL of 100% methanol was added to solubilize the dye. Adherence of the remaining control cells was considered to be 100%. Absorbance was read at 550 nm. The results were expressed as absorbance.

2.8. Phagocytic Activity of J774A.1 Macrophages. This test was performed according to [12]. In brief, J774A.1 macrophages were plated on 13 mm diameter glass coverslips (Glass Tecnica, Brazil) in 24-well plates at a density of 2×10^5 cells per coverslip and allowed to attach for 2 h at 37°C under a 5% CO_2 atmosphere. Nonadherent cells were removed by washing with PBS. Cell monolayers were cultured for 1 h with RPMI-1640 supplemented with 100 μg/mL gentamicin, 2 mM L-glutamine, and 10% FBS at 37°C and 5% CO_2 and then incubated with RPMI (control) or different concentrations of BaTX-I or II or $BaPLA_2$ (6 μg/mL) diluted in RPMI. After washing in cold PBS the monolayers were incubated for 40 min at 37°C and 5% CO_2 with serum-opsonized zymosan, prepared as described below, and unbound particles were removed by washing with PBS. Cells were fixed with 2.5% glutaraldehyde for 15 min at room temperature and the coverslips were mounted on microscope slides. The extent of phagocytosis was quantified by contrast phase microscopic observation. At least 200 macrophages were counted in each determination and those containing three or more internalized particles were considered positive for phagocytosis. Results were presented as the percentage of cells positive for phagocytosis.

To prepare serum-opsonized zymosan, zymosan particles, obtained from yeast cell walls, were suspended in PBS at a concentration of particles of 5.7 mg/mL. For opsonization, 2 mL of zymosan particles was mixed with 2 mL normal mouse serum and incubated for 30 min at 37°C. The serum-opsonized zymosan particles were then centrifuged at 200 g for 10 min and suspended in PBS in preparation for the phagocytosis assay. Mouse serum from swiss mice used in the present study was approved by the Tropical Pathology Research Institute Ethics Committee on Animal Use (number 08/2008).

2.9. Staining and Counting of Lipid Bodies. Macrophages were plated on 13 mm diameter glass coverslips (Glass Tecnica, Brazil) in 24-well plates at a density of 2×10^5 cells per coverslip and allowed to attach for 2 h at 37°C in a 5% CO_2 atmosphere. Nonadherent cells were removed by washing with PBS. Cell monolayers were incubated with supplemented RPMI (control) or BaTX-I or BaTX-II or $BaPLA_2$ (6 μg/mL) diluted in RPMI for 60 min at 37°C and 5% CO_2. In a set of experiments, J774A.1 macrophages were submitted to phagocytosis according to item 2.8 and then the lipid bodies were stained. After washing in cold PBS the monolayers were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 15 min at room temperature, rinsed in 0.1 M cacodylate buffer, pH 7.4, stained in 1.5% OsO_4 (30 min), rinsed in dH_2O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, restained in 1.5% OsO_4 (3 min), rinsed in dH_2O, and then dried and mounted on microscope slides. The morphology of fixed cells was observed, and lipid bodies were counted using phase contrast microscopy with an objective lens at a magnification of 100 in 50 consecutively scanned macrophages. The results were expressed as the mean number of lipid bodies per cell.

2.10. Production of Superoxide Anion by J774A.1 on Glass Coverslips. In this test, J774A.1 the concentration of macrophages

was adjusted to $2 \times 10^5/100\ \mu$L and they were incubated with 100 μL RPMI containing 0.1% NBT (control) or 100 μL of different concentrations of BaTX-I or BaTX-II or $BaPLA_2$ (6 μg/mL), diluted in RPMI containing 0.1% NBT, and incubated for 1 h at 37°C and 5% CO_2. At the end of the incubation period, the cells were centrifugated in a cytospin for 5 min at 180 ×g and then fixed with methanol for 5 min. Finally, the cells were stained with 0.1% safranin for 5 min. At least 100 macrophages were counted and those containing blue formazan crystals were considered positive. The results were expressed in a percentage.

2.11. Lysosomal Volume. This test, which evaluates the vesicles of the endocytic compartment by lysosomal retention of neutral red, was performed according to [35]. J774A.1 macrophages were incubated for 15, 30, and 60 minutes in the presence of RPMI (control) or BaTX-I or BaTX-II or $BaPLA_2$ (6 μg/mL) at 37°C in a humidified atmosphere (5% CO_2). Then, the cells were washed by centrifugation and incubated with 0.04% neutral red (stock solution 2% = 20 mg dissolved in 1 mL DMSO). After this the macrophages were washed again by centrifugation and the dye was solubilized by adding 200 μL of extraction solution (acetic acid 10% and 40% ethanol in distilled water). The cells were incubated for 30 minutes and then the samples were read on a spectrophotometer at 550 nm. The results were expressed in percentage, considering the control group to have 100% absorbance.

2.12. Tumor Necrosis Factor-α (TNF-α) Quantification. J774A.1 macrophages ($2 \times 10^5/50\ \mu$L) were incubated with BaTX-I or BaTX-II or $BaPLA_2$ (6 μg/mL) or RPMI (control) or mouse INF-γ (1 μg/mL, positive control) for 4 hours at 37°C in a humidified atmosphere (5% CO_2). After centrifugation the supernatant was used to determine TNF-α levels by specific EIA, as described by [36]. Briefly, 96-well plates were coated with 100 μL of the first capture monoclonal antibody antitumor necrosis factor-α (4 μg/mL) and incubated for 18 hours at 37°C. Following this period, the plate was washed with washer buffer (PBS/Tween20). After that, 200 μL of blocking buffer, containing 5% bovine serum albumin (BSA) in PBS/Tween20, was added to the wells and the plates were incubated for 1 hour at 37°C. Following this period, the wells were washed and 50 μL of either samples or standard was dispensed into each well and the plates incubated for 2 hours at 37°C. After this period, the plate was washed and 100 μL of detection antibody TNF-α (250 ng/mL) was added for 2 hours at 37°C. After incubation and washing, 100 μL of streptavidin-peroxidase was added, followed by incubation and addition of the substrate (100 μL/mL 3,3′,5,5′-tetramethylbenzidine). Finally, sulfuric acid (50 μL) was added to stop the reaction. Absorbances at 540 and 450 nm were recorded and concentrations of TNF-α were estimated from standard curves prepared with recombinant TNF-α. The results were expressed as pg/mL of this cytokine.

2.13. Statistical Analyses. Means and S.E.M. of all data were obtained and compared using two way ANOVA, followed by a Tukey test with significance probability levels of less than 0.05.

3. Results

3.1. Isolation and Biochemical Characterization of Myotoxins. *Bothrops atrox* crude venom (100 mg) was applied on a CM-Sepharose ion-exchange column, previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, and then eluted with a continuous gradient up to a concentration of 1.0 M of NaCl. Fraction CM-11, with myotoxic activity, was named BaTX-I (Figures 1(a) and 1(c)). The fractions CM-6 and CM-1, with phospholipase A_2 activity, were further fractionated on reverse phase C18 (Figure 1(b)) and Butyl-Sepharose (Figure 2(a)) columns, respectively. The fraction CM-6-3 (Figure 1(b)), with PLA_2 and myotoxic activities, was named BaTX-II (Figure 1(d)). Fraction CM-1-6 was applied on a RP-HPLC C18 column and resolved into one main peak with only PLA_2 activity, named $BaPLA_2$ (Figures 2(b) and 2(c)). The homogeneity of these proteins was further demonstrated by SDS-PAGE. The purified myotoxins consisted of a single polypeptide chain with an apparent approximate molecular weight of 14,500 Da.

3.2. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Macrophage Viability. To test the toxicityof PLA_2s on J774A.1 macrophages, the effect of 1 and 4 hours of incubation of several concentrations of BaTX-I or BaTX-II or $BaPLA_2$ was investigated. As shown in Figure 3, the incubation of secretory PLA_2s at concentrations of 1.5, 3, and 6 μg/mL for 1 h and 4 h (data not shown) did not affect J774A.1 macrophage viability.

3.3. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Macrophage Adhesion. To investigate the effect of BaTX-I, BaTX-II, and $BaPLA_2$ on macrophage adhesion, the cells were incubated with 6 μg/mL of either PLA_2s (a noncytotoxic concentration) or RPMI (control). As shown in Figure 4(a), the toxins did not affect the adhesion of the J774A.1 macrophages.

3.4. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Macrophage Detachment. To test whether these secretory PLA_2s of *Bothrops atrox* snake venom affect the ability of J774A.1 macrophages to detach, the cells were incubated with BaTX-I or BaTX-II or $BaPLA_2$ at a noncytotoxic concentration (6 μg/mL) or RPMI (control). As shown in Figure 4(b), the studied PLA_2s did not affect the ability of the macrophages to detach.

3.5. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Phagocytosis by Macrophages. In order to assess the ability of BaTX-I, BaTX-II, and $BaPLA_2$ to stimulate complement receptor-mediated phagocytosis, the uptake of serum-opsonized zymosan particles was determined in adherent J774A.1 macrophages treated with noncytotoxic concentration of toxins or with RPMI (control). As shown in Figure 5, macrophages incubated with RPMI showed an average phagocytosis of serum-opsonized zymosan particles of 37%. Incubation of macrophages with BaTX-I induced a significant increase in J774A.1 macrophage phagocytosis of serum-opsonized zymosan at a concentration of 6 μg/mL. On the other hand, incubation of macrophages with BaTX-II and $BaPLA_2$, at a concentration of

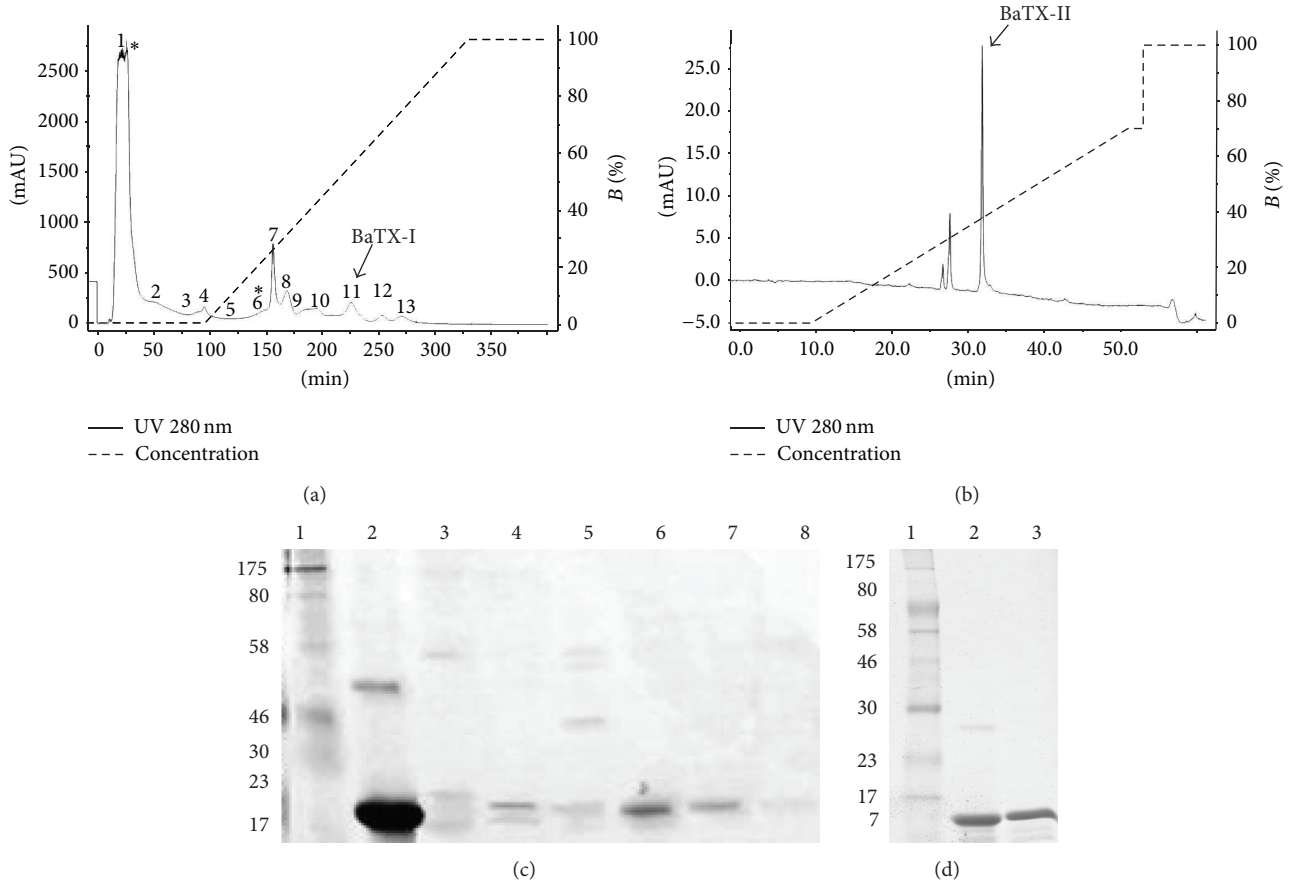

Figure 1: (a) Chromatographic profile of *Bothrops atrox* venom. The crude venom of *B. atrox* after being solubilized was applied on CM-Sepharose (400 mm × 10 mm) previously equilibrated with Tris 50 mM pH 7.4 and eluted under a gradient of NaCl (0–1 M) in the same buffer, in 5 column volumes. The fractions with PLA_2 activity are identified with (∗). (b) High performance chromatographic profile. Fraction 6 obtained from CM-Sepharose was solubilized in 0.1% TFA (solvent A) and applied on a C18 column (discovery 25 mm × 46 mm, 5 μ, 300 Å) previously equilibrated with the same buffer and eluted with 0.1% TFA in 99.9% acetonitrile (solvent B) under a gradient 0–70% and flow of 1 mL/min. Both chromatograms were monitored with absorbance at 280 nm. (c) SDS-PAGE 12. 5%: samples: 1 molecular weight marker; 2 BthTX-I; 3 fraction 8; 4 fraction 9; 5 fraction 10; 6 11 fraction (BaTX-I); 7 fraction 12. (d) SDS-PAGE 12.5% in reducing conditions: electrophoretic analysis of basic Asp49 PLA_2 from *B. atrox*. Samples: 1 molecular weight; 2 BthTX-I; 3 BaTX-II.

6 μg/mL, resulted in 44.6 ± 3.8% and 41.3 ± 2.3%, of phagocytosis of serum-opsonized zymosan, respectively. These values were not significant compared to control.

3.6. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Superoxide Anion (O_2^-) Production by Macrophages. To investigate the ability of BaTX-I, BaTX-II, and $BaPLA_2$ to induce the production of the superoxide anion by J774A.1 macrophages, the cells were incubated with a noncytotoxic concentration of BaTX-I, BaTX-II, or $BaPLA_2$, or with RPMI (control), in the presence of NBT. As shown in Figure 6, J774A.1 macrophages incubated with RPMI showed an average superoxide anion production of 37 ± 2.8%. Incubation of macrophages with BaTX-I, BaTX-II, and $BaPLA_2$ at a concentration of 6 μg/mL, induced significant production of O_2^- by J774A.1 macrophages.

3.7. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Macrophage Lipid Bodies. To determine whether these enzymes induce the formation of lipid bodies in J774A.1 macrophages, the cells were incubated with noncytotoxic BaTX-I, BaTX-II, or $BaPLA_2$, or with RPMI (control) for 1 h. After that, the cells were submitted to phagocytosis and lipid bodies were counted. As shown in Figure 7, the incubation of macrophages with the toxins increased the number of lipid bodies compared to the control. In the cells submitted to phagocytosis, the amount of lipid bodies was significantly higher than cells not submitted to phagocytosis.

3.8. Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on Macrophage Lysossomal Volume. To assess the effect of these secretory PLA_2s on macrophage lysossomal volume, the cells were stimulated with noncytotoxic concentrations of BaTX-I, BaTX-II, or $BaPLA_2$, or with RPMI (control) for 15, 30, and 60 min. As shown in Figure 8(a), incubation with the toxin BaTX-I at a concentration of 6 μg/mL for 15 min increased the lysossomal volume in J774A.1 macrophages compared to the control. The toxins BaTX-II and $BaPLA_2$ did not affect

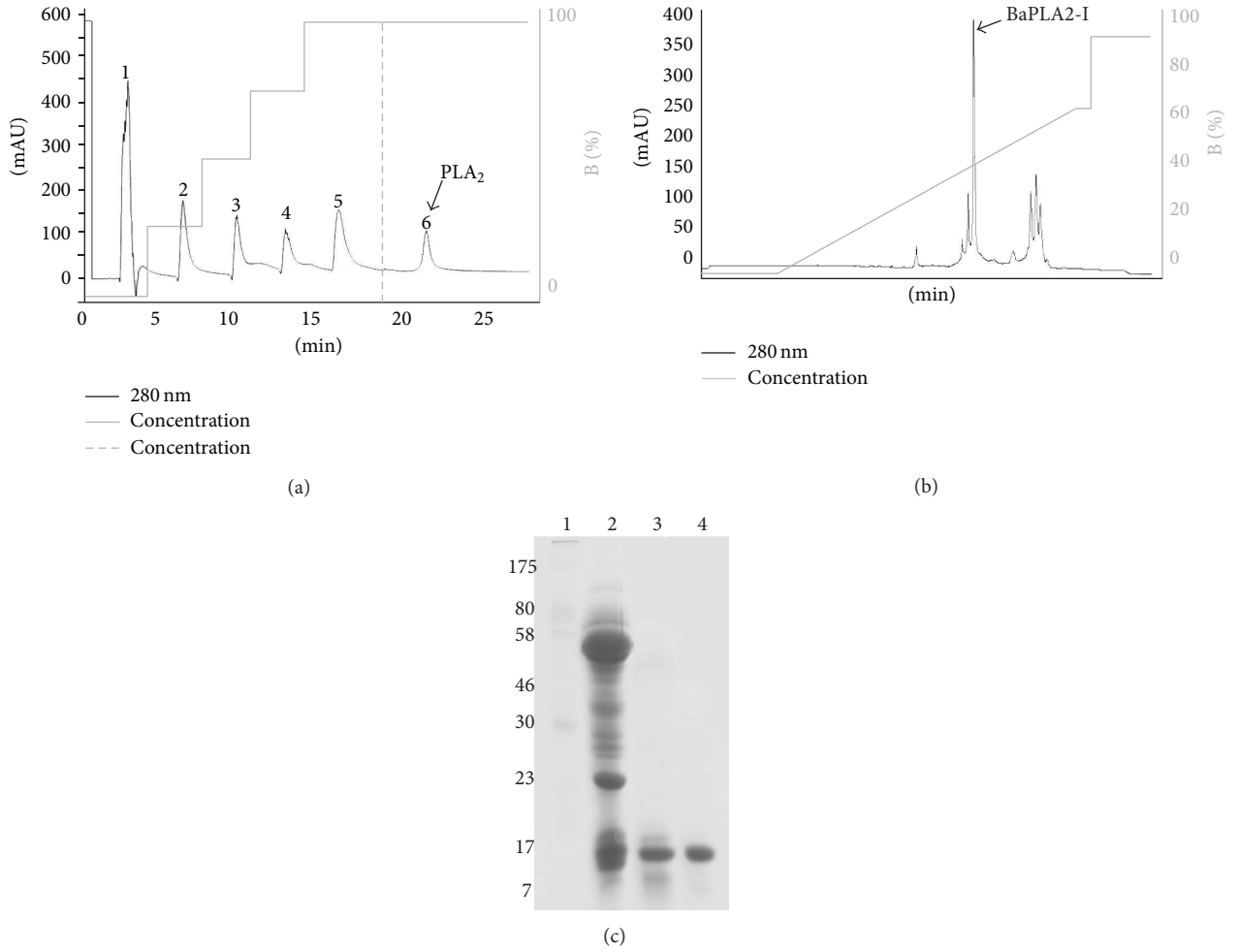

Figure 2: (a) Chromatography profile on hydrophobic interaction resin (Butyl-Sepharose HP 50 mm × 10 mm). Fractions 1, 2, and 3 obtained from CM-Sepharose chromatography were pooled and solubilized in 20 mM Ambic plus 4 M NaCl (buffer A) and applied on a Butyl-Sepharose HP column previously equilibrated with the same buffer and eluted with 20 mM Ambic (buffer B) using a step gradient of 0, 25, 50, 75, and 100%. Subsequently, a step using Milli-Q water was performed. (b) High performance chromatographic profile. Fraction 6 obtained from a Butyl-Sepharose HP column was solubilized in 0.1% TFA (solvent A) and applied on a C18 column (discovery 25 mm × 46 mm, 5 μ, 300 Å) previously equilibrated with the same buffer and eluted with 0.1% TFA in 99.9% acetonitrile (solvent B) under a gradient of 0–70% and flow of 1 mL/min. Both elutions were monitored with absorbance at 280 nm.

macrophage lysossomal volume at this time. However, after incubation for 30 min, the three toxins induced a significant increase in macrophage lysossomal volume, as shown in Figure 8(b). Incubation of the cells with secretory PLA_2s at a concentration of 6 μg/mL for 1 h did not affect the lysossomal volume in macrophages, as shown in Figure 8(c).

3.9. Effect of BaTX-I, BaTX-II, and BaPLA$_2$ on TNF-α Production by Macrophages . To investigate the effect of these secretory PLA_2s on TNF-α production by macrophages, the cells were stimulated with IFN-γ (1 μg/mL, positive control) or noncytotoxic concentrations of BaTX-I or BaTX-II or $BaPLA_2$ or RPMI (control) for a period of 4 hours. Soon after, the supernatant was collected and used to determine TNF-α concentration using an enzyme immunoassay. As shown in Figure 9, the incubation with the enzymatically active toxins BaTX-II and $BaPLA_2$ induced an increase in TNF-α production by macrophages, although BaTX-II was more effective than $BaPLA_2$ in maintaining this effect. The enzymatically inactive toxin BaTX-I did not induce any significant increase in the production of TNF-α in the studied period.

4. Discussion

Both acidic and basic PLA_2s can be found in snake venoms in variable proportions depending on the snake species. The basic PLA_2 isoforms appear to have the highest toxicity and the acidic PLA_2 isoform usually has higher catalytic activity. To date, all acidic PLA_2s purified from Viperidae venoms present an Asp residue at position 49 [37, 38]. In this study, we purified two basic myotoxic PLA_2s from *B. atrox* venom, named BaTX-I (Lys-49), and BaTX-II (Asp-49) and an acidic

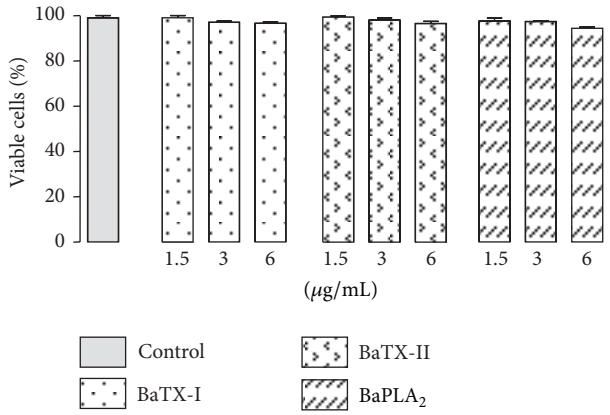

Figure 3: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on macrophage viability. 2×10^5 J774A.1 macrophages were incubated with different concentrations of toxins or RPMI (control) for 60 minutes at 37°C in a humidified atmosphere of 5% CO_2. The viability of macrophages was assessed by a Trypan blue exclusion test. Values represent the mean ± S.E.M. from 3 independent experiments.

PLA_2 named $BaPLA_2$ (Asp-49), by one-, two-, or three-step chromatography. The quality of the final sample is crucial for further studies involving structure and function. Several PLA_2s from *Bothrops* venoms have been exhaustively purified using a combination of chromatographic methods: gel filtration, ion exchange, RP-HPLC, and affinity chromatography [10, 39].

Based on this, we performed a study in order to evaluate the effect of three $sPLA_2$s BaTX-I (Lys-49), BaTX-II (Asp-49), and $BaPLA_2$ (Asp-49) on the functionality of the murine macrophage cell line J774A.1.

For this, we assessed, first, the cytotoxic activity of these phospholipases. The viability assay using the trypan blue exclusion method demonstrated that toxins at concentrations of 1.5, 3, and 6 μg/mL showed no cytotoxic effect on macrophages during 1 hour of incubation. Thus, for the realization of the other proposed experiments, the highest concentration, 6 μg/mL, was chosen. It is noteworthy that the same concentration was used in the study of macrophage activation by Zuliani et al. [12] and Setúbal et al. [19]. The three PLA_2s used had the same effect on macrophages. Still, it is worth noting that the acidic or basic character of these toxins did not influence the results.

Corroborating this data, Lomonte and Gutierrez [40] showed that two basic PLA_2s isolated from the venom of *Bothrops asper* (MT-II and MT-III) exert cytotoxic effects only at high concentrations. Also, in the study by Zuliani et al. [12] it was shown that thioglycollate elicited murine peritoneal macrophages incubated with concentrations less than 25 μg/mL of MT-II and MT-III, for a period of one hour, did not show compromised viability. The study also showed that the enzymatically active toxin MT-III (Asp-49) is more toxic than the enzymatically inactive toxin MT-II (Lys-49). In addition, Setúbal et al. [19] examined the effects of BaltTX-I and BaltTX-II on thioglycollate elicited murine peritoneal macrophage viability. These two basic PLA_2 did not affect macrophage viability, indicating their low toxicity on this cell type.

Macrophages, a professional phagocyte, play an important role in the innate immune response against invading pathogens in the resolution of inflammation and maintenance of tissue homeostasis. This cell type has a central role in the inflammatory reaction, producing microbicidal agents and exerting its main function: phagocytosis. For this to occur efficiently, macrophages need to adhere to a substrate and then modify their cell morphology, gaining a more flattened shape. This step is very important and allows the macrophage to spread and increase its contact surface area with the particle to be phagocytosed. Thus, our study evaluated the effect of the three phospholipases on the ability of macrophages to adhere and to detach. The results showed that BaTX-I, BaTX-II, and $BaPLA_2$ did not affect the ability of macrophages to adhere and to detach from support in the period studied.

The literature reports that PLA_2s have an important role in cell adhesion. The use of inhibitors of PLA_2s such as p-bromophenacyl bromide reduced monocyte adhesion and affected the expression of Mac-1 in neutrophils [41, 42] as well as the spreading of these cells [43]. On the other hand, two nontoxic enzymes with high PLA_2 activity isolated from *Cerastes cerastes* snake venomcalled CC-PLA_2-I and CC-PLA_2-II inhibited the migration and adhesion of IGR39 melanoma and HT1080 fibrosarcoma cells to fibrinogen and fibronectin [44]. Bazaa et al. [45] demonstrated the action of another PLA_2, called MVL-PLA_2, isolated from *Macrovipera lebetina* Transmediterranea. In this study, the enzyme was also able to inhibit the adhesion and migration of tumor cells to fibrinogen and fibronectin, but not to collagen type I. These effects were mediated by the specific inhibition of $\alpha 5\beta 1$, $\alpha v\beta 6$, and $\alpha v\beta 3$ integrins. These data show that integrins may be a specific target for phospholipases' mechanism of action in tumor cell lines. Despite the ability of detachment, our data showed that cells remained viable after incubation with toxins remaining adhered to the plate surface. It is important for the process of phagocytosis to occur effectively. Then, macrophages need to adhere to a substrate to subsequently modify their cellular morphology from a round shape into a flattened shape [46].

Phagocytosis is an extremely complex process and may be mediated by several receptors such as the receptor for the Fc portion of IgG (FcγRs), the mannose receptor (MR), the β-glucan receptor (Dectin-1), and complement receptor type 3 (CR3). Accordingly, we evaluated the effect of PLA_2s on phagocytosis via the complement receptor, using opsonized zymosan particles. Our results showed that only BaTX-I, a Lys-49 toxin, induced a significant increase in phagocytosis via the complement receptor. The others toxins, BaTX-II and $BaPLA_2$, both Asp-49, did not affect this process. The acidic or basic character does not seem to affect this parameter. These results are in agreement with those obtained by Zuliani et al. [12]. The authors showed that MT-II, a Lys-49 isolated from the venom of *Bothrops asper*, also considerably increases phagocytosis via the complement receptor, while MT-III, an Asp-49, only stimulates phagocytosis via mannose receptors. Similar results were obtained by Setúbal et al. [19]. These

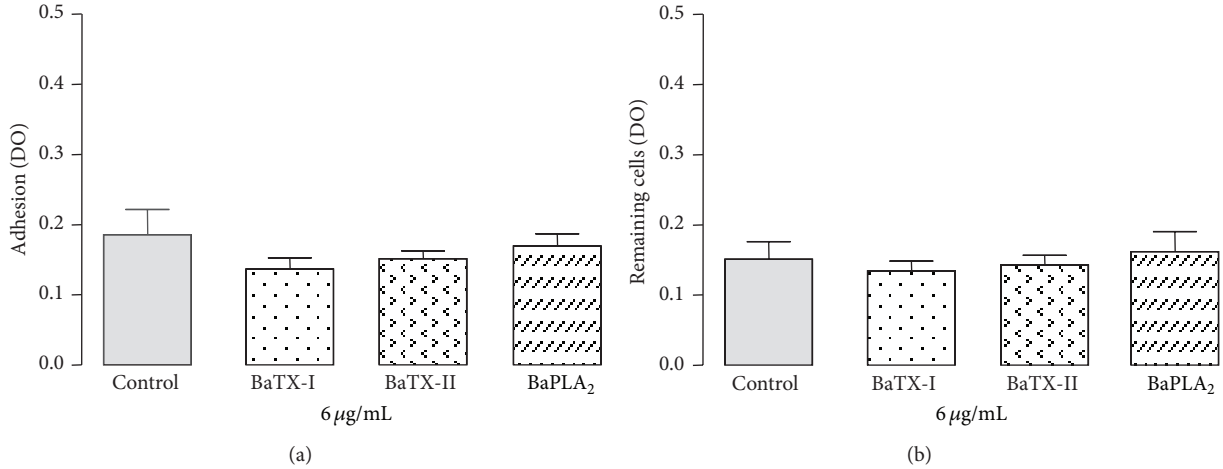

FIGURE 4: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on J774A.1 macrophage adhesion and detachment. For the adhesion test (a), 2×10^5 macrophages were incubated for 60 minutes with toxins (6 μg/mL) or RPMI (control) at 37°C in a humidified atmosphere of 5% CO_2. For the detachment test (b), macrophages were incubated with RPMI for only 24 hours and then for 60 minutes with toxins (6 μg/mL) or RPMI (control) at 37°C in a humidified atmosphere of 5% CO_2. Adhered cell levels were determined by optical density (550 nm) being proportional to the amount of incorporated Giemsa dye. Values represent the mean ± S.E.M. from 3 independent experiments.

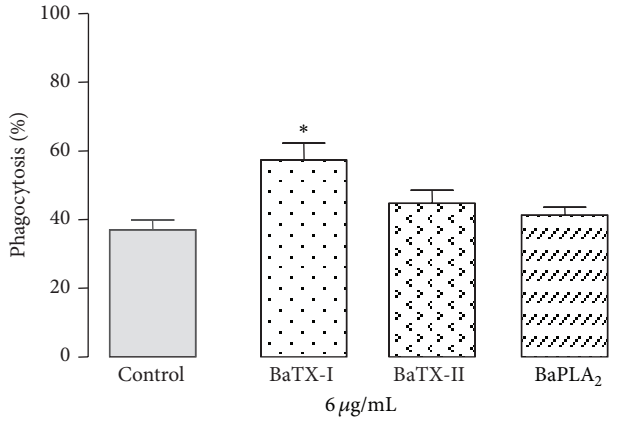

FIGURE 5: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on phagocytosis of opsonized zymosan by J774A.1 macrophages. 2×10^5 macrophages were incubated for 60 minutes with toxins (6 μg/mL) or RPMI (control) at 37°C in a humidified atmosphere of 5% CO_2 before addition of opsonized zymosan particles. Values represent the mean ± S.E.M. from 3 independent experiments $^*P \leq 0.05$ compared with control (ANOVA).

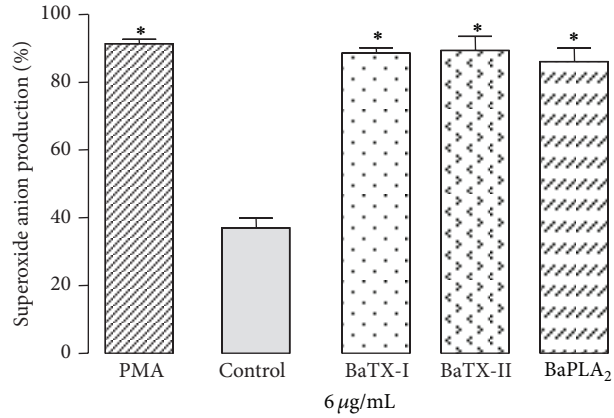

FIGURE 6: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on superoxide production by J774A.1 macrophages. 2×10^5 macrophages were incubated with RPMI, 0.1% NBT, 10 uL of PMA (500 ng/mL) (positive control) or toxins (6 μg/mL) for 60 minutes at 37°C in a humidified atmosphere of 5% CO_2 for the formation of formazan crystals resulting from the reduction of NBT by superoxide. The crystals were solubilized and the absorbance of the supernatant was determined at 620 nm in a spectrophotometer. Data were expressed as O.D. Values represent the mean ± S.E.M. from 3 independent experiments. $^*P \leq 0.05$ compared with control (ANOVA).

authors showed that BaltTX-I, a Lys49, but not BaltTX-II, an Asp-49 enzyme, is able to directly stimulate phagocytosis via the complement receptor. Thus, it is important to note that enzymatic activity does not seem to be essential for increasing the phagocytic capacity via CR3, since only catalytically inactive phospholipases were able to affect this parameter. The mechanism by which Lys49 PLA_2 homologs activate the process of phagocytosis has not been clarified. There are two hypotheses for the action of these enzymatically inactive toxins. One is the interaction of the enzyme with the receptor, leading to its internalization. The other hypothesis is that the PLA_2s have the ability to induce perturbations in the cell membrane, which activate signaling pathways culminating in an increase of phagocytosis. Thus, further studies are needed to elucidate the mechanism of action of these enzymes, which makes them extremely interesting from a scientific standpoint.

Concomitantly with phagocytosis, there is an increase in the oxidative metabolism of macrophages, called a respiratory burst that is mediated by the NADPH oxidase enzyme complex. The high O_2 consumption associated with rapid

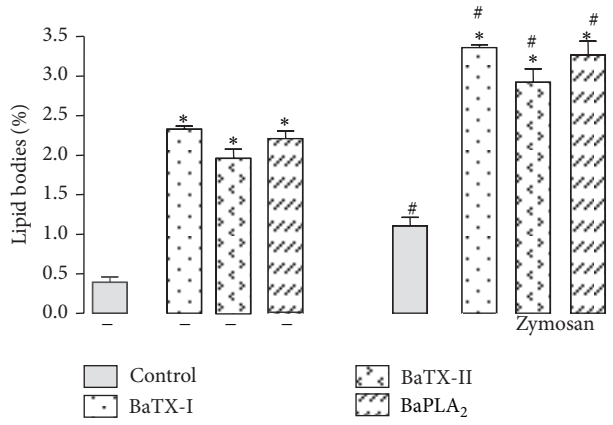

Figure 7: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on lipid body formation by J774A.1 macrophages. 2×10^5 macrophages were incubated with toxins (6 μg/mL) or RPMI (control) for 60 minutes at 37°C in a humidified atmosphere of 5% CO_2 before the addition of opsonized zymosan particles. The number of lipid bodies stained with osmium tetroxide within macrophages was determined using phase-contrast microscopy. Values represent the mean ± S.E.M. from 3 independent experiments. $^*P \leq 0.05$ compared with control and $^\#P \leq 0.05$ compared with control submitted to phagocytosis (ANOVA).

activation of NADPH oxidase triggers the production of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which are potent microbicidal agents [47]. Therefore, this study evaluated the PLA_2 action on superoxide anion production by J774A.1 macrophages. The results showed that all three toxins significantly increased superoxide anion release compared with the unstimulated control. The increase was similar to that observed in cells incubated with PMA, a positive control. There was no difference between the Asp49 and Lys49 phospholipases A_2, suggesting that catalytic activity may not be essential to superoxide anion production. Once again, it is clear that the enzyme's acidic or basic character does not appear to contribute to this effect.

According to Setúbal et al. [19] the excessive release of superoxide can be harmful to tissues involved in inflammation. In this study the authors showed that BaltTX-I and BaltTX-II, isolated from *Bothrops alternatus* venom, induced a significant release of superoxide anion compared with the negative control (RPMI medium), but there was no difference when compared to PMA (a positive control), indicating that the myotoxins are able to activate the respiratory burst. Moreover, Zuliani et al. [12] showed that MT-II and MT-III, isolated from *B. asper* venom, induce the release of H_2O_2, another reactive oxygen intermediate produced from the superoxide anion. In this case, MT-III's activity was more pronounced than that of MT-II. This increase in microbicidal agent production induced by PLA_2s may be considered one of the key points for the development of the intense local inflammatory process observed in *Bothrops* envenoming.

It is possible that the increase in phagocytic index is also associated with an increase in the endocytic compartment, contributing to an increase in the microbicidal activity of the macrophages, since destabilization of lysosomal membranes involves an increase in lysosome and phagolysosome fusion, often associated with stress derived from the action of prooxidant agents and pathological conditions. Accordingly, experiments were performed to assess whether the toxins are able to induce an increase in lysosomal volume. The macrophages were incubated with toxins for 15, 30, and 60 minutes. In evaluating the time course of the effect of the three toxins, it can be seen that increasing the endocytic compartment of macrophages occurs quite sharply in the first 15 minutes after stimulation and that enzymatic activity is not essential for this effect, since only BaTX-I, which is enzymatically inactive, changed this parameter. The enzymatically active phospholipases BaTX-II and $BaPLA_2$ were only able to induce an increase in lysosomal volume after 30 minutes of incubation. During this period, BaTX-I was also effective in maintaining this effect. After being stimulated for 1 hour with phospholipases, macrophages showed no change in their endocytic compartment. This may suggest that, after a period of 30 minutes, the PLA_2 action decreases and the volume of the cells' lysosomal compartments returns to their baseline level. Despite the fact that the lipolytic activity of PLA_2 often affects several membrane structures including lysosomes, in our studies, enzymatic activity does not appear to interfere with the increase in lysosomal volume, nor does the acidic or basic character of these enzymes. Studies by Burlando et al. [26] showed that a calcium-independent $cPLA_2$ is one of the agents responsible for the lysosomal membrane destabilization induced by estradiol. The destabilization of the membrane leads to the fusion of the lysosome with other organelles such as the endosome. This process, as evidenced by Mayorga et al. [48], involves the action of a PLA_2. Thus, the data suggest that these enzymes can induce changes in the vacuole-lysosomal system.

The literature shows that, in the presence of inflammation, activated leukocytes induced an increase of organelles called lipid bodies. These organelles compartmentalize different groups of proteins involved in lipid metabolism and in cell signaling, in addition to containing the complex enzyme COX and 5-LO, and even AA, $cPLA_2$, MAPK, and PI3K [49, 50]. Thus, the actions of BaTX-I, BaTX-II, and $BaPLA_2$ on lipid body formation in J774A.1 macrophages were evaluated. The data obtained show that all three phospholipases were able to increase the number of lipid bodies present in the macrophages. This action occurred independently of enzymatic activity and no statistical difference was observed between the toxins. Similar results were obtained by Leiguez et al. [51] and Giannotti et al. [52]. These authors showed that MT-III, an Asp49, and MT-II, a Lys49, both isolated from *B. asper* venom were able to induce a marked increase in lipid bodies formation in macrophages. Therefore, we evaluated the action of these PLA_2s in macrophages undergoing phagocytosis. The results showed that the number of lipid bodies inside macrophages that phagocytosed was greater than that found in macrophages which were not subjected to the process. The BaTX-I was slightly more efficient than the others in the maintenance of this effect. In relation to enzymatic activity, the three phospholipases were able to

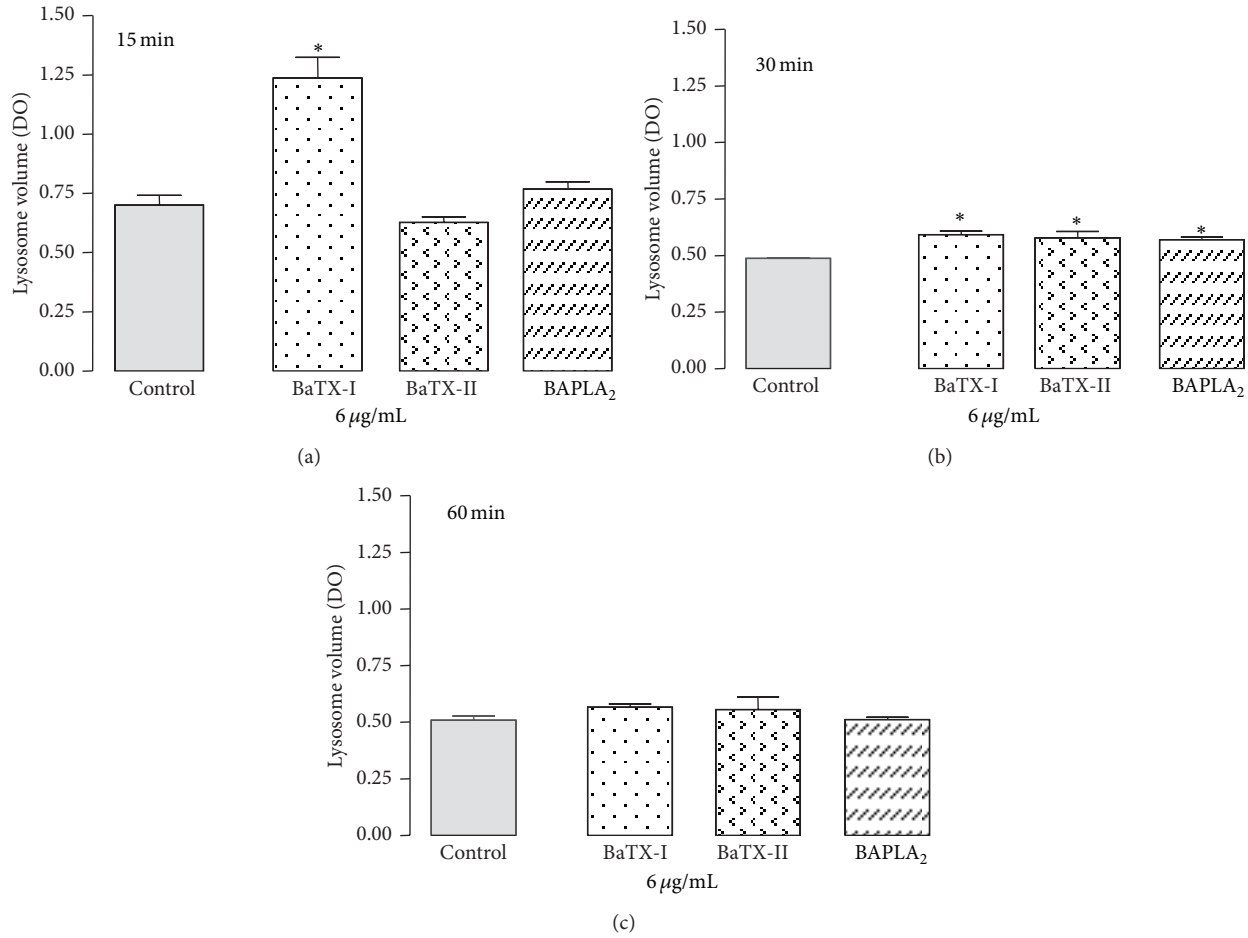

Figure 8: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on J774A.1 macrophage lysosomal volume. 2×10^5 macrophages were incubated with the toxins (6 μg/mL) or RPMI (control) for 15, 30, and 60 minutes at 37°C in a humidified atmosphere of 5% CO_2. After that, the macrophages were incubated with 0.04% neutral red. The incorporated dye was solubilized and the absorbance of the supernatant was determined at 505 nm in a spectrophotometer. Values represent the mean ± S.E.M. from 3 independent experiments. $^*P \leq 0.05$ compared with control (ANOVA).

induce these events regardless of catalytic ability. Again, there was no difference in the acidic or basic character of these enzymes. The data suggest that the activation of complement receptors during phagocytosis stimulates lipid body formation in macrophages, also induced by the action of $sPLA_2$s. Thus, PLA_2s, in addition to releasing the AA of membrane phospholipids, can hydrolyze phosphatidylcholine and cause lyso-PAF release, which is the precursor of platelet activating factor (PAF). Data from the literature indicate that this inflammatory mediator, with platelet aggregating activity, is involved in signaling pathways that favor the formation of lipid bodies and the release of mediators such as LTC_4 and PGE_2 [50]. Additional studies to determine the role of lipid bodies in the process of phagocytosis and the mechanisms of formation of these organelles are required. These details also have significance for the functionality of the leucocytes during inflammatory reactions.

Besides the increase in the number of lipid bodies, activated macrophages show an increased production of cytokines with important roles in inflammatory responses. One of them is TNF-α, a potent inflammatory mediator capable of inducing proliferation of fibroblasts producing other cytokines, activating leukocytes, and stimulating the generation of reactive oxygen species. Thus, we evaluated the action of the three phospholipases, BaTX-I, BaTX-II, and $BaPLA_2$, on TNF-α production by J774A.1 macrophages. The data showed that BaTX-II and $BaPLA_2$ induced a significant increase in TNF-α production by macrophages after 4 hours of incubation, and BaTX-II was somewhat more effective than $BaPLA_2$ in inducing the production of this cytokine. Both toxins are enzymatically active Asp49 homologs, whereas the first is basic and the latter is acidic. The results also show that BaTX-I, an enzymatically inactive Lys49 PLA_2 homolog, did not induce any increase in the production of this cytokine in the studied period. These data are in agreement with Zuliani et al. [11], in which MT-III, an Asp49, isolated from *B. asper*, induced an increase in the production of TNF-α in the peritoneal fluid of mice 1 hour after injection. MT-II, a Lys49, induced a small increase in this parameter only after 6 hours of incubation. Increased production of

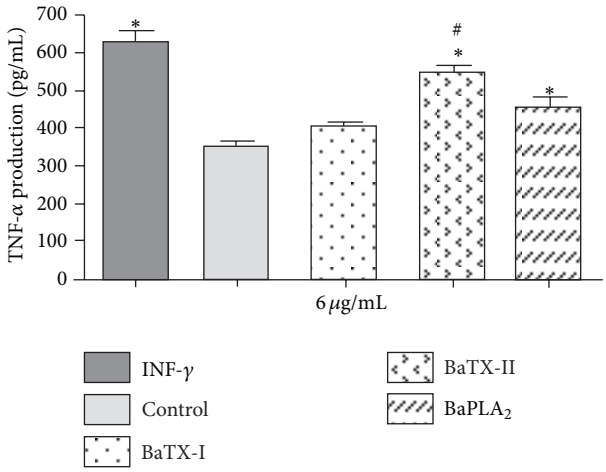

Figure 9: Effect of BaTX-I, BaTX-II, and $BaPLA_2$ on J774A.1 macrophage TNF-α production. 2×10^5 macrophages were incubated with RPMI (control) or INF-γ (1 μg/mL) (positive control) or toxins (6 μg/mL) for 4 hours. The concentrations of TNF-α in the supernatant were quantified by specific EIA. The results were expressed as pg/mL of TNF-α and represent the mean ± S.E.M from 3 independent experiments. $^*P \leq 0.05$ compared to control and $^\#P \leq 0.05$ compared to BaTX-II (ANOVA).

cytokines, particularly TNF-α, can be regulated by increased production of reactive oxygen species in a mechanism dependent on NF-kB. Zuliani et al. [12] demonstrated the ability of these isolated myotoxins to induce a significant increase in H_2O_2 production, suggesting that high levels of this reactive oxygen may play an important role in regulating the production of TNF-α.

It is important to note that TNF-α binds to its cellular TNF-α receptor 1 (TNFR1), which triggers signaling cascades that activate NF-κB and AP-1 transcription factors [53, 54]. This cytokine has also been reported to increase reactive oxygen species (ROS) production from mitochondria, plasma-membrane NADPH oxidase and lipoxygenase [55–58]. It is possible that BaTX-II and $BaPLA_2$ stimulate ROS production through TNF-α production probably through the engagement of a subset of Toll-like receptors (TLR1, TLR2, and TLR4) different from BaTX-I that stimulates ROS through phagocytosis.

Taken together, our data indicate that the all studied PLA_2s do not interfere with adhesion or detachment of J774A.1 macrophages. BaTX-I, but not BaTX-II and $BaPLA_2$, stimulates opsonized zymosan phagocytosis via a complement receptor. However, all of the PLA_2s stimulated an increase in superoxide production and lysosome volume by J774A.1 macrophages after 30 min of incubation. Moreover, BaTX-I, BaTX-II, and $BaPLA_2$ increases the number of lipid bodies on macrophages submitted to phagocytosis and not submitted to phagocytosis. Only, BaTX-II and $BaPLA_2$ induced the release of TNF-α by J774A.1 macrophages. It is important to note that PLA_2 activity is not essential for triggering these effects. Certain regions of the toxin molecules, other than the catalytic site, can interact with cell membranes, leading to the activation of macrophages. These studies have added knowledge about the mechanisms of action of PLA_2s isolated from *B. atrox*, contributing to better characterization of macrophage function.

Regarding the acidic or basic character of these enzymes, there is no evident difference in the activity of phospholipases used in this study. Many of the PLA_2s isolated from *Bothrops* venoms are basic proteins. Acidic PLA_2s are still being studied, but the literature shows that these enzymes can induce various pharmacological effects such as platelet aggregation and hypotension. Acidic phospholipases A_2, in general, are not myotoxic, but this effect may appear on a smaller scale compared to basic phospholipases, which show intense myotoxicity. However, acidic phospholipases have higher catalytic activity [59]. Additional studies with these enzymes, both acidic and basic, are still needed so that we can determine a possible correlation between toxicity and their pharmacological potential.

Conflict of Interests

There is no conflict of interests statement.

Acknowledgments

This study was supported by Conselho Nacional de Desenvolvimento Cientíifico e Tecnolóogico (CNPq), Coordenação de Aperfeiçoamento de Nível Superior (CAPES, NanoBiotec), MCTI, INCT-InpetAM, and Secretaria de Desenvolvimento do Estado de Rondonia (SEPLAN-RO/CNPq), Brazil. Juliana Pavan Zuliani was a recipient of productivity Grant 303974/2008-7 from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Juliana Loca Furtado was the beneficiary of CAPES by Master's fellowship. Amy Hamaker provided the English editing of the paper.

References

[1] C. A. T. Souza, A. M. Kayano, S. S. Setúbal et al., "Local and systemic biochemical alterations induced by Bothrops atrox snake venom in mice," *Journal of Venom Research*, vol. 3, pp. 28–34, 2012.

[2] J. E. Burke and E. A. Dennis, "Phospholipase A_2 biochemistry," *Cardiovascular Drugs and Therapy*, vol. 23, no. 1, pp. 49–59, 2009.

[3] D. A. Six and E. A. Dennis, "The expanding superfamily of phospholipase A_2 enzymes: classification and characterization," *Biochimica et Biophysica Acta*, vol. 1488, no. 1-2, pp. 1–19, 2000.

[4] M. Murakami, Y. Taketomi, C. Girard, K. Yamamoto, and G. Lambeau, "Emerging roles of secreted phospholipase A_2 enzymes: lessons from transgenic and knockout mice," *Biochimie*, vol. 92, no. 6, pp. 561–582, 2010.

[5] E. Valentin and G. Lambeau, "What can venom phospholipases A_2 tell us about the functional diversity of mammalian secreted phospholipases A_2?" *Biochimie*, vol. 82, no. 9-10, pp. 815–831, 2000.

[6] M. Murakami and I. Kudo, "Phospholipase A_2," *Journal of Biochemistry*, vol. 131, no. 3, pp. 285–292, 2002.

[7] I. I. Kaiser, J. M. Gutierrez, D. Plummer, S. D. Aird, and G. V. Odell, "The amino acid sequence of a myotoxic phospholipase

from the venom of Bothrops asper," *Archives of Biochemistry and Biophysics*, vol. 278, no. 2, pp. 319–325, 1990.

[8] R. M. Kini, "Phospholipase A_2—A complex multifunctional protein puzzle," in *Venom Phospholipase A_2 Enzymes. Structure, Function and Mechanism*, R. Manjunatha Kini, Ed., p. 1, John Wiley & Sons, New York, NY, USA, 1997.

[9] C. F. P. Teixeira, E. C. T. Landucci, E. Antunes, M. Chacur, and Y. Cury, "Inflammatory effects of snake venom myotoxic phospholipases A_2," *Toxicon*, vol. 42, no. 8, pp. 947–962, 2003.

[10] A. M. Soares, M. R. M. Fontes, and J. R. Giglio, "Phospholipase A_2 myotoxins from Bothrops snake venoms: structure-function relationship," *Current Organic Chemistry*, vol. 8, no. 17, pp. 1677–1690, 2004.

[11] J. P. Zuliani, C. M. Fernandes, S. R. Zamuner, J. M. Gutiérrez, and C. F. P. Teixeira, "Inflammatory events induced by Lys-49 and Asp-49 phospholipases A_2 isolated from Bothrops asper snake venom: role of catalytic activity," *Toxicon*, vol. 45, no. 3, pp. 335–346, 2005.

[12] J. P. Zuliani, J. M. Gutiérrez, L. L. Casais E Silva, S. C. Sampaio, B. Lomonte, and C. D. F. Pereira Teixeira, "Activation of cellular functions in macrophages by venom secretory Asp-49 and Lys-49 phospholipases A_2," *Toxicon*, vol. 46, no. 5, pp. 523–532, 2005.

[13] J. Gutiérrez and B. Lomonte, "Phospholipase A_2 myotoxins from Bothrops snake venoms," *Toxicon*, vol. 33, no. 11, pp. 1405–1424, 1995.

[14] J. M. Gutiérrez and B. Lomonte, "Phospholipase A_2 myotoxins from *Bothrops*snake venoms," in *Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism*, R. M. Kini, Ed., pp. 321–352, 1997.

[15] A. Gambero, E. C. T. Landucci, M. H. Toyama et al., "Human neutrophil migration in vitro induced by secretory phospholipases A_2: a role for cell surface glycosaminoglycans," *Biochemical Pharmacology*, vol. 63, no. 1, pp. 65–72, 2002.

[16] A. Gambero, S. M. Thomazzi, A. C. O. Cintra, E. C. T. Landucci, G. De Nucci, and E. Antunes, "Signalling pathways regulating human neutrophil migration induced by secretory phospholipases A_2," *Toxicon*, vol. 44, no. 5, pp. 473–481, 2004.

[17] E. C. T. Landucci, R. C. Castro, M. F. Pereira et al., "Mast cell degranulation induced by two phospholipase A_2 homologues: dissociation between enzymatic and biological activities," *European Journal of Pharmacology*, vol. 343, no. 2-3, pp. 257–263, 1998.

[18] V. Moreira, J. M. Gutiérrez, A. M. Soares, S. R. Zamunér, E. Purgatto, and C. D. F. P. Teixeira, "Secretory phospholipases A_2 isolated from Bothrops asper and from Crotalus durissus terrificus snake venoms induce distinct mechanisms for biosynthesis of prostaglandins E2 and D2 and expression of cyclooxygenases," *Toxicon*, vol. 52, no. 3, pp. 428–439, 2008.

[19] S. S. Setúbal, A. S. Pontes, J. L. Furtado et al., "Action of two phospholipases A_2 purified from Bothrops alternatus snake venom on macrophages," *Biochemistry*, vol. 78, pp. 194–203, 2013.

[20] M. Naito, "Macrophage heterogeneity in development and differentiation," *Archives of Histology and Cytology*, vol. 56, no. 4, pp. 331–351, 1993.

[21] A. Aderem and T. A. Underhill, "The cell biology of macrophage activation," *Annual Review of Immunology*, vol. 17, pp. 593–623, 1999.

[22] W. J. Brown, K. Chambers, and A. Doody, "Phospholipase A_2 (PLA_2) enzymes in membrane trafficking: mediators of membrane shape and function," *Traffic*, vol. 4, no. 4, pp. 214–221, 2003.

[23] B. M. Babior, "NADPH oxidase: an update," *Blood*, vol. 93, no. 5, pp. 1464–1476, 1999.

[24] B. M. Babior, "NADPH oxidase," *Current Opinion in Immunology*, vol. 16, no. 1, pp. 42–47, 2004.

[25] B. M. Babior, J. D. Lambeth, and W. Nauseef, "The neutrophil NADPH oxidase," *Archives of Biochemistry and Biophysics*, vol. 397, no. 2, pp. 342–344, 2002.

[26] B. Burlando, B. Marchi, I. Panfoli, and A. Viarengo, "Essential role of Ca^{2+}-dependent phospholipase A_2 in estradiol-induced lysosome activation," *American Journal of Physiology–Cell Physiology*, vol. 283, no. 5, pp. C1461–C1468, 2002.

[27] P. T. Bozza, R. C. N. Melo, and C. Bandeira-Melo, "Leukocyte lipid bodies regulation and function: contribution to allergy and host defense," *Pharmacology and Therapeutics*, vol. 113, no. 1, pp. 30–49, 2007.

[28] C. A. Dinarello, "The proinflammatory cytokines interleukin-1 and tumor necrosis factor and treatment of the septic shock syndrome," *Journal of Infectious Diseases*, vol. 163, no. 6, pp. 1177–1184, 1991.

[29] S. H. Andrião-Escarso, A. M. Soares, V. M. Rodrigues et al., "Myotoxic phospholipases A_2 in *Bothrops* snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*," *Biochimie*, vol. 82, no. 8, pp. 755–763, 2000.

[30] S. H. Andrião-Escarso, A. M. Soares, M. R. M. Fontes et al., "Structural and functional characterization of an acidic platelet aggregation inhibitor and hypotensive phospholipase A_2 from *Bothrops jararacussu* snake venom," *Biochemical Pharmacology*, vol. 64, no. 4, pp. 723–732, 2002.

[31] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[32] J. M. Gutierrez, C. L. Ownby, and G. V. Odell, "Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake Bothrops asper (Fer-de-Lance)," *Toxicon*, vol. 22, no. 5, pp. 719–731, 1984.

[33] A. M. Soares, S. H. Andrião-Escarso, Y. Angulo et al., "Structural and functional characterization of myotoxin I, a Lys49 phospholipase A_2 homologue from *Bothrops moojeni* (Caissaca) snake venom," *Archives of Biochemistry and Biophysics*, vol. 373, no. 1, pp. 7–15, 2000.

[34] S. S. Setubal, A. S. Pontes, J. L. Furtado, A. M. Kayano, R. G. Stábeli, and J. P. Zuliani, "Effect of *Bothrops alternatus* snake venom on macrophage phagocytosis and superoxide production: participation of protein kinase C," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 17, no. 4, pp. 430–441, 2011.

[35] R. K. Pipe, J. A. Coles, and S. R. Farley, "Assay for measuring immune response in the mussel *Mytilus edulis*," *Techniques in the Immunology*, vol. 4, pp. 93–100, 1995.

[36] J. H. Schumacher, A. O'Garra, B. Shrader et al., "The characterization of four monoclonal antibodies specific for mouse IL-5 and development of mouse and human IL-5 enzyme-linked immunosorbent," *Journal of Immunology*, vol. 141, no. 5, pp. 1576–1581, 1988.

[37] J. Fernández, J. M. Gutiérrez, Y. Angulo et al., "Isolation of an acidic phospholipase A_2 from the venom of the snake Bothrops asper of Costa Rica: biochemical and toxicological characterization," *Biochimie*, vol. 92, no. 3, pp. 273–283, 2010.

[38] S. S. Teixeira, L. B. Silveira, F. M. N. da Silva et al., "Molecular characterization of an acidic phospholipase A_2 from Bothrops

pirajai snake venom: synthetic C-terminal peptide identifies its antiplatelet region," *Archives of Toxicology*, vol. 85, no. 10, pp. 1219–1233, 2011.

[39] R. G. Stábeli, R. Simões-Silva, and A. M. Kayano, "Purification of phospholipases A_2 from American snake venoms," in *Chromatography—the Most Versatile Method of Chemical Analysis*, A. Calderon, Ed., pp. 1–34, 2012.

[40] B. Lomonte and J. M. Gutierrez, "A new muscle damaging toxin, myotoxin II, from the venom of the snake Bothrops asper (terciopelo)," *Toxicon*, vol. 27, no. 7, pp. 725–733, 1989.

[41] P. B. Jacobson and D. J. Schrier, "Regulation of CD11b/CD18 expression in human neutrophils by phospholipase A_2," *Journal of Immunology*, vol. 151, no. 10, pp. 5639–5652, 1993.

[42] J. B. Lefkowith, M. R. Lennartz, M. Rogers, A. R. Morrison, and E. J. Brown, "Phospholipase activation during monocyte adherence and spreading," *Journal of Immunology*, vol. 149, no. 5, pp. 1729–1735, 1992.

[43] S. I. Galkina, G. F. Sud'ina, and L. B. Margolis, "Regulation of intracellular pH by phospholipase A_2 and protein kinase C upon neutrophil adhesion to solid substrata," *FEBS Letters*, vol. 393, no. 1, pp. 117–120, 1996.

[44] R. Zouari-Kessentini, J. Luis, A. Karray et al., "Two purified and characterized phospholipases A_2 from *Cerastes cerastes* venom, that inhibit cancerous cell adhesion and migration," *Toxicon*, vol. 53, no. 4, pp. 444–453, 2009.

[45] A. Bazaa, N. Marrakchi, M. El Ayeb, L. Sanz, and J. J. Calvete, "Snake venomics: comparative analysis of the venom proteomes of the Tunisian snakes *Cerastes cerastes*, *Cerastes vipera* and *Macrovipera lebetina*," *Proteomics*, vol. 5, no. 16, pp. 4223–4235, 2005.

[46] P. R. Taylor, G. D. Brown, D. M. Reid et al., "The β-glucan receptor, dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineages," *Journal of Immunology*, vol. 169, no. 7, pp. 3876–3882, 2002.

[47] B. M. Babior, R. S. Kipnes, and J. T. Curnutte, "Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent," *The Journal of Clinical Investigation*, vol. 52, no. 3, pp. 741–744, 1973.

[48] L. S. Mayorga, M. I. Colombo, M. Lennartz et al., "Inhibition of endosome fusion by phospholipase A_2 (PLA2) inhibitors points to a role for PLA2 in endocytosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 21, pp. 10255–10259, 1993.

[49] P. T. Bozza, J. L. Payne, S. G. Morham, R. Langenbach, O. Smithies, and P. F. Weller, "Leukocyte lipid body formation and eicosanoid generation: cyclooxygenase-independent inhibition by aspirin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 20, pp. 11091–11096, 1996.

[50] P. T. Bozza, W. Yu, and P. F. Weller, "Mechanisms of formation and function of eosinophil lipid bodies: inducible intracellular sites involved in arachidonic acid metabolism," *Memorias do Instituto Oswaldo Cruz*, vol. 92, pp. 135–140, 1997.

[51] E. Leiguez, J. P. Zuliani, A. M. Cianciarullo, C. M. Fernandes, J. M. Gutiérrez, and C. Teixeira, "A group IIA-secreted phospholipase A_2 from snake venom induces lipid body formation in macrophages: the roles of intracellular phospholipases A_2 and distinct signaling pathways," *Journal of Leukocyte Biology*, vol. 90, no. 1, pp. 155–166, 2011.

[52] K. C. Giannotti, E. Leiguez, V. Moreira et al., "A Lys49 phospholipase A_2, isolated from *Bothrops asper* snake venom, induces lipid droplet formation in macrophages which depends on distinct signaling pathways and the C-terminal region," *BioMed Research International*, vol. 2013, Article ID 807982, 14 pages, 2013.

[53] G. Chen and D. V. Goeddel, "TNF-R1 signaling: a beautiful pathway," *Science*, vol. 296, no. 5573, pp. 1634–1635, 2002.

[54] A. Ashkenazi, "Targeting death and decoy receptors of the tumour-necrosis factor superfamily," *Nature Reviews Cancer*, vol. 2, no. 6, pp. 420–430, 2002.

[55] O. Micheau and J. Tschopp, "Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes," *Cell*, vol. 114, no. 2, pp. 181–190, 2003.

[56] T. Hennet, C. Richter, and E. Peterhans, "Tumour necrosis factor-α induces superoxide anion generation in mitochondria of L929 cells," *Biochemical Journal*, vol. 289, no. 2, pp. 587–592, 1993.

[57] B. Meier, H. H. Radeke, S. Selle et al., "Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-α," *Biochemical Journal*, vol. 263, no. 2, pp. 539–545, 1989.

[58] V. B. O'Donnell, S. Spycher, and A. Azzi, "Involvement of oxidants and oxidant-generating enzyme(s) in tumour-necrosis-factor-α-mediated apoptosis: role for lipoxygenase pathway but not mitochondrial respiratory chain," *Biochemical Journal*, vol. 310, no. 1, pp. 133–141, 1995.

[59] P. Rosenberg, E. Condrea, B. E. Rapuano, K. R. Soons, and C. C. Yang, "Dissociation of pharmacological and enzymatic activities of snake venom phospholipases A_2 by modification of carboxylate groups," *Biochemical Pharmacology*, vol. 32, no. 23, pp. 3525–3530, 1983.

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 408068, 7 pages
http://dx.doi.org/10.1155/2014/408068

Research Article

Inhibitory Effect of Plant *Manilkara subsericea* against Biological Activities of *Lachesis muta* Snake Venom

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Received 28 August 2013; Revised 9 December 2013; Accepted 10 December 2013; Published 8 January 2014

Academic Editor: Leonardo A. Calderon

Snake venom is composed of a mixture of substances that caused in victims a variety of pathophysiological effects. Besides antivenom, literature has described plants able to inhibit injuries and lethal activities induced by snake venoms. This work describes the inhibitory potential of ethanol, hexane, ethyl acetate, or dichloromethane extracts and fractions from stem and leaves of *Manilkara subsericea* against *in vivo* (hemorrhagic and edema) and *in vitro* (clotting, hemolysis, and proteolysis) activities caused by *Lachesis muta* venom. All the tested activities were totally or at least partially reduced by *M. subsericea*. However, when *L. muta* venom was injected into mice 15 min first or after the materials, hemorrhage and edema were not inhibited. Thus, *M. subsericea* could be used as antivenom in snakebites of *L. muta*. And, this work also highlights Brazilian flora as a rich source of molecules with antivenom properties.

1. Introduction

Snakebites are a public health problem that affects mainly poor people who live in rural areas of Africa, Asia, Latin America, and Oceania [1, 2]. According to World Health Organization, it is estimated that up to five million people are bitten by snakes every year, and of these, 100,000 deaths occur annually, with 400,000 amputations and other severe health consequences (such as infection, tetanus, and scarring). Poor access to health care and scarcity of antivenoms increase the severity of the injuries and their outcomes. Snake venoms are a complex mixture of proteins, including metalloproteinase, serine protease, phospholipase A_2, disintegrins, C-type lectins, myotoxins, and others [3, 4]. *Lachesis muta* (Bushmaster) is the longest venomous snake in the Americas and is distributed in the equatorial forests east of the Andes, ranging from eastern Ecuador, Colombia, Peru, northern Bolivia, and eastern and northern Venezuela, to Guyana, French Guyana, Surinam, and northern Brazil. Within their range, they are often abundant and are an important cause of snakebites [5]. *L. muta* snakebites are mainly characterized by systemic (generalized bleeding, coagulopathy, renal failure, and shock) and local effects (pain, hemorrhage, edema, and necrosis) [6, 7]. In South America, *B. jararaca* has a higher incidence of accidents (95%) than *L. muta* (2%), but, on the other hand, *L. muta* bites led to more severe symptoms (listed above) and have lethality indexes three times higher than *B. jararaca* [8]. Thus, reducing these indexes is necessary and must be fast.

Although antivenoms are effective against systemic effects, they do not inhibit the local effects. And, a few countries produce antivenom of adequate quality as well. Moreover,

antivenoms may produce side effects and their cost of production may be expensive. In fact, worldwide the production of antivenoms is a global challenge. Due to all these limitations, the search for alternative or complementary treatment for snakebites deserves attention.

Plants are a rich source of pharmacologically active molecules that have been used for native people to treat several diseases, including snakebites. Several molecules have been isolated and tested against some lethal activities induced by snake venoms [9–11]. *Manilkara subsericea* (Mart.) Dubard (Sapotaceae), popularly known in Brazil as guracica, is an endemic species with edible fruits from the Brazilian Atlantic forest and grows in sandy coastal plains [12]. A chemical composition study of genus *Manilkara* has shown the presence of triterpenes [13, 14], saponins [15], and flavonoids [16]. The extracts of *Manilkara* displayed antimicrobial [13, 17, 18], antiparasitic [19, 20], anticholinesterase [21], against pest insects [22], or antitumoral [23] properties.

The present work investigates the ability of different ethanolic extracts of leaves and stem of *M. subsericea* and solvent-partitioned fractions to neutralize some *in vitro* and *in vivo* biological activities induced by *L. muta* snake venom.

2. Materials and Methods

2.1. Chemicals. All solvents were of the best analytical grade. Dimethylsulfoxide (DMSO) and azocasein were obtained from Sigma Chemical Co. (St. Louis, Missouri).

2.2. Plant Material and Extraction. Manilkara subsericea (Mart.) Dubard (Sapotaceae) was collected in January 2009 at the Jurubatiba Sandbank National Park, located in the Brazilian state of Rio de Janeiro (220° 16′ 4.93″ S–41° 38′ 8.50″ W), under license number 13659-2 from Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis—BAMA/SISBIO. The material was identified by Professor Dr. Marcelo Guerra Santos, a member of Department of Botany of State University of Rio de Janeiro (UERJ) and a voucher specimen was deposited in the Herbarium of UERJ and assigned under the number 13416.

The leaves (1.930 kg) and stems (0.960 kg) of *M. subsericea* were dried at 40°C for two days, ground, and macerated with ethanol 96% (v/v) at room temperature until exhaustion. Then, both materials were filtered and solvent evaporated using a rotary evaporator, yielding 530 g of ethanolic extract of leaves (ETL) and 169.3 g ethanolic extract of stems (ETS). These extracts were sequentially washed with solvents of increasing polarities (hexane and ethyl acetate). After complete removal of solvent under reduced pressure, using a rotary evaporator, 21.6 g hexane-soluble (HEL) and 11.5 g ethyl acetate-soluble (EAL) fractions from leaves and 12.1 g hexane (HES) and 13.7 g ethyl acetate (EAS) fractions from stems were obtained. All materials were dissolved in DMSO (30% v/v) to perform the biological assays.

2.3. Venom and Animals. Lachesis muta lyophilized venom was kindly supplied from Fundação Ezequiel Dias (FUNED), Brazil. Venom was diluted in physiological saline (1 mg/mL) and stored at −20°C until experiments. Male Swiss mice (18–20 g) were obtained from the Center of Laboratory Animals (NAL) of the Federal Fluminense University (UFF). All experiments were approved by the UFF Institutional Committee for Ethics in Animal Experimentation (protocol number 212) that were in accordance with the guidelines of the Brazilian Committee for Animal Experimentation (COBEA).

2.4. Proteolytic Activity. Proteolytic activity of *L. muta* venom was determined according to the method of Garcia et al. [24], using azocasein as substrate (0.2%, w/v, in 20 mM Tris-HCl, 8 mM $CaCl_2$, and pH 8.8). Aliquots of *L. muta* venom (5–50 μg/mL) were incubated with 0.4 mL azocasein at 37°C for 90 min in a total volume of 1.2 mL. The enzymatic reaction was stopped by adding trichloroacetic acid (5%, v/v, final concentration). The tubes were centrifuged at 15,000 ×g for 3 min. Then, the supernatant was removed and mixed with 2 N NaOH, and the tubes were read at A 420 nm (spectrophotometer Hitachi U-5100) to measure the release of azo dye and recorded from the samples. An effective concentration (EC) was defined as the amount of venom (μg/mL) able to produce a variation of 0.2 units at A 420 nm. For the inhibitory experiments, one EC of *L. muta* venom (8.75 μg/mL) was incubated at room temperature with the extracts or fractions of *M. subsericea* at 1 : 10 venom : plant (w/w). Then, proteolytic activity was determined accordingly. Positive control experiments were performed by incubating *L. muta* venom with saline or DMSO (1% v/v).

2.5. Clotting Activity. A pool of citrated normal human plasma (diluted with equal volume of saline) was obtained from the local blood bank (University Hospital Antônio Pedro of UFF) and was mixed with *L. muta* snake venom (2.5–70 μg/mL) and the clotting time was monitored using an Amelung coagulometer, model KC4A (Labcon, Germany). The concentration of venom (μg/mL) that clotted plasma in 60 seconds was considered as the minimum coagulant dose (MCD). To evaluate the inhibitory effect, the extracts or fractions of *M. subsericea* were incubated at room temperature for 30 minutes with one MCD of venom (25 μg/mL) at 1 : 10 venom : plant (w/w), and then the mixture was added to plasma and clotting time was recorded. Negative control experiments were performed in parallel by mixing extracts or DMSO (0.5% v/v) with plasma in the absence of venom.

2.6. Hemolytic Indirect Activity. Hemolytic activity of *L. muta* venom was determined by the indirect hemolytic test using human erythrocytes and hen's egg yolk emulsion as substrate [25]. One minimum indirect hemolytic dose (MIHD) was defined as the lowest amount of *L. muta* venom (μg/mL) able to produce 100% hemolysis. To verify the inhibitory action of *M. subsericea*, one MIDH (17.6 μg/mL) was incubated at room temperature for 30 minutes with *M. subsericea* extracts or fractions at 1 : 10 venom : plant (w/w) ratio. Then, the hemolytic assay was performed. One hundred percent of hemolysis was obtained after lysing erythrocytes with distilled water. Negative control experiments were performed

in parallel by mixing extracts or DMSO (1% v/v) with erythrocytes in the absence of venom.

2.7. Hemorrhagic Activity. Hemorrhagic lesions produced by *L. muta* crude venom were quantified using a procedure described by Kondo et al. [26], with minor modifications. Briefly, samples of venom (100 μL) were injected intradermally (i.d.) into abdominal skin of mice. Two hours later, animals were euthanized and the skin was removed, stretched, and inspected for visual changes in the inner surface or subcutaneous layers to localize hemorrhagic spots. Hemorrhage was quantified as the minimum hemorrhagic dose (MHD), defined as the amount of venom (μg/g) able to produce a hemorrhagic halo of 10 millimeters (mm) and that was the positive control. To evaluate the inhibitory effect, the extracts or fractions were incubated with two MHD (1.56 μg/g) of venom at room temperature for 30 minutes at 1 : 10 venom : plant ratio (w/w). This mixture was injected i.d. into animals and hemorrhagic activity evaluated, as described above. In another set of experiments, *L. muta* venom was injected i.d. 15 min first or after injection of *M. subsericea* extract or fractions. Hemorrhage was expressed as the mean diameter (in millimeters) of the hemorrhagic halo induced by venom in the absence and presence of the extract. Negative control experiments were performed in parallel by injecting i.d. extracts or DMSO (1% v/v) in the absence of venom.

2.8. Edematogenic Activity. Edema-inducing activity of *L. muta* venom was determined according to Yamakawa et al. [27], with modifications. Groups of mice received subcutaneously (s.c.) 50 μL of *L. muta* venom (0.74 μg/g) in the right paw, while the left paw received 50 μL of saline, DMSO (1% v/v), or extracts. One hour after injection, edema was evaluated as the percentage increase in weight of the right paw compared to the left one. Antiedematogenic activity was performed by incubating the extracts or fractions of *M. subsericea* with *L. muta* venom for 30 min at room temperature at 1 : 10 venom : plant ratio (w/w), and then mixture was injected s.c. into mice. Also, venom was also injected s.c. prior to or after the extract. Positive control experiments were performed by incubating *L. muta* venom with saline or DMSO (1% v/v) prior to injection.

2.9. Statistical Analysis. Results are expressed as means ± SEM of indicated number of animals or experiments performed. Student's t-test was used and P values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. The L. muta Venom-Induced Proteolysis. The crude ethanolic extract of stem (ETS) and leaves (ETL) of *M. subsericea* (87.5 μg/mL) inhibited 96% and 93%, respectively, the proteolytic activity of *L. muta* venom (8.75 μg/mL) (Figure 1). Further, ETS and ETL were partitioned using two solvents of increasing polarities, hexane, and ethyl acetate, yielding hexane-soluble and ethyl acetate-soluble fractions, respectively. The ethyl acetate-soluble fraction of stem (EAS) inhibited 99% proteolysis induced by *L. muta* venom, whereas

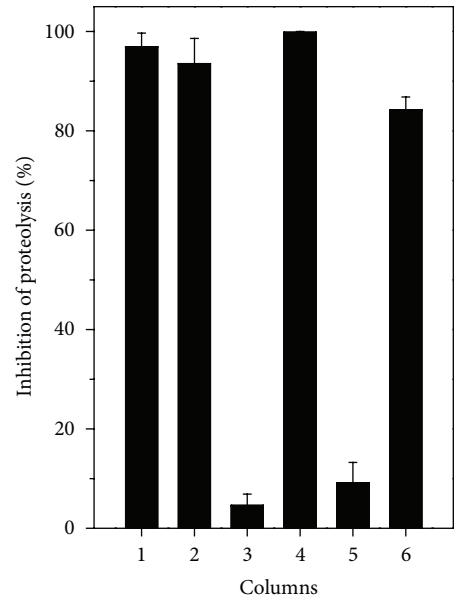

Figure 1: Effect of *M. subsericea* extracts on proteolysis induced by *L. muta* venom. *L. muta* venom (8.75 μg/mL) was incubated with extracts of *M. subsericea* (87.5 μg/mL) for 30 minutes; then proteolysis activity was measured as described in the Methods. Columns are *L. muta* mixed with (1) ethanolic extract of stem; (2) ethanolic extract of leaves; (3) hexane-soluble fraction of stem; (4) ethyl acetate-soluble fraction of stem; (5) hexane-soluble fraction of leaves; (6) ethyl acetate-soluble fraction of leaves. Data are expressed as means ± S.E.M of three individual experiments (n = 3).

the hexane one (HES) inhibited 4%. And, ethyl acetate-soluble (EAL) and hexane-soluble (HEL) fractions of the leaves inhibited 84% and 9% proteolytic activity, respectively (Figure 1).

3.2. The L. muta Venom-Induced Clotting. ETL (250 μg/mL) fully prevented *L-muta*-induced clotting and ETS delayed clotting so that it took around eight times longer, when compared to control value (*L. muta* incubated with DMSO or saline) that clotted plasma in around 60 seconds (Figure 2). When venom was incubated with EAS or EAL, plasma clotted around 300 and 150 seconds, respectively (Figure 2). On the other hand, HES or HEL did not inhibit clotting induced by venom. DMSO (0.5% v/v, final concentration) did not interfere on clotting time induced by *L. muta* venom (Figure 2).

3.3. The L. muta Venom-Induced Hemolysis. The extracts ETL or EAS (176 μg/mL) inhibited 100% hemolysis induced by *L. muta* venom, while ETS or HES inhibited circa of 95% and HEL and ETL inhibited 75% and 60% hemolysis, respectively (Figure 3).

3.4. The L. muta Venom-Induced Hemorrhage. L. muta venom (1.56 μg/g) injected into mice induced a hemorrhage halo of 20 mm that represents two MHD. But, in mice injected with venom mixed with ETL, ETS, EAS, or EAL (15.6 μg/g),

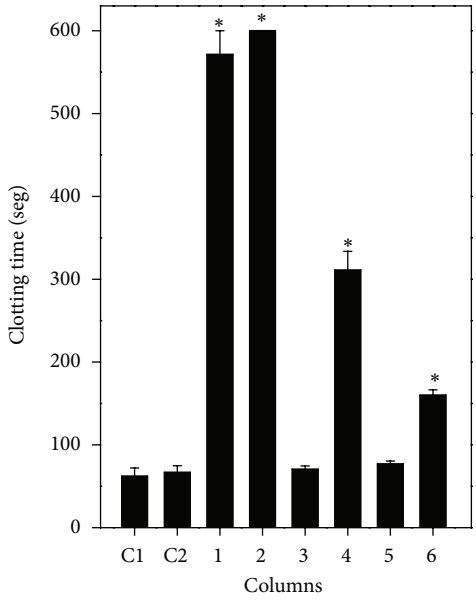

Figure 2: Effect of *M. subsericea* extracts on clotting induced by *L. muta* venom. *L. muta* venom (25 μg/mL) was incubated for 30 minutes with *M. subsericea* extracts (250 μg/mL); then mixtures were added to plasma and clotting monitored, as described in the Methods. Columns are *L. muta* venom incubated with NaCl (C1); with DMSO (C2), with ethanolic extract of stem (1) or leaves (2); with hexane- (3) or ethyl acetate- (4) soluble fractions of stem; with hexane- (5) or ethyl acetate- (6) soluble fraction of leaves. Data are expressed as means ± S.E.M of three individual experiments ($n = 3$). Significance level ($P < 0.05$) when compared to C1 or C2 columns. # means that plasma did not clot until 600 seconds of observation.

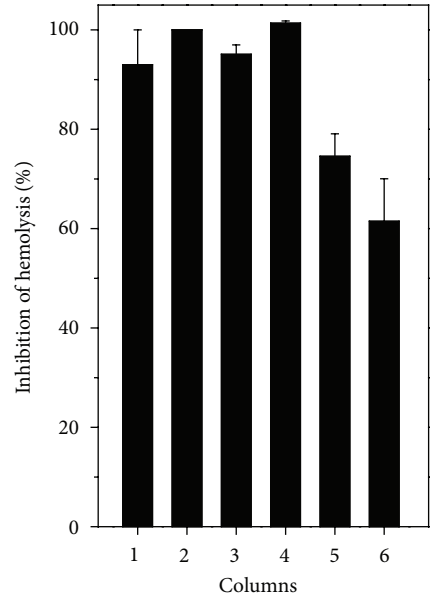

Figure 3: Effect of *M. subsericea* extracts on hemolysis induced by *L. muta* venom. *L. muta* venom (17.6 μg/mL) was incubated with *M. subsericea* extracts (176 μg/mL) for 30 minutes, and then hemolytic activity was performed. *L. muta* was incubated with ethanolic extracts of stem (1) or leaves (2); with hexane- (3) or ethyl acetate- (4) soluble fraction of stem; with hexane- (5) or ethyl acetate- (6) soluble fraction of leaves. Data are expressed as means ± S.E.M of three individual experiments ($n = 3$).

the mice were fully protected from hemorrhage induced by the venom (Figure 4). HEL inhibited the hemorrhage halo by 46% such activity and HES did not protect mice from *L. muta*-induced hemorrhage (Figure 4). However, when *L. muta* venom was injected into mice 15 minutes prior to or after ETS or ETL, protection was not observed anymore (results not shown).

3.5. The L. muta Venom-Induced Edema. ETL (7.3 μg/g) fully inhibited the formation of edema induced by *L. muta* venom (0.73 μg/g), and ETS inhibited around 70% (Figure 5). HES and EAS inhibited edema below 5% and for EAS or EAL, 40 and 60% inhibition was observed. DMSO (1% v/v) neither interfered on hemorrhagic or edema of venom nor induced hemorrhage.

4. Discussion

Snakebites represent an important public health problem, mainly in rural and poor areas of developing countries, and because of that, they are considered neglected diseases according to World Health Organization. *L. muta* snakebites cause pain, edema, necrosis, hemorrhage, vascular disturbances, and death. Since the antiserum treatment is not so effective against local effects, it is necessary to search for alternative or complementary methods in order to efficiently neutralize such effects. Native people have used plants and their metabolites to treat several diseases, including snake bite [9], and the literature has proven their efficacy as well as their metabolites against some harmful activities of snake venoms [28].

Thus, in this work, we tested the ability of ethanolic crude extracts from stem and leaves of *M. subsericea* and also their soluble fractions (hexane-soluble and ethyl acetate-soluble) to inhibit some toxic and harmful activities induced by *L. muta* venom. Overall, extracts of the stem and leaves of *M. subsericea* as well as its fractions inhibited such activities. Their inhibition occurred around 90% the proteolytic activity of *L. muta* venom that has been associated with some symptoms that follow snakebites, as hemorrhage and/or clotting. As seen, extracts of stem or leaves inhibited clotting and hemorrhage induced by *L. muta* venom and thus would prevent bleeding or vascular disturbances caused by *L. muta* bites. ETL inhibited clotting more efficiently than ETS, but EAS was the most powerful to prevent clotting. In addition, ETS, ETL, EAS, and EAL inhibited 100% hemorrhage induced by *L. muta*. Hemorrhage induced by snake venoms may occur through activating blood clotting and/or degrading specific proteins (as fibrinogen) of the blood cascade system. Metalloproteases and serine proteases are responsible for such harmful effects. Data from the literature state that plants or their products, mainly those enriched in phenolic compounds [29], inhibit hemorrhage by chelating metal

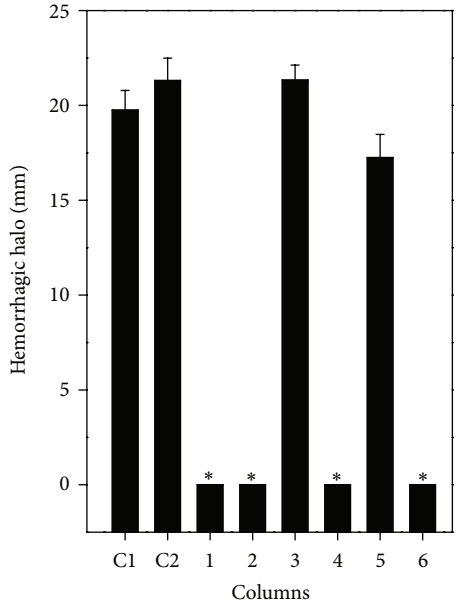

Figure 4: Effect of *M. subsericea* on hemorrhage induced by *L. muta* venom. *L. muta* venom (1.56 μg/g) was incubated for 30 minutes with *M. subsericea* extracts (15.6 μg/g); then mixture was injected into mice and hemorrhage evaluated, as described in the Methods. *L. muta* was incubated with saline (C1) or DMSO (C2); with ethanolic extracts of stem (1) or leaves (2); with hexane- (3) or ethyl acetate- (4) soluble fraction of stem; with hexane- (5) or ethyl acetate- (6) soluble fraction of leaves. Data are expressed as means ± S.E.M of three individual experiments ($n = 4$). Significance level ($P < 0.05$) when compared to C1 or C2 columns.

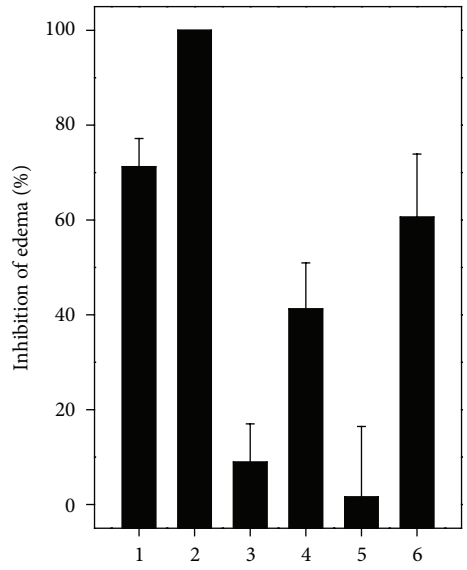

Figure 5: Effect of *M. subsericea* on *L. muta* venom-induced edema. *L. muta* venom (0.73 μg/g) was incubated for 30 minutes with *M. subsericea* extracts (7.3 μg/g); then mixtures were injected into mice and edema was evaluated, as described in the Methods. *L. muta* venom was incubated with ethanolic extracts of stem (1) or leaves (2); with hexane- (3) or ethyl acetate- (4) soluble fraction of stem; with hexane- (5) or ethyl acetate- (6) soluble fraction of leaves. Data are expressed as means ± S.E.M of three individual experiments ($n = 4$).

ions that are essential for the action of metalloproteases and serine proteases [9, 30, 31]. Other plants constituents, as flavonoids, xanthenes, and terpenoids, may also bind to the catalytic site of enzymes and, in turn, inhibit their enzymatic activity [32, 33]. Previous reports have identified components isolated from the root of *Hemidesmus indicus* [34] or from *Vitex negundo* and *Emblica officinalis* [35] able to neutralize lethal, hemorrhagic, clotting, and inflammatory activities of Viper venom, probably through an antioxidant action [36]. Phospholipases A_2 promote several harmful activities, including hemolysis, platelet aggregation, edema, and myotoxicity [25, 37, 38], and as seen, *M. subsericea* interfered on some of these effects. ETS, ETL, HES, and HEL of *M. subsericea* inhibited 100% hemolysis, but only ETL fully protected mice from edema. ETS or EAL inhibited edema above 60% and EAS inhibited it by 40%. Polar and nonpolar secondary metabolites have been described as responsible for the inhibition of PLA_2 in snake venom [39]. Besides plants, other natural products are inhibitors of PLA_2 enzymes, such as diterpenes from the marine brown algae *Spatoglossum schröederi* [31] or *Canistrocarpus cervicornis* [40]. In addition, these algae also inhibited clotting and hemorrhagic activities induced by *L. muta* venom [31, 40]. Proteolysis, edema, and hemorrhage contribute to and/or are responsible for the local effects observed in victims after snakebite, which lead to amputation or morbidity in victims. Previous reports have indicated the presence of beta- and alpha-amyrin esters, such as their acetates, caprylates, and caproates in the hexanic extracts of leaves and stems [13, 22] that had antivenom properties. Moreover, spectroscopic analysis indicates the presence of some flavonoids, as quercetin, myricetin, and kaempferol-like in the ethyl acetate-soluble fraction of stems or leaves of *M. subsericea* (unpublished data). It should be mentioned that these substances displayed antivenom activity [9] and may be partially responsible for the inhibitory properties observed at the present study.

5. Conclusion

Since *M. subsericea* extracts inhibited *in vivo* (hemorrhagic and edematogenic) and *in vitro* (hemolytic, proteolytic, and clotting) harmful activities induced by *L. muta* venom, they may have a potential to the development of an alternative or complementary treatment for snakebites caused by *L. muta*.

Conflict of Interests

The authors declares that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are grateful to Dra. Olga Maria Diniz Pereira for providing the blood bags and to Brazilian funding agencies

(FAPERJ and CNPq) and UFF/PROPPi. Authors also want to thank Dr. Norman Ratcliffe, from Swansea University, United Kingdom for kindly revising the paper.

References

[1] J. M. Gutiérrez, H. W. Fan, C. L. M. Silvera, and Y. Angulo, "Stability, distribution and use of antivenoms for snakebite envenomation in Latin America: report of a workshop," *Toxicon*, vol. 53, no. 6, pp. 625–630, 2009.

[2] J. M. Gutiérrez, G. León, and T. Burnouf, "Antivenoms for the treatment of snakebite envenomings: the road ahead," *Biologicals*, vol. 39, no. 3, pp. 129–142, 2011.

[3] J. J. Calvete, L. Sanz, Y. Ângulo, B. Lomonte, and J. M. Gutiérrez, "Venoms, venomics, antivenomics," *FEBS Letters*, vol. 583, no. 5, pp. 1736–1743, 2009.

[4] K. J. Clemetson, "Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors," *Toxicon*, vol. 56, no. 7, pp. 1236–1246, 2010.

[5] J. A. Campbell and W. W. Lamar, *The Venomous Reptiles of Latin America*, Cornell University Press, Ithaca, NY, USA, 1989.

[6] J. M. Gutiérrez, "Comprendiendo los venenos de serpientes: 50 años de investigaciones en América Latina," *Revista de Biologia Tropical*, vol. 50, no. 2, pp. 377–394, 2002.

[7] A. M. Soares, M. R. M. Fontes, and J. R. Giglio, "Phospholipase A2 myotoxins from Bothrops snake venoms: structure-function relationship," *Current Organic Chemistry*, vol. 8, no. 17, pp. 1677–1690, 2004.

[8] World Health Organization, *Guidelines for the Production Control and Regulation of Snake Antivenom Immunoglobulins*, WHO, Geneva, Switzerland, 2010.

[9] W. B. Mors, M. C. do Nascimento, B. M. R. Pereira, and N. A. Pereira, "Plant natural products active against snake bite—the molecular approach," *Phytochemistry*, vol. 55, no. 6, pp. 627–642, 2000.

[10] M. S. Santhosh, M. Hemshekhar, K. Sunitha et al., "Snake venom induced local toxicities: plant secondary metabolites as an auxiliary therapy," *Mini-Reviews in Medicinal Chemistry*, vol. 13, no. 1, pp. 106–123, 2013.

[11] J. Vásquez, S. L. Jiménez, I. C. Gómez et al., "Snakebites and ethnobotany in the Eastern region of Antioquia, Colombia-the traditional use of plants," *Journal of Ethnopharmacology*, vol. 146, no. 2, pp. 449–455, 2013.

[12] R. Gomes, M. C. B. Pinheiro, H. A. de Lima, and L. D. R. Santiago-Fernandes, "Biologia floral de *Manilkara subsericea* e de *Sideroxylon obtusifolium* (Sapotaceae) em restinga," *Revista Brasileira de Botânica*, vol. 33, no. 2, pp. 271–283, 2010.

[13] C. P. Fernandes, A. L. Corrêa, J. F. R. Lobo et al., "Triterpene esters and biological activities from edible fruits of *Manilkara subsericea* (Mart.) Dubard, Sapotaceae," *BioMed Research International*, vol. 2013, Article ID 280810, 7 pages, 2013.

[14] G. Misra and C. R. Mitra, "*Mimusops manilkara*, constituents of fruit and seed," *Phytochemistry*, vol. 8, no. 1, pp. 249–252, 1969.

[15] C. Lavaud, G. Massiot, M. Becchi, G. Misra, and S. K. Nigam, "Saponins from three species of *Mimusops*," *Phytochemistry*, vol. 41, no. 3, pp. 887–893, 1996.

[16] J. Ma, X.-D. Luo, P. Protiva et al., "Bioactive novel polyphenols from the fruit of *Manilkara zapota* (Sapodilla)," *Journal of Natural Products*, vol. 66, no. 7, pp. 983–986, 2003.

[17] A. Cáceres, H. Menéndez, E. Méndez et al., "Antigonorrhoeal activity of plants used in Guatemala for the treatment of sexually transmitted diseases," *Journal of Ethnopharmacology*, vol. 48, no. 2, pp. 85–88, 1995.

[18] R. Nair and S. Chanda, "Antimicrobial activity of *Terminalia catappa*, *Manilkara zapota* and *Piper betel* leaf extract," *Indian Journal of Pharmaceutical Sciences*, vol. 70, no. 3, pp. 390–393, 2008.

[19] S. Muelas-Serrano, J. J. Nogal, R. A. Martínez-Díaz, J. A. Escario, A. R. Martínez-Fernández, and A. Gómez-Barrio, "*In vitro* screening of American plant extracts on *Trypanosoma cruzi* and *Trichomonas vaginalis*," *Journal of Ethnopharmacology*, vol. 71, no. 1-2, pp. 101–107, 2000.

[20] J. Desrivot, J. Waikedre, P. Cabalion et al., "Antiparasitic activity of some New Caledonian medicinal plants," *Journal of Ethnopharmacology*, vol. 112, no. 1, pp. 7–12, 2007.

[21] C. P. Fernandes, A. L. Corrêa, R. A. S. Cruz et al., "Anticholinesterasic activity of *Manilkara subsericea* (Mart.) Dubard triterpenes," *Latin American Journal of Pharmacy*, vol. 30, no. 8, pp. 1631–1634, 2011.

[22] C. P. Fernandes, A. Xavier, J. P. Pacheco et al., "Laboratory evaluation of the effects of *Manilkara subsericea* (Mart.) Dubard extracts and triterpenes on the development of *Dysdercus peruvianus* and *Oncopeltus fasciatus*," *Pest Management Science*, vol. 69, no. 2, pp. 292–301, 2013.

[23] G. M. Cragg, D. J. Newman, and S. S. Yang, "'Natural product extracts of plant and marine origin having antileukemia potential' the NCI experience," *Journal of Natural Products*, vol. 69, no. 3, pp. 488–498, 2006.

[24] E. S. Garcia, J. A. Guimarães, and J. L. Prado, "Purification and characterization of a sulfhydryl-dependent protease from *Rhodnius prolixus* midgut," *Archives of Biochemistry and Biophysics*, vol. 188, no. 2, pp. 315–322, 1978.

[25] A. L. Fuly, O. L. Machado, E. W. Alves, and C. C. Carlini, "Mechanism of inhibitory action on platelet activation of a phospholipase A_2 isolated from *Lachesis muta* (Bushmaster) snake venom," *Thrombosis and Haemostasis*, vol. 78, no. 5, pp. 1372–1380, 1997.

[26] H. Kondo, S. Kondo, H. Ikegawa, and R. Murata, "Studies on the quantitative method for determination of hemorrhagic activity of habu snake venom," *Japanese Journal of Medical Science and Biology*, vol. 13, no. 1-2, pp. 43–52, 1960.

[27] M. Yamakawa, M. Nozani, and Z. Hokama, "Fractionation of sakishima-habu (*Trimeresurus elegans*) venom and lethal hemorrhagic, and edema forming activities of the fractions," in *Toxins: Animal, Plant and Microbial*, A. Ohsaka, K. Hayashi, and Y. Sawai, Eds., pp. 97–120, Plenum Press, New York, NY, USA, 1976.

[28] R. C. de Paula, E. F. Sanchez, T. R. Costa et al., "Antiophidian properties of plant extracts against *Lachesis muta* venom," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 16, no. 2, pp. 311–323, 2010.

[29] J. Leanpolchareanchai, P. Pithayanukul, and R. Bavovada, "Anti-necrosis potential of polyphenols against snake venoms," *Immunopharmacology and Immunotoxicology*, vol. 31, no. 4, pp. 556–562, 2009.

[30] B. O. Owuor and D. P. Kisangau, "Kenyan medicinal plants used as antivenin: a comparison of plant usage," *Journal of Ethnobiology and Ethnomedicine*, vol. 2, article 7, pp. 1–8, 2006.

[31] T. F. S. Domingos, F. A. Ortiz-Ramirez, R. C. Villaça et al., "Inhibitory effect of a Brazilian marine brown alga *Spatoglossum schröederi* upon biological activities of *Lachesis muta* snake venom," *Brazilian Journal of Pharmacognosy*, vol. 22, no. 4, pp. 741–747, 2012.

[32] B. Havsteen, "Flavonoids, a class of natural products of high pharmacological potency," *Biochemical Pharmacology*, vol. 32, no. 23, pp. 1141–1148, 1983.

[33] Z. E. Selvanayagam, S. G. Gnanavendhan, K. Balakrishna et al., "Ehretian one, a novel quinonoid xanthene from *Ehretia buxifolia* with antisnake venom activity," *Journal of Natural Products*, vol. 59, no. 7, pp. 664–667, 1996.

[34] M. I. Alam, B. Auddy, and A. Gomes, "Isolation, purification and partial characterization of viper venom inhibiting factor from the root extract of the Indian medicinal plant sarsaparilla (*Hemidesmus indicus* R.br.)," *Toxicon*, vol. 32, no. 12, pp. 1551–1557, 1994.

[35] M. I. Alam and A. Gomes, "Snake venom neutralization by Indian medicinal plants (*Vitex negundo* and *Emblica officinalis*) root extracts," *Journal of Ethnopharmacology*, vol. 86, no. 1, pp. 75–80, 2003.

[36] A. Bhattacharya, A. Chatterjee, S. Ghosal, and S. K. Bhattacharya, "Antioxidant activity of active tannoid principles of *Emblica officinalis* (amla)," *Indian Journal of Experimental Biology*, vol. 37, no. 7, pp. 676–680, 1999.

[37] J. M. Gutiérrez and B. Lomonte, "Phospholipases A_2: unveiling the secrets of a functionally versatile group of snake venom toxins," *Toxicon*, vol. 62, no. 5, pp. 27–39, 2013.

[38] V. C. Carregari, R. S. Floriano, L. Rodrigues-Simioni et al., "Biochemical, pharmacological, and structural characterization of new basic PLA_2 Bbil-TX from *Bothriopsis bilineata* snake venom," *BioMed Research International*, vol. 2013, Article ID 61264, 12 pages, 2013.

[39] R. P. Samy, P. Gopalakrishnakone, and V. T. K. Chow, "Therapeutic application of natural inhibitors against snake venom phospholipase A_2," *Bioinformation*, vol. 8, pp. 48–57, 2012.

[40] L. A. Moura, E. F. Sanchez, E. M. Bianco, R. C. Pereira, V. L. Teixeira, and A. L. Fuly, "Antiophidian properties of a dolastane diterpene isolated from the marine brown alga *Canistrocarpus cervicornis*," *Biomedicine & Preventive Nutrition*, vol. 1, no. 1, pp. 61–66, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 153045, 8 pages
http://dx.doi.org/10.1155/2013/153045

Review Article

Snake Venom PLA_2s Inhibitors Isolated from Brazilian Plants: Synthetic and Natural Molecules

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Received 31 May 2013; Accepted 19 August 2013

Academic Editor: Fernando Albericio

Ophidian envenomation is an important health problem in Brazil and other South American countries. In folk medicine, especially in developing countries, several vegetal species are employed for the treatment of snakebites in communities that lack prompt access to serum therapy. However, the identification and characterization of the effects of several new plants or their isolated compounds, which are able to inhibit the activities of snake venom, are extremely important and such studies are imperative. Snake venom contains several organic and inorganic compounds; phospholipases A_2 (PLA_2s) are one of the principal toxic components of venom. PLA_2s display a wide variety of pharmacological activities, such as neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant, hemorrhagic, and edema-inducing effects. PLA_2 inhibition is of pharmacological and therapeutic interests as these enzymes are involved in several inflammatory diseases. This review describes the results of several studies of plant extracts and their isolated active principles, when used against crude snake venoms or their toxic fractions. Isolated inhibitors, such as steroids, terpenoids, and phenolic compounds, are able to inhibit PLA_2s from different snake venoms. The design of specific inhibitors of PLA_2s might help in the development of new pharmaceutical drugs, more specific antivenom, or even as alternative approaches for treating snakebites.

1. Introduction

Venomous snakebites represent an important risk for public health worldwide, especially in tropical regions where these accidents are more common. Snake venom is composed by a mixture of inorganic ions (calcium potassium, iron, cobalt, copper, and magnesium), organic compounds like carbohydrate, serotonin, histamine, bradykinin potentiating peptide, disintegrins, and proteins with or without catalytic activity (L-amino acid oxidases, lectins, hyaluronidases, serine proteases, metalloproteases, and phospholipases A_2) [1]. The phospholipase A_2 enzymes (PLA_2s, E.C. 3.1.1.4, and phosphatide sn-2 acylhydrolases) are one of the most important enzymes for its effect. The PLA_2 class includes several polypeptides with similar enzymatic functions; however, these proteins exert a variety of relevant toxic actions, such as neurotoxicity and myotoxicity [2].

Secreted phospholipases A_2 ($sPLA_2$s) catalyze the hydrolysis of glycerophospholipids in sn-2 position and promote the release of lysophospholipids and fatty acids, such as the arachidonic acid. The arachidonic acid is a precursor of prostaglandins and leukotrienes, and it is involved in inflammatory process characterized by increase by microvascular permeability and oedema formation, leukocyte recruitment into tissues, nociception, and release of inflammatory mediators which mimic a number of systemic and local inflammatory disorders in humans [1–5]. In addition, the

excess levels of $sPLA_2$s were associated with many physiopathological processes as cerebral illnesses, cardiovascular disorders, cancers, asthma, respiratory distress syndrome, and progression of tonsillitis [4–8]. On the other hand, the increased $sPLA_2$ activity is observed in some brain tumours, in chronic neurological disorders associated with neurodegenerative diseases, such as neural trauma, Alzheimer's, and Parkinson's diseases, and may serve as a marker of increases in permeability of the blood-cerebrospinal fluid barrier [9, 10].

PLA_2s show considerable identity in their amino acid sequence [11, 12], but the three-dimensional structure similarity among group II sPLA2s is considerably higher, and this fact shows the importance of the 3D structure for the biological activities [2, 13–16]. Venom of different snake species is used as sources of PLA_2, due to the abundance of these enzymes and the fact that the purification of these molecules is relatively simple [17–19].

The apparent contradiction between structural uniformity and functional diversity, exhibited by PLA_2s, has attracted much interest from the scientific community. According to Ohno and collaborators [20], this diversity of pharmacological and toxic effects may have been evolutionarily acquired by positive Darwinian selection of the coding exons of these activities.

Due to a high degree of structural similarity between the $sPLA_2$s from snake venom and the human, it is a prerequisite to use the snake venom PLA_2 inhibitors for the design of new drugs for human diseases because the new inhibitory drugs must be related to the transitional state of the enzyme [2, 21]. Small variations among PLA_2 isoforms may be used for the study of structural and functional relationships of these proteins. Moreover, research regarding natural and synthetic inhibitors that are able to neutralize the toxic effects promoted by these enzymes is being carried out in an attempt to explain the physiopathological mechanisms of these molecules [22–24]. Furthermore, knowledge about the mechanism of toxicity exhibited by these proteins may assist the discovery and development of new anti-inflammatory drugs, cellular lesions, and therapies for several diseases, including Parkinson's, Alzheimer's, and even cancer [12, 25–29].

Treatment of snakebites is still carried out using traditional antivenom therapy [30]. However, although antivenom therapy is effective for the majority of cases, some side effects exist for these treatments, including adverse reactions on the skin, gastrointestinal tract, and respiratory and circulatory systems [31, 32]. Moreover, snake antivenom therapy is usually unable to prevent the progress of local effects [30]. Given the limitations of traditional therapy, research focusing on the interactions between PLA_2s and their natural or synthetic inhibitors could allow the development of alternative treatments for the toxic and pharmacological effects of snake bites [23, 33]. Plant extracts have become a promising alternative to substitute traditional snake antivenom, which often are unavailable in emergency situations [34, 35]. After studying plants commonly used to treat snakebites in South America, Soares and collaborators [34] reported 56 vegetal species that exhibited anti-inflammatory activity caused by crude snake venom or by their isolated components.

2. PLA_2 Inhibitors Isolated from Plants

Plants are used in traditional medicine to treat the effects of venomous snake bites. Pharmacological studies have shown that fractions of these plant extracts have anti-inflammatory, antiviral, and antivenom properties [36, 37]. The effect of specific molecules from these plant extracts may be attributed to the presence of multiple factors, such as low molecular weight of chemical compounds and the abundance of chemical and pharmacological properties [33].

Borges et al. [38] reported that the aqueous extract of *Casearia sylvestris* (Flacourtiaceae), a native vegetal species found in Brazilian open pastures, had the ability to inhibit myotoxic, anticoagulant, and edema-inducing activities from *Bothrops moojeni*, *B. pirajai*, *B. neuwiedi,* and *B. jararacussu* venom and its Asp49 and Lys49-PLA_2 isolated toxins. In addition, Borges and collaborators [39] emphasized that *C. sylvestris* was able to neutralize hemorrhagic activity caused by the *B. pirajai*, *B. jararacussu*, *B. asper*, *B. moojeni,* and *B. neuwiedi* venom. Cavalcante and collaborators [22] showed that the *C. sylvestris* aqueous extract demonstrated protective effects against muscle damage induced by two Lys49-PLA_2 toxins (PrTX-I from *B. pirajai* and BthTX-I from *B. jararacussu* snake venom) and prevented the neuromuscular blockage induced by all PLA_2 toxins.

Mandevilla velutina (Apocynaceae) is a perennial plant from the Brazilian *cerrado* that has been studied for its anti-inflammatory activity, as well as its antagonist effect on bradykinin, a vasodilator [40]. These authors reported that the aqueous extract of this plant was an effective inhibitor of phospholipase A_2 activity and some toxic effects, such as hemorrhage, caused by venom from snakes of the *Bothrops* and *Crotalus* genus. In a posterior study, the same research group reported that extracts from *Mandevilla illustris* were able to completely inhibit the activity of the Crotoxin B, the basic Asp49-PLA_2, isolated from *Crotalus durissus terrificus* venom [41].

The antihemorrhagic properties of the aqueous extract of *Pentaclethra macroloba* (Fabaceae), an ethnomedicinal plant found in the Amazon region, were evaluated against snake venom and displayed a full inhibition of hemorrhagic and nucleolytic activities induced by several snake venom. Additionally, a partial inhibition of myotoxic, lethal, enzymatic and edema activities of snake venom, and their isolated PLA_2s was observed [42].

Almeida and collaborators [43] showed that the aqueous extract of *Tabernaemontana catharinensis* (Apocynaceae), which is encountered in some countries of South America, was able to inhibit the crotoxin complex, isolated from *C. d. terrificus* venom, and was also able to partially neutralize the myotoxicity of *B. jararacussu* snake venom and its basic PLA_2s [44].

The aqueous extract of the aerial parts of *Bauhinia forficate* (Fabaceae), a species native to Asia and well adapted and developed in several regions of Brazil, was assayed against the fibrinogenolytic and coagulant activities of *C. d. terrificus* and *B. jararacussu* crude venom and was found to neutralize these effects. Moreover, the extract efficiently inhibited the

edema induced by *C. d. terrificus* venom and its isolated PLA_2 [45].

Mendes and collaborators [46] reported that the aqueous extract of *Schizolobium parahyba* (Fabaceae), a plant found in the Mata Atlântica of southeastern Brazil, contains compounds that can inhibit some enzymatic and biological activities induced by *Bothrops pauloensis* (current *Bothropoides paulensis*) and *C. d. terrificus* snake venom as well as by their isolated neuwiedase toxins (metalloproteinase), BnSP-7 (basic Lys49-PLA_2 from *B. paulensis* venom), and Crotoxin B.

The ethanolic extract of the aerial parts of *Blutaparon portulacoides* (Amaranthaceae), an herbaceous plant that occurs mainly in the Atlantic bush, caused a reduction in edema formation and in the leukocyte influx induced by Lys49-PLA_2 and isolated from *B. jararacussu* venom [47].

In 2005, Maiorano and collaborators [48] evaluated aqueous extracts prepared from dried or fresh roots, stems, or leaves of *Mikania glomerata* (Asteraceae), a plant found in the Mata Atlântica in Southeastern Brazil and popularly known as "Guaco." The *M. glomerata* extract efficiently neutralized different toxic, pharmacological, and enzymatic effects induced by *Bothrops* and *Crotalus* snake venom. The phospholipase A_2 activity and the edema induced by *C. d. terrificus* venom were inhibited by approximately 100 and 40%, respectively, and this inhibition was also partially observed with the *Bothrops* venom. The hemorrhagic activities of *B. alternatus*, *B. moojeni*, *B. neuwiedi*, and *B. jararacussu* venom were significantly inhibited by *M. glomerata* extract, while the clotting activities of *C. d. terrificus*, *B. jararacussu*, and *B. neuwiedi* venom were totally inhibited. In addition, Floriano and collaborators [49] reported clinical and laboratory alterations in mice caused by the combination of *M. glomerata* leaf extract and antiophidian serum against *C. d. terrificus* venom.

Nazato and collaborators [50] found that the methanolic extract of the bark from *Dipteryx alata* (Fabaceae) (a native species of the Brazilian Savanna, found principally in Minas Gerais, Goiás, Federal District, and Mato Grosso) decreased the neurotoxicity and myotoxicity of *B. jararacussu* crude venom. In another study by Puebla and collaborators [51], the extract from *D. alata* was fractionated and its compounds were evaluated against the neuromuscular blockade caused by *B. jararacussu* venom.

The ability of the ethanolic extract of *Hypericum brasiliense* (Hypericaceae), a plant found mainly in the southeastern and southern regions of Brazil, to neutralize some effects induced by *B. jararaca* venom was investigated using biological assays. *H. brasiliense* extracts were able to inhibit some pharmacological effects such as lethality, edema, hemorrhage, hemolysis and, proteolysis, as well as fibrinogen or plasma clotting [52].

In 2012, Dey and De [53] published a review that evaluated several pharmacological studies on plant efficacies against snakebites. The authors compiled studies from a number of plants or their fractionsthat were active against snake venom and concluded that folk knowledge is relevant. However, clinical tests should be performed with these plant extracts or fractions to assess the effect of the compounds used for the treatment of snakebites.

Recently, Samy et al. [54] published an extensive revision on the therapeutic application of natural inhibitors of snake venom PLA_2s, covering molecules from the primary metabolism of different organisms, such as glycoproteins (PLIs), peptides, and lipids, as well as from secondary metabolism, exemplified by terpenoids, alkaloids, flavonoids, and other molecules. These authors concluded that the biotechnological potential of PLA_2 inhibitors may provide therapeutic molecular models with antiophidian activity to supplement conventional serum therapy or for the development of novel antivenom therapeutics. Additionally, inhibitors isolated from medicinal plants may also be an essential tool in isolated communities [23, 54].

3. Structural Characterization of PLA_2 Inhibitors

The main classes of PLA_2 inhibitors are the phenolic compounds, which include flavonoids, coumestans and alkaloids, steroids and terpenoids (mono-, di-, and triterpenes), and polyphenols (vegetable tannins). There is also mention in the literature of other molecules such as carbohydrates, lipids, and proteins, although this paper emphasizes molecules originating from plant secondary metabolism.

3.1. Phenolic Compounds

3.1.1. Flavonoids. Polyphenolic secondary metabolites are commonly able to bind to biological polymers, and some of these have been shown to inhibit PLA_2s. Examples include quercetin, a strong lipoxygenase inhibitor, naringenin, artemetin, kaempferol, and galangin, among several other flavonoids. Primetin (Figure 1), 5,8-dihydroxyflavone, isolated from *Primula* sp. (Primulaceae), is known for its ability to inhibit toxins from snake venom; its structural form may be seen in Figure 1. Flavonoids usually exert their inhibitory effect via hydrophobic interactions with the A and B rings and aromatic or hydrophobic amino acid residues in the protein [36, 55, 56].

3.1.2. Coumestans. *Eclipta alba* (Asteraceae) is a native plant from Brazil and other tropical and subtropical areas of the world whose medicinal properties are widely known. *E. alba* was genetically engineered using *Agrobacterium rhizogenes* LB9402 to enhance the production of secondary wedelolactone metabolites, which are coumestan compounds with activity against basic PLA_2s. This mutant strain was found to reduce the phospholipase A_2 activities and myotoxic and neurotoxic effects of the *C. d. terrificus* and *B. jararacussu* snake venom [37, 57]. Analogs of wedelolactone molecule (Figure 1) were able to antagonize the release of creatine kinase induced by *B. jararacussu* venom even at concentrations as low as 30 μM [58, 59].

3.1.3. Alkaloids. Batina and collaborators [60] isolated an alkaloid from *Tabernaemontana catharinensis* (Apocynaceae) named 12-methoxy-4-methylvoachalotine (Figure 1) and

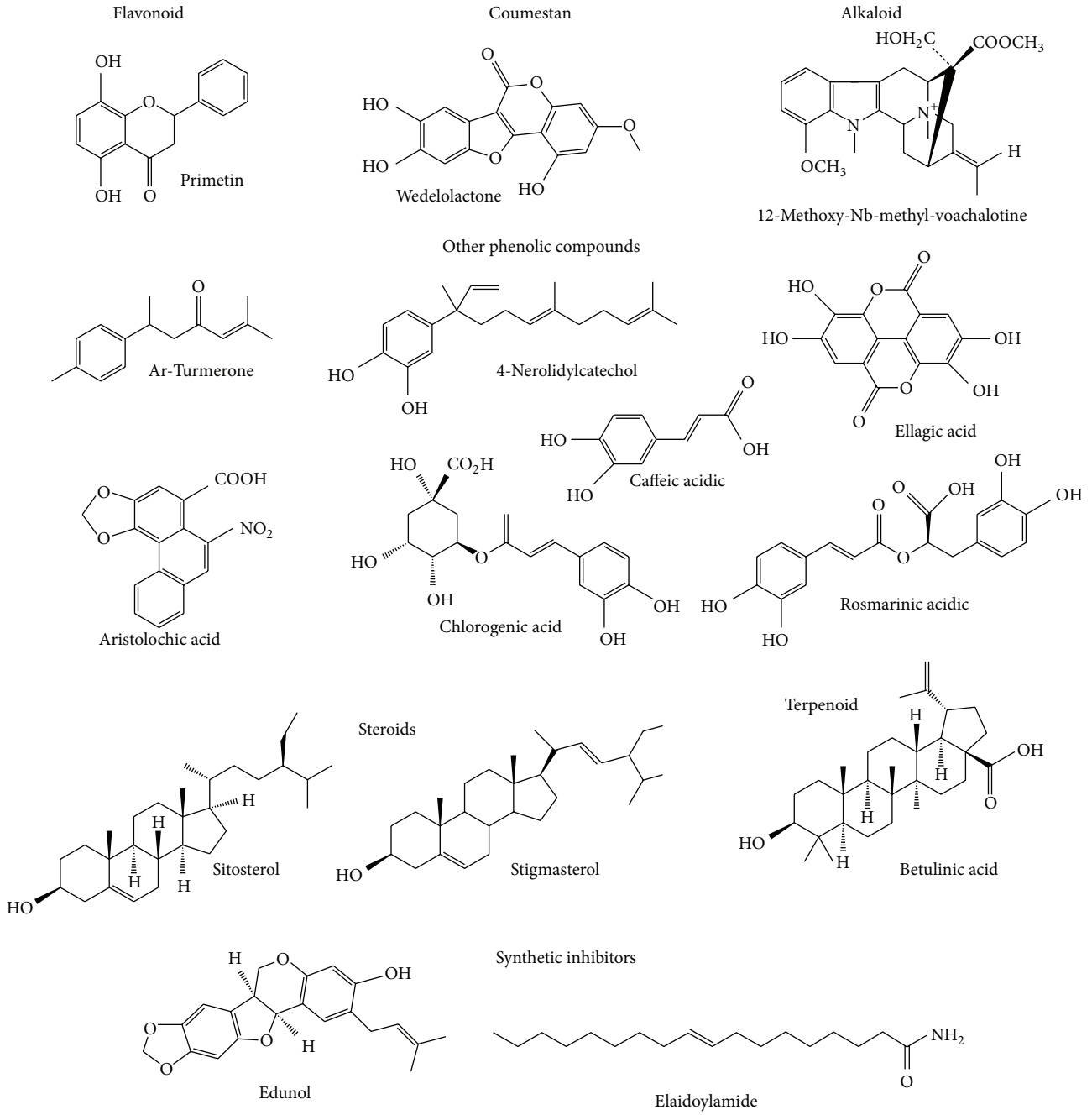

Figure 1: Structures of bioactive compounds with inhibitory potential against the snake venom or its phospholipase A_2 fraction. Draw using ACD/ChemSketch program (http://www.acdlabs.com/).

reported a strong inhibitory effect against lethality and myotoxic activities induced by *C. d. terrificus* venom.

3.1.4. Other Phenolic Compounds. Ar-Turmerone (Figure 1) is a phenolic compound isolated from the *Curcuma longa* (Zingiberaceae) plant that has a strong effect against the hemorrhage and lethality caused by *B. jararaca* and *C. d. terrificus* snake venom [61].

Extracts from *Piper umbellatum* and *P. peltatum* (Piperaceae)were shown to inhibit the myotoxic activities of PLA_2s isolated from *Bothrops* snake venom [62]. Fractionation of these plant extracts revealed that 4-nerolidylcatechol, a hydroxylated phenolic compound (Figure 1), was responsible for at least part of the inhibitory effect against groups I, II, and III of PLA_2s.

In 2008, Da Silva and collaborators [63] studied the half maximal inhibitory concentration (IC_{50}) of ellagic acid (Figure 1), extracted from *C. sylvestris,* against BthTX-II, a basic Asp49-PLA_2 from *B. jararacussu* snake venom, and concluded that this compound was effective at competitively inhibiting the induction of edema, myotoxicity, and enzymatic activities, incurred by this PLA_2.

The first structural analysis of aristolochic acid (Figure 1), isolated from *Aristolochia* sp. (Aristolochiaceae), was performed by Vishwanath and Gowda [64]. In this study, the interaction of aristolochic acid, an alkaloid, with PLA_2 from *Vipera russelli* was characterized as noncompetitive inhibitive. This compound has also been shown to reduce the induction of edema by this enzyme. Additionally, Vishwanath et al. [65] emphasized that the interaction between aristolochic acid, from *Aristolochia radix,* and three PLA_2s from *Trimeresurus flavoviridis* resulted in the inhibition of hemolytic and edema induction by competitive inhibition. Chandra and collaborators [66] reported the crystal structure of the complex formed between the PLA_2 isolated from *V. russelli* venom and aristolochic acid. In this study, the interaction between aristolochic acid and PLA_2 was competitive, and the final model consists of a dimer of PLA_2 and one molecule of aristolochic acid located in the binding site of molecule A; this interaction was stabilized by three hydrogen bonds and hydrophobic contacts.

Chlorogenic and caffeic acids (Figure 1) can interact with proteins via hydrophobic contacts and hydrogen bonds, inhibiting enzyme function and acting as antidotes. Strong interactions may induce conformational changes in the protein structure [26]. In 2011, Shimabuku and collaborators [67] crystallized PrTX-I (basic Lys49-PLA_2 from *B. pirajai* snake venom) in the presence of the inhibitor, caffeic acid, and the electron-density map which unambiguously indicated the presence of three caffeic acid molecules interacting with the C-terminus of the protein.

Rosmarinic acid (Figure 1) is a hydroxylated phenolic compound isolated from *Cordia verbenacea* (Boraginaceae). This compound demonstrates antimyotoxic properties and inhibits edema induced by crude *B. jararacussu* snake venom and its basic PLA_2s [36, 55]. The three-dimensional structure of the PrTX-I, rosmarinic acid complex, was elucidated by Santos and collaborators [68], where rosmarinic acid was observed located at the entrance of the hydrophobic channel monomer A of the PrTX-I dimer via an interaction between hydrogen bonds and hydrophobic contacts in the same monomer. Interactions were also observed between rosmarinic acid and a residue of the C-terminal region of the monomer B. The interaction between the rosmarinic acid molecule with the hydrophobic channel (monomer A) and the C-terminal region (myotoxic site, monomer B) suggests two mechanisms of myotoxicity inhibition [68].

3.2. Steroid Compounds. Sterol and cholesterol molecules present well-known antidote activities against snake venom. Steroids can form complexes that are stabilized via Van der Waals interactions, as well as by hydrophobic interactions [37]. Antimyotoxic and antihemorrhagic effects of the *Eclipta prostrata* (Asteraceae) extract and its components, sitosterol and stigmasterol (Figure 1), were observed against *B. jararaca*, *B. jararacussu*, and *Lachesis muta* snake venom [37, 69]. Previously, Mors [70] reported that sitosterol and stigmasterol, isolated from *E. prostrata,* prevented the lethality of the *C. d. terrificus* venom in a dose-dependent manner.

3.3. Terpenoids. The neoclerodane, diterpenoid, isolated from the aerial parts of *Baccharis trimera* (Asteraceae), demonstrate anti-hemorrhagic and antiprotolithic properties against *Bothrops* snake venom [71]

Several pentacyclic triterpenes, such as oleanolic acid, lupeol, ursolic acid, taraxerol, taraxasterol, α,β-amyrin, and friedeline, exhibit activity against snake venom [37]. Triterpenoids, isolated from *Betula alba* (Betulaceae), including pentacyclic triterpenes betulin and betulinic acid (Figure 1), exhibited antiphospholipasic A_2 activity. Docking (*in silico* experiments) indicated betulinic acid as the best PLA_2 inhibitor, due to its direct insertion in the catalytic site on the enzyme, with a very low energy value [55].

3.4. Synthetic Inhibitors. Edunol (Figure 1) is a pterocarpan with a chemical structure similar to those of the inhibitors extracted from the roots of *Harpalyce brasiliana* (Fabaceae). Edunol was obtained via chemical synthesis, and the compound showed anti-myotoxic, anti-proteolytic, and anti-PLA_2 activities against *B. jararacussu* crude venom [55, 72].

Elaidoylamide, the amide of trans-9-octadecenoic acid (Figure 1), is a powerful synthetic inhibitor of a neurotoxic Asp49-PLA_2 from *Vipera ammodytes meridionalis* venom. In 2003, Georgieva and collaborators [73] isolated the neurotoxic complex from *V. a. meridionalis* venom, dissociated the basic PLA_2 from the complex, and crystallized it with elaidoylamide. This final structure contained two identical homodimers and one molecule of elaidoylamide bound simultaneously to the substrate-binding sites of each homodimer [74].

Villar and collaborators [33] demonstrated that synthetic inhibitor derivatives from nitrostyrene that contain typical nitro groups at the *ortho*-, *meta*-, and *para*- positions on the aromatic ring were more efficient against the enzymatic, edematogenic, and myotoxic activities of PLA_2s from *B. jararacussu* venom. Da Silva and collaborators [75, 76], performing molecular modeling studies between Asp49-PLA_2 from *C. adamanteus* venom and synthetic derivatives polyhydroxy phenolic compounds, concluded that some conformations of these groups might positively influence enzymatic activity inhibition.

Isolated inhibitors (natural or synthetic) can be important tools for understanding the mechanisms of action of PLA_2s from snake venom, and, consequently, these results might be helpful for the design of a drug that specifically inhibits PLA_2s. However, the synthesis of compounds analogous to their natural equivalents, based on chemical characteristics or with minor structural modifications, is often necessary. The synthesis of compounds could be justified by the low amount of these compounds available in vegetal extracts or to adjust some specific chemical characteristics. For this reason, some researchers have isolated and characterized new compounds or produced synthetic analogues for use in the commercial production of pharmaceutical drugs.

Acknowledgments

The authors gratefully acknowledge financial support from Coordenação de Aperfeiçoamento de Nível Superior

(CAPES), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

[1] D. C. I. Koh, A. Armugam, and K. Jeyaseelan, "Snake venom components and their applications in biomedicine," *Cellular and Molecular Life Sciences*, vol. 63, no. 24, pp. 3030–3041, 2006.

[2] R. K. Arni and R. J. Ward, "Phospholipase A_2—a structural review," *Toxicon*, vol. 34, no. 8, pp. 827–841, 1996.

[3] C. D. Funk, "Prostaglandins and Leukotrienes: advances in eicosanoid biology," *Science*, vol. 294, no. 5548, pp. 1871–1875, 2001.

[4] F. S. Markland Jr, "Snake venoms and the hemostatic system," *Toxicon*, vol. 36, no. 12, pp. 1749–1800, 1998.

[5] S. L. da Silva, M. Comar Jr., K. M. T. Oliveira et al., "Molecular modeling of the inhibition of enzyme PLA_2 from snake venom by dipyrone and 1-phenyl-3-methyl-5-pyrazolone," *International Journal of Quantum Chemistry*, vol. 108, no. 13, pp. 2576–2585, 2008.

[6] Z. Mallat, G. Lambeau, and A. Tedgui, "Lipoprotein-associated and secreted phospholipases A_2 in cardiovascular disease: roles as biological effectors and biomarkers," *Circulation*, vol. 122, no. 21, pp. 2183–2200, 2010.

[7] D. de Luca, A. Minucci, P. Cogo et al., "Secretory phospholipase A_2 pathway during pediatric acute respiratory distress syndrome: a preliminary study," *Pediatric Critical Care Medicine*, vol. 12, no. 1, pp. e20–e24, 2011.

[8] R. Ezzeddini, M. Darabi, B. Ghasemi et al., "Circulating phospholipase A_2 activity in obstructive sleep apnea and recurrent tonsillitis," *International Journal of Pediatric Otorhinolaryngology*, vol. 76, no. 4, pp. 471–474, 2012.

[9] S. Chalbot, H. Zetterberg, K. Blennow et al., "Blood-cerebrospinal fluid barrier permeability in Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 25, no. 3, pp. 505–515, 2011.

[10] A. A. Farooqui, M. L. Litsky, T. Farooqui, and L. A. Horrocks, "Inhibitors of intracellular phospholipase A_2 activity: their neurochemical effects and therapeutical importance for neurological disorders," *Brain Research Bulletin*, vol. 49, no. 3, pp. 139–153, 1999.

[11] S. S. Teixeira, L. B. Silveira, F. M. N. da Silva et al., "Molecular characterization of an acidic phospholipase A_2 from Bothrops pirajai snake venom: synthetic C-terminal peptide identifies its antiplatelet region," *Archives of Toxicology*, vol. 85, no. 10, pp. 1219–1233, 2011.

[12] L. Romero, S. Marcussi, D. P. Marchi-Salvador et al., "Enzymatic and structural characterization of a basic phospholipase A_2 from the sea anemone *Condylactis gigantea*," *Biochimie*, vol. 92, no. 8, pp. 1063–1071, 2010.

[13] L. C. Corrêa, D. P. Marchi-Salvador, A. C. O. Cintra, S. V. Sampaio, A. M. Soares, and M. R. M. Fontes, "Crystal structure of a myotoxic Asp49-phospholipase A_2 with low catalytic activity: insights into Ca^{2+} independent catalytic mechanism," *Biochimica et Biophysica Acta*, vol. 1784, no. 4, pp. 591–599, 2008.

[14] D. P. Marchi-Salvador, L. C. Corrêa, A. J. Magro, C. Z. Oliveira, A. M. Soares, and M. R. M. Fontes, "Insights into the role of oligomeric state on the biological activities of crotoxin: crystal structure of a tetrameric phospholipase A_2 formed by two isoforms of crotoxin B from *Crotalus durissus terrificus* venom," *Proteins*, vol. 72, no. 3, pp. 883–891, 2008.

[15] D. P. Marchi-Salvador, C. A. H. Fernandes, L. B. Silveira, A. M. Soares, and M. R. M. Fontes, "Crystal structure of a phospholipase A_2 homolog complexed with *p*-bromophenacyl bromide reveals important structural changes associated with the inhibition of myotoxic activity," *Biochimica et Biophysica Acta*, vol. 1794, no. 11, pp. 1583–1590, 2009.

[16] C. A. H. Fernandes, D. P. Marchi-Salvador, G. H. M. Salvador et al., "Comparison between apo and complexed structures of bothropstoxin-I reveals the role of Lys122 and Ca^{2+}-binding loop region for the catalytically inactive Lys49-PLA_2s," *Journal of Structural Biology*, vol. 171, no. 1, pp. 31–43, 2010.

[17] J. L. C. Cardoso, F. O. S. França, F. H. Wen, C. M. S. Málaque, and V. Haddad Jr, *Animais peçonhentos no Brasil: biologia clinica e terapêutica dos acidentes*, Sarvier, São Paulo, Brazil, 2003.

[18] D. C. S. Damico, M. A. C. Höfling, M. Cintra et al., "Pharmacological study of edema and myonecrosis in mice induced by venom of the bushmaster snake (*Lachesis muta muta*) and its basic Asp49 phospholipase A_2 (LmTX-I)," *The Protein Journal*, vol. 27, no. 6, pp. 384–391, 2008.

[19] D. C. S. Damico, T. Vassequi-Silva, F. D. Torres-Huaco et al., "LmrTX, a basic PLA_2 (D49) purified from *Lachesis muta rhombeata* snake venom with enzymatic-related antithrombotic and anticoagulant activity," *Toxicon*, vol. 60, pp. 773–781, 2012.

[20] M. Ohno, R. Ménez, T. Ogawa et al., "Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution?" *Progress in Nucleic Acid Research and Molecular Biology*, vol. 59, pp. 307–364, 1998.

[21] E. Valentin and G. Lambeau, "What can venom phospholipases A_2 tell us about the functional diversity of mammalian secreted phospholipases A_2?" *Biochimie*, vol. 82, no. 9-10, pp. 815–831, 2000.

[22] W. L. G. Cavalcante, T. O. Campos, M. dal Pai-Silva et al., "Neutralization of snake venom phospholipase A_2 toxins by aqueous extract of *Casearia sylvestris* (Flacourtiaceae) in mouse neuromuscular preparation," *Journal of Ethnopharmacology*, vol. 112, no. 3, pp. 490–497, 2007.

[23] S. Marcussi, C. D. Sant'Ana, C. Z. Oliveira et al., "Snake venom phospholipase A_2 inhibitors: medicinal chemistry and therapeutic potential," *Current Topics in Medicinal Chemistry*, vol. 7, no. 8, pp. 743–756, 2007.

[24] C. Z. Oliveira, D. L. Menaldo, S. Marcussi et al., "An α-type phospholipase A_2 inhibitor from *Bothrops jararacussu* snake plasma: structural and functional characterization," *Biochimie*, vol. 90, no. 10, pp. 1506–1514, 2008.

[25] B. S. Cummings, J. McHowat, and R. G. Schnellmann, "Phospholipase A_2s in cell injury and death," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 294, no. 3, pp. 793–799, 2000.

[26] C. Araya and B. Lomonte, "Antitumor effects of cationic synthetic peptides derived from Lys49 phospholipase A_2 homologues of snake venoms," *Cell Biology International*, vol. 31, no. 3, pp. 263–268, 2007.

[27] B. Lomonte, Y. Angulo, and E. Moreno, "Synthetic peptides derived from the C-Terminal region of Lys49 phospholipase A_2 homologues from viperidae snake venoms: biomimetic activities and potential applications," *Current Pharmaceutical Design*, vol. 16, no. 28, pp. 3224–3230, 2010.

[28] R. Perumal Samy and P. Gopalakrishnakone, "Therapeutic potential of plants as anti-microbials for drug discovery," *Evidence-Based Complementary and Alternative Medicine*, vol. 7, no. 3, pp. 283–294, 2010.

[29] C. Z. Oliveira, N. A. Santos-Filho, D. L. Menaldo et al., "Structural and functional characterization of a γ-type phospholipase A_2 inhibitor from *Bothrops jararacussu* Snake Plasma," *Current Topics in Medicinal Chemistry*, vol. 11, no. 20, pp. 2509–2519, 2011.

[30] J. M. Gutiérrez, B. Lomonte, G. León, A. Rucavado, F. Chaves, and Y. Angulo, "Trends in snakebite envenomation therapy: scientific, technological and public health considerations," *Current Pharmaceutical Design*, vol. 13, no. 28, pp. 2935–2950, 2007.

[31] M. García, M. Monge, G. León et al., "Effect of preservatives on IgG aggregation, complement-activating effect and hypotensive activity of horse polyvalent antivenom used in snakebite envenomation," *Biologicals*, vol. 30, no. 2, pp. 143–151, 2002.

[32] J. M. Schmidt, "Antivenom therapy for snakebites in children: is there evidence?" *Current Opinion in Pediatrics*, vol. 17, no. 2, pp. 234–238, 2005.

[33] J. A. F. P. Villar, F. T. D. Lima, C. L. Veber et al., "Synthesis and evaluation of nitrostyrene derivative compounds, new snake venom phospholipase A_2 inhibitors," *Toxicon*, vol. 51, no. 8, pp. 1467–1478, 2008.

[34] A. M. Soares, A. H. Januário, M. V. Lourenço, A. M. S. Pereira, and P. S. Pereira, "Neutralizing effects of Brazilian plants against snake venoms," *Drugs of the Future*, vol. 29, no. 11, pp. 1105–1117, 2004.

[35] M. L. da Silva, S. Marcussi, R. S. Fernandes et al., "Anti-snake venom activities of extracts and fractions from callus cultures of *Sapindus saponaria*," *Pharmaceutical Biology*, vol. 50, no. 3, pp. 366–375, 2012.

[36] F. K. Ticli, L. I. S. Hage, R. S. Cambraia et al., "Rosmarinic acid, a new snake venom phospholipase A_2 inhibitor from *Cordia verbenacea* (Boraginaceae): antiserum action potentiation and molecular interaction," *Toxicon*, vol. 46, no. 3, pp. 318–327, 2005.

[37] W. B. Mors, M. C. Nascimento, B. M. R. Pereira, and N. A. Pereira, "Plant natural products active against snake bite—the molecular approach," *Phytochemistry*, vol. 55, no. 6, pp. 627–642, 2000.

[38] M. H. Borges, A. M. Soares, V. M. Rodrigues et al., "Effects of aqueous extract of *Casearia sylvestris* (Flacourtiaceae) on actions of snake and bee venoms and on activity of phospholipases A_2," *Comparative Biochemistry and Physiology B*, vol. 127, no. 1, pp. 21–30, 2000.

[39] M. H. Borges, A. M. Soares, V. M. Rodrigues et al., "Neutralization of proteases from Bothrops snake venoms by the aqueous extract from *Casearia sylvestris* (Flacourtiaceae)," *Toxicon*, vol. 39, no. 12, pp. 1863–1869, 2001.

[40] R. Biondo, A. M. S. Pereira, S. Marcussi, P. S. Pereira, S. C. França, and A. M. Soares, "Inhibition of enzymatic and pharmacological activities of some snake venoms and toxins by *Mandevilla velutina* (Apocynaceae) aqueous extract," *Biochimie*, vol. 85, no. 10, pp. 1017–1025, 2003.

[41] R. Biondo, A. M. Soares, B. W. Bertoni, S. C. França, and A. M. S. Pereira, "Direct organogenesis of *Mandevilla illustris* (Vell) Woodson and effects of its aqueous extract on the enzymatic and toxic activities of *Crotalus durissus terrificus* snake venom," *Plant Cell Reports*, vol. 22, no. 8, pp. 549–552, 2004.

[42] J. O. da Silva, J. S. Coppede, V. C. Fernandes et al., "Antihemorrhagic, antinucleolytic and other antiophidian properties of the aqueous extract from *Pentaclethra macroloba*," *Journal of Ethnopharmacology*, vol. 100, no. 1-2, pp. 145–152, 2005.

[43] L. Almeida, A. C. O. Cintra, E. L. G. Veronese et al., "Anticrotalic and antitumoral activities of gel filtration fractions of aqueous extract from *Tabernaemontana catharinensis* (Apocynaceae)," *Comparative Biochemistry and Physiology C*, vol. 137, no. 1, pp. 19–27, 2004.

[44] E. L. G. Veronese, L. E. Esmeraldino, A. P. F. Trombone et al., "Inhibition of the myotoxic activity of *Bothrops jararacussu* venom and its two major myotoxins, BthTX-I and BthTX-II, by the aqueous extract of *Tabernaemontana catharinensis* A. DC. (Apocynaceae)," *Phytomedicine*, vol. 12, no. 1-2, pp. 123–130, 2005.

[45] C. Z. Oliveira, V. A. Maiorano, S. Marcussi et al., "Anticoagulant and antifibrinogenolytic properties of the aqueous extract from *Bauhinia forficata* against snake venoms," *Journal of Ethnopharmacology*, vol. 98, no. 1-2, pp. 213–216, 2005.

[46] M. M. Mendes, C. F. Oliveira, D. S. Lopes et al., "Anti-snake venom properties of *Schizolobium parahyba* (Caesalpinoideae) aqueous leaves extract," *Phytotherapy Research*, vol. 22, no. 7, pp. 859–866, 2008.

[47] I. C. Pereira, A. M. Barbosa, M. J. Salvador et al., "Anti-inflammatory activity of *Blutaparon portulacoides* ethanolic extract against the inflammatory reaction induced by *Bothrops jararacussu* venom and isolated myotoxins BthTX-I and II," *Journal of Venomous Animals and Toxins Including Tropical Diseases*, vol. 15, no. 3, pp. 527–545, 2009.

[48] V. A. Maiorano, S. Marcussi, M. A. F. Daher et al., "Antiophidian properties of the aqueous extract of *Mikania glomerata*," *Journal of Ethnopharmacology*, vol. 102, no. 3, pp. 364–370, 2005.

[49] R. S. Floriano, R. M. B. Nogueira, M. Sakate et al., "Effect of *Mikania glomerata* (Asteraceae) leaf extract combined with anti-venom serum on experimental *Crotalus durissus* (Squamata: Viperidae) envenomation in rats," *Revista de Biología Tropical*, vol. 57, no. 4, pp. 929–937, 2009.

[50] V. S. Nazato, L. Rubem-Mauro, N. A. G. Vieira et al., "*In vitro* antiophidian properties of *Dipteryx alata*Vogel bark extracts," *Molecules*, vol. 15, no. 9, pp. 5956–5970, 2010.

[51] P. Puebla, Y. Oshima-Franco, L. M. Franco et al., "Chemical constituents of the bark of *Dipteryx alata* vogel, an active species against *Bothrops jararacussu* venom," *Molecules*, vol. 15, no. 11, pp. 8193–8204, 2010.

[52] M. Assafim, E. C. Coriolano, S. E. Benedito et al., "*Hypericum brasiliense* plant extract neutralizes some biological effects of *Bothrops jararaca* snake venom," *Journal of Venom Research*, vol. 2, pp. 11–16, 2011.

[53] A. Dey and J. N. De, "Phytopharmacology of antiophidian botanicals: a review," *International Journal of Pharmacology*, vol. 8, no. 2, pp. 62–79, 2012.

[54] R. P. Samy, P. Gopalakrishnakone, and V. T. Chow, "Therapeutic application of natural inhibitors against snake venom phospholipase A_2," *Bioinformation*, vol. 8, pp. 48–57, 2012.

[55] A. M. Soares, F. K. Ticli, S. Marcussi et al., "Medicinal plants with inhibitory properties against snake venoms," *Current Medicinal Chemistry*, vol. 12, no. 22, pp. 2625–2641, 2005.

[56] J. Lättig, M. Böhl, P. Fischer et al., "Mechanism of inhibition of human secretory phospholipase A_2 by flavonoids: rationale for lead design," *Journal of Computer-Aided Molecular Design*, vol. 21, no. 8, pp. 473–483, 2007.

[57] L. C. Diogo, R. S. Fernandes, S. Marcussi et al., "Inhibition of snake venoms and phospholipases A_2 by extracts from native and genetically modified *Eclipta alba*: isolation of active coumestans," *Basic & Clinical Pharmacology & Toxicology*, vol. 104, no. 4, pp. 293–299, 2009.

[58] A. J. M. da Silva, P. A. Melo, N. M. V. Silva et al., "Synthesis and preliminary pharmacological evaluation of coumestans with

different patterns of oxygenation," *Bioorganic & Medicinal Chemistry Letters*, vol. 11, no. 3, pp. 283–286, 2001.

[59] R. C. S. Nunomura, V. G. Oliveira, S. L. da Silva, and S. M. Nunomura, "Characterization of bergenin in Endopleura uchi bark and its anti-inflammatory activity," *Journal of the Brazilian Chemical Society*, vol. 20, no. 6, pp. 1060–1064, 2009.

[60] M. F. Batina, A. C. O. Cintra, E. L. G. Veronese et al., "Inhibition of the lethal and myotoxic activities of *Crotalus durissus terrificus* venom by *Tabernaemontana catharinensis*: identification of one of the active components," *Planta Medica*, vol. 66, no. 5, pp. 424–428, 2000.

[61] L. A. F. Ferreira, O. B. Henriques, A. A. S. Andreoni et al., "Antivenom and biological effects of ar-turmerone isolated from *Curcuma longa* (Zingiberaceae)," *Toxicon*, vol. 30, no. 10, pp. 1211–1218, 1992.

[62] V. Núñez, V. Castro, R. Murillo, L. A. Ponce-Soto, I. Merfort, and B. Lomonte, "Inhibitory effects of *Piper umbellatum* and *Piper peltatum* extracts towards myotoxic phospholipases A_2 from *Bothrops* snake venoms: isolation of 4-nerolidylcatechol as active principle," *Phytochemistry*, vol. 66, no. 9, pp. 1017–1025, 2005.

[63] S. L. da Silva, A. K. Calgarotto, J. S. Chaar, and S. Marangoni, "Isolation and characterization of ellagic acid derivatives isolated from *Casearia sylvestris* SW aqueous extract with anti-PLA_2 activity," *Toxicon*, vol. 52, no. 6, pp. 655–666, 2008.

[64] B. S. Vishwanath and T. V. Gowda, "Interaction of aristolochic acid with *Vipera russelli* phospholipase A_2: its effect on enzymatic and pathological activities," *Toxicon*, vol. 25, no. 9, pp. 929–937, 1987.

[65] B. S. Vishwanath, R. M. Kini, and T. V. Gowda, "Characterization of three edema-inducing phospholipase A_2 enzymes from habu (*Trimeresurus flavoviridis*) venom and their interaction with the alkaloid aristolochic acid," *Toxicon*, vol. 25, no. 5, pp. 501–515, 1987.

[66] V. Chandra, J. Jasti, P. Kaur, A. Srinivasan, C. H. Betzel, and T. P. Singh, "Structural basis of phospholipase A_2 inhibition for the synthesis of prostaglandins by the plant alkaloid aristolochic acid from a 1.7 Å crystal structure," *Biochemistry*, vol. 41, no. 36, pp. 10914–10919, 2002.

[67] P. S. Shimabuku, C. A. H. Fernandes, A. J. Magro, T. R. Costa, A. M. Soares, and M. R. M. Fontes, "Crystallization and preliminary X-ray diffraction analysis of a Lys49-phospholipase A_2 complexed with caffeic acid, a molecule with inhibitory properties against snake venoms," *Acta Crystallographica F*, vol. 67, no. 2, pp. 249–252, 2011.

[68] J. I. Santos, F. F. Cardoso, A. M. Soares, M. dal Pai Silva, M. Gallacci, and M. R. M. Fontes, "Structural and functional studies of a bothropic myotoxin complexed to rosmarinic acid: new insights into Lys49-PLA_2 inhibition," *PLoS One*, vol. 6, no. 12, Article ID e28521, pp. 1–11, 2011.

[69] A. D. L. Souza, E. Rodrigues-Filho, A. Q. L. Souza et al., "Koninginins, phospholipase A_2 inhibitors from endophytic fungus *Trichoderma koningii*," *Toxicon*, vol. 51, no. 2, pp. 240–250, 2008.

[70] W. B. Mors, M. C. do Nascimento, J. P. Parente, M. H. da Silva, P. A. Melo, and G. Suarez-Kurtz, "Neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of the plant *Eclipta prostrata* (Asteraceae)," *Toxicon*, vol. 27, no. 9, pp. 1003–1009, 1989.

[71] A. H. Januário, S. L. Santos, S. Marcussi et al., "Neo-clerodane diterpenoid, a new metalloprotease snake venom inhibitor from *Baccharis trimera* (Asteraceae): anti-proteolytic and anti-hemorrhagic properties," *Chemico-Biological Interactions*, vol. 150, no. 3, pp. 243–251, 2004.

[72] A. J. M. da Silva, A. L. Coelho, A. B. C. Simas et al., "Synthesis and pharmacological evaluation of prenylated and benzylated pterocarpans against snake venom," *Bioorganic & Medicinal Chemistry Letters*, vol. 14, no. 2, pp. 431–435, 2004.

[73] D. N. Georgieva, W. Rypniewski, M. Perbandt, M. Jain, N. Genov, and C. Betzel, "Crystallization and preliminary X-ray diffraction studies of a toxic phospholipase A_2 from the venom of *Vipera ammodytes meridionalis* complexed to a synthetic inhibitor," *Biochimica et Biophysica Acta*, vol. 1650, no. 1-2, pp. 1–3, 2003.

[74] D. N. Georgieva, W. Rypniewski, A. Gabdoulkhakov, N. Genov, and C. Betzel, "Asp49 phospholipase A_2-elaidoylamide complex: a new mode of inhibition," *Biochemical and Biophysical Research Communications*, vol. 319, no. 4, pp. 1314–1321, 2004.

[75] S. L. da Silva, A. K. Calgarotto, V. Maso et al., "Molecular modeling and inhibition of phospholipase A_2 by polyhydroxy phenolic compounds," *European Journal of Medicinal Chemistry*, vol. 44, no. 1, pp. 312–321, 2009.

[76] E. S. de Alvarenga, S. A. Silva, L. C. A. Barosa et al., "Synthesis and evaluation of sesquiterpene lactone inhibitors of phospholipase A_2 from *Bothrops jararacussu*," *Toxicon*, vol. 57, no. 1, pp. 100–108, 2011.

Hindawi Publishing Corporation
BioMed Research International
Volume 2013, Article ID 903292, 12 pages
http://dx.doi.org/10.1155/2013/903292

Research Article

Rapid Purification and Procoagulant and Platelet Aggregating Activities of Rhombeobin: A Thrombin-Like/Gyroxin-Like Enzyme from *Lachesis muta rhombeata* Snake Venom

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Received 4 June 2013; Accepted 29 June 2013

Academic Editor: Saulo Luís da Silva

We report a rapid purification method using one-step chromatography of SVSP Rhombeobin (LMR-47) from *Lachesis muta rhombeata* venom and its procoagulant activities and effects on platelet aggregation. The venom was fractionated by a single chromatographic step in RP-HPLC on a C8 Discovery BIO Wide Pore, showing high degree of molecular homogeneity with molecular mass of 47035.49 Da. Rhombeobin showed amidolytic activity upon BAρNA, with a broad optimum pH (7–10) and was stable in solution up to 60°C. The amidolytic activity was inhibited by serine proteinase inhibitors and reducing agents, but not chelating agents. Rhombeobin showed high coagulant activity on mice plasma and bovine fibrinogen. The deduced amino acid sequence of Rhombeobin showed homology with other SVSPs, especially with LM-TL (*L. m. muta*) and Gyroxin (*C. d. terrificus*). Rhombeobin acts, *in vitro*, as a strong procoagulant enzyme on mice citrated plasma, shortening the APTT and PT tests in adose-dependent manner. The protein showed, "*ex vivo*", a strong defibrinogenating effect with 1 μg/animal. Lower doses activated the intrinsic and extrinsic coagulation pathways and impaired the platelet aggregation induced by ADP. Thus, this is the first report of a venom component that produces a venom-induced consumptive coagulopathy (VICC).

1. Introduction

Snakes of the genus *Lachesis*, commonly known as bushmasters, or surucucu in Brazil, can be found in the tropical forests of Central and South Americas. There are three different *Lachesis* species: *L. stenophrys*, *L. melanocephala* (both found in Central America), and *L. muta*. In Brazil, two subspecies can be found: *L. muta muta* in the Amazon Forest and *L. muta rhombeata* in the Atlantic Forest along the eastern coast of Brazil [1].

Poisoning by *Lachesis* is characterized by local damage, pain, edema, hemorrhage, and myonecrosis, as well as systemic complications, such as coagulation disorders, hemolysis, neurotoxicity, renal failure, diarrhea, hypotension, and bradycardia, among others [2–5]. *Lachesis m. rhombeata* crude venom shows lower lethal and hemorrhagic activities when compared with *L. m. muta* venom, while both venoms induce similar edema-forming and myotoxic activities. However, *L. m. rhombeata* venom exhibits higher coagulant and defibrinogenating effects [6].

Snake venom serine proteinases (SVSPs) belong to the trypsin S1 family of SA clan, the largest family of peptidases [7, 8]. SVSPs are among the best-characterized venom enzymes affecting the haemostatic system. They act on a variety of components of the coagulation cascade, on the fibrinolytic and kallikrein-kinin systems, and on platelets to cause an imbalance of the haemostatic system of the prey [8].

So far, two serine proteinases were isolated from *Lachesis m. rhombeata*: a thrombin-like/gyroxin-like enzyme denominated LMR-47 [9] and LMR kininogenin [10]. However, none of these proteins were fully characterized biochemically or had their effects studied over the haemostatic system. Since the *L. m. rhombeata* crude venom induces strong effects over the haemostatic system, it was decided to investigate the participation of the SVSP LMR-47 in those effects induced by the envenomation of this snake species.

In this study, we report a one-step method of purification, prediction of primary structure, and "*in vivo*" effects over the coagulation cascade as well as over the platelet aggregation of SVTLE LMR-47, which will suggest its possible role in relation to the effects on the haemostatic system of *Lachesis m. rhombeata* crude venom. Also, we propose to rename this protein as Rhombeobin according to its thrombin-like activity.

2. Materials and Methods

2.1. Venom and Reagents. *Lachesis m. rhombeata* venom was gently donated by Dr. Rodrigo C. G. de Souza from Serra Grande Center for *Lachesis muta* Breeding, with IBAMA Authorization no. 24945-1. All chemicals and reagents were of analytical or sequencing grade. Bovine fibrinogen was purchased from Amour Pharmaceutical, CA, USA.

2.2. Animals. Four-five-week male Swiss mice were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). Mice were housed at room temperature on a 12 h light/dark cycle and had free access to food and water. All procedures were performed according to the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA) and were approved by the university's Committee for Ethics in Animal Experimentation (CEEA/UNICAMP) no. 1790-1.

2.3. Venom Fractionation. Five mg of crude venom of *L. m. rhombeata* was dissolved in 0.2 mL of solvent A (0.1% (v/v) trifluoroacetic acid). The resulting solution was clarified by centrifugation, and the supernatant was further submitted to a reversed-phase chromatography on a C8 Discovery BIO Wide Pore (25 cm × 4.6 mm × 10 μm). Fractions were eluted using a stepwise gradient of solvent B (66% acetonitrile in solvent A) (0%–55% B for 26 min, followed by 55%–65% B over 20 min, and 65%–100% B over 14 min). The flow rate of 1.0 mL/min was constant, and the resulting fractions were manually collected. The elution profile was monitored at 280 nm, and the collected fractions were lyophilized and conserved at −20°C. The homogeneity of the final material was assessed by a rechromatography on the same column with a linear gradient (0%–100%) of solvent B.

2.4. SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on purified Rhombeobin according to Laemmli [20]. The molecular mass markers used were (in kDa)the following: phosphorylase B, 94; albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; soybean trypsin inhibitor, 20; and lysozyme, 14.

2.5. Determination of Protein Concentration. Protein concentration was determined by the method of Bradford [21] and standardized with bovine serum albumin.

2.6. Amidolytic Activity and Determination of Kinetic Parameters. Amidolytic activity was measured using the synthetic substrate N-benzoyl-L-arginine ρ-nitroanilide (BAρNA) modified for 96-well plates. The standard assay mixture contained 50 μL of buffer (10 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, and 100 mM NaCl), 200 μL of substrate solution (1 mM), 15 μL of water, and 5 μL of fractions or enzyme (Rhombeobin) in a final volume of 270 μL. The reaction was carried out in a VERSAMAX microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) for 30 min at 37°C, with the absorbance being read at 410 nm. The results were expressed as the initial velocity of the reaction (V_0) calculated based on the amount of ρ-nitroaniline released [22]. The temperature and pH effect on amidolytic activity of Rhombeobin was examined by incubating the overall mixture reaction at different temperatures (10–80°C) and pH (5–11). At different BAρNA concentrations (0.0095–10 mM), the K_m and V_{max} of Rhombeobin were determined under optimal pH and temperature. The effects of inhibitors EDTA (5 mM), EGTA (5 mM), PMSF (2 mM), SBT-I (1 mg/mL), and DTT (5 mM) were tested by preincubating the sample with these compounds for 30 min at 37°C prior to the standard test. The inhibition results were expressed as percentage of residual activity.

2.7. Fibrinogenolytic Activity. Fibrinogenolytic activity was determined by mixing 10 μL of Rhombeobin with 200 μL of bovine fibrinogen solution (2 mg/mL in 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$, and 100 mM NaCl) at 37°C, at different incubation times (5 min–24 h) and different concentrations of enzyme (0.632–10 μg). The reaction was stopped with 150 μL of denaturizing solution (Tris-HCl 0.05 M, pH 6.5, urea 10 M, b-mercaptoethanol 10% (v/v), glycerol 10% (v/v), and bromophenol blue 0.05% (v/v)), incubated for 15 minutes at room temperature. Fibrinogenolytic activity was evaluated by SDS-PAGE using 12.5% polyacrylamide gels as described by Laemmli [20].

2.8. Determination of Minimum Coagulant Dose (MCD). The MCD over bovine fibrinogen (MCD-F) or plasma (MCD-P) of Rhombeobin was determined according to Theakston and Reid [23]. Briefly, 0.25 to 10 μg of Rhombeobin (20 μL PBS) were added to 200 μL of bovine fibrinogen solution (2 mg/mL) or mice citrated plasma at 37°C. The solutions

were mixed thoroughly, and the clotting times were recorded. The MCDs for bovine fibrinogen and plasma were calculated by plotting Log of Rhombeobin concentration versus Log of the clotting times. The MCD was defined as the least amount of toxin (in mg of dry weight per liter of test solution) that clots citrated plasma or bovine fibrinogen solution in 60 seconds at 37°C.

2.9. Determination of the Molecular Mass of the Purified Protein by Mass Spectrometry. An aliquot (4.5 μL, 0,1% formic acid) of Rhombeobin (10 μg) was injected by C18 (100 mm × 100 mm) RP-UPLC (nanoACQUITY UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nL/min. The gradient was 0%–50% acetonitrile in 0.1% formic acid over 45 min. The instrument operation and the processing parameters were set according to Damico et al. [24].

2.10. Sequencing Procedures. The reduced (5 mM dTT for 25 min at 56°C) and alkylated (14 mM iodoacetamide, 30 min) protein was digested with trypsin (Promega-Sequencing Grade Modified) or SV-8 (Promega-Sequencing Grade Modified). The digestion procedures, the peptide separation, and the mass analysis of the resulting peptides were made following the methodology described by Damico et al. [24].

Raw data files from LC-MS/MS runs were processed using Masslynx 4.1 software package (Waters) and were analyzed using the MASCOT search engine version 2.3 (Matrix Science, Ltd.) against the snakes database, using the following parameters: peptide mass tolerance of ±0.1 Da, fragment mass tolerance of ±0.1 Da, oxidation as variable modification in methionine, and trypsin as enzyme.

2.11. Evaluation of Gyroxin Syndrome. Rhombeobin gyroxin-like activity was determined according to Seki et al. [25]. Briefly, 25 μL of enzyme (1–20 μg protein in PBS) was injected iv. into adult mice weighing 25 ± 2 g. After injection, it was determined whether the animals showed the characteristic equilibrium loss and, eventually, typical rolling movements as described by Barrabin et al. [26]. The animals were observed 24 h after injection in order to rule out the contamination of the purified fraction with lethal toxins.

2.12. Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT). Male Swiss mice were anesthetized (xylazine, 2%–16% mg/kg; ketamine, 10%–100% mg/kg), and blood was collected from cava vein in citrate 3.2% and and centrifuged at 1500 ×g for 15 min at 25°C; after blood withdrawn was mice were euthanized by anesthesia deepening. For APTT, a 50 μL, of aliquot plasma was warmed to 37°C for 2 min, 50 μL, of APTT reagent was added, of, after a 2 min incubation at 37°C, 0.25 M of $CaCl_2$ was added, and the clotting time was determined. For the PT test, 100 μL of CLOT PT reagent was incubated for 4 min at 37°C, and 50 μL of plasma was added, triggering the reaction. These analyses were performed in triplicate, using the APTT and PT Kit CLOT BIOS diagnosis (CLOT, Brazil) in a CLOTimer coagulometer (CLOT, Brazil). To assay APTT and PT *in vitro*, mice plasma (45 μL) was preincubated with 5 μL of a different Rhombeobin (2, 5, 10, and 20 μg/mL); then the standard analyses for APTT and PT were followed. To assay APTT and PT *ex vivo,* Swiss mice were injected i.v. with Rhombeobin (0.1 and 1 μg/animal), and after 30, 60, and 90 min, blood was withdrawn and processed as described above.

2.13. Effects of Rhombeobin on the Mice Plasma Fibrinogen. The *ex vivo* test was performed according to Maruñak et al. [27] and modified as follows. Groups of mice with different exposure times (30, 60, and 90 min) and one control group, each one composite of four mice weighing 25 ± 2 g, were injected i.v. with 25 μL of PBS (control group) or Rhombeobin (0.1 and 1 μg/animal). After the times of exposure, blood samples and poor platelet plasma were obtained as described in Section 2.12. The fibrinogen content was determined using the Wiener diagnostic kit, and the results were expressed as percentage of the remaining fibrinogen compared with the control group.

2.14. Platelet Aggregation Assay. Washed platelets suspensions from mice were obtained according to Theakston and Reid [23]. Platelet aggregation was performed in an optical aggregometer (Chrono-log, Kordia Life Sciences, Leiden, Belgium) at 37°C with 400 μL of washed platelets placed in glass cuvettes containing a disposable stir bar for constant stirring. Platelet aggregation was induced using ADP (20 μM) or thrombin (0.05 U/mL) as agonist. The *ex vivo* effects of Rhombeobin over platelet aggregation were studied by injecting i.v. 25 μL of PBS or enzyme (0.1 or 1 μg/animal) in adult mice (25 ± 2 g). After 1 h, the mice were anesthetized, and washed platelets were obtained.

2.15. Statistical Analyses. Results were reported as mean ± SEM. The significance of differences among means was assessed by analysis of variance followed by Tuckey's test. The statistical analyses were made using the Origin 8 SR2 v8.0891 (B891; OriginLab Corporation, Northampton, MA, USA). Program $P < 0.05$ was considered statistically significant.

3. Results

The fractionation of *Lachesis muta rhombeata* crude venom in an RP-HPLC system (C8 column) results in twenty-three main fractions named F1 to F23 (Figure 1(a)). Each fraction was assayed for amidolytic and coagulant activities, and both were found only in F-16 fraction. Rechromatography of F-16 fraction in an RP-HPLC, with a linear gradient, results in a single symmetric peak eluted with 57% of buffer B and a retention time of 36.23 ± 2.2 min (Figure 1(b)); SDS-PAGE showed that this fraction in nonreduced and reduced conditions is a single-chain protein, with a relative molecular mass of 45 kDa (Figure 1(b): insert). The protein homogeneity of F-16 was confirmed by ESI-MS, and it was shown that fraction F-16 is protein with a real molecular mass of 47035.49 Da (Figure 1(c)).

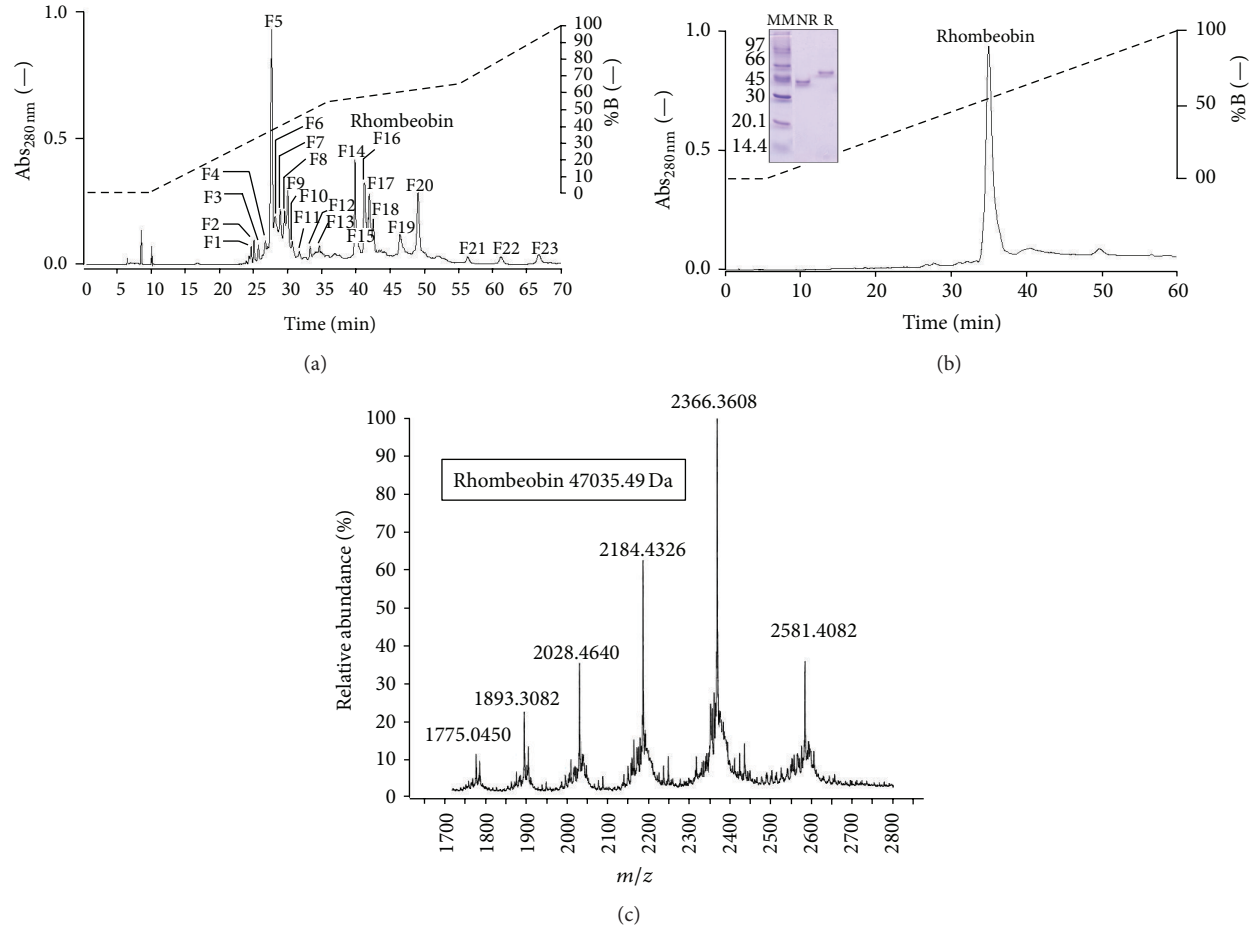


Figure 1: (a) Elution profile of *L. muta rhombeata* venom by the reversed phase HPLC on C8 Discovery BIO Wide Pore. Fraction 16 (F16) that contained both amidolytic and thrombin-like activities (Rhombeobin) from *L. muta rhombeata* venom is indicated. (b) Elution profile of F8 following RP-HPLC on C8 Discovery BIO Wide Pore. Insert: SDS-PAGE in nonreduced (NR) and reduced (R) conditions. (c) Molecular mass determination of the native Rhombeobin by nanoelectrospray tandem mass spectrometry, using a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) with output mass range of 40.000–50.000 Da at a "resolution" of 0.1 Da/channel.

The fraction F-16 was named Rhombeobin, and it showed an arginine amidase activity toward the chromogenic substrate BApNA with K_m and V_{max} of 0.8×10^{-3} M and 1.06 ± 0.106 nmol/min/mg, respectively (Figure 2(b)). The optimum temperature was between 40 and 50°C, and the remarkable stability in the pH was in the range of 7 to 10. The serineproteinase inhibitor PMSF and the reducing agent DTT completely abrogated Rhombeobin enzymatic activity, while SBT-I partially reduced ($P = 0.0269$) the enzymatic activity (Figure 2(c)). However, EDTA and EGTA did not show any effects (Figure 2(c)).

Rhombeobin is a strong thrombin-like enzyme since it can clot a bovine fibrinogen solution with an MCD-F of 18.3 mg/L of mice citrated plasma, with an MCD-P of 7.3 mg/L (Figure 2(d)).

Rhombeobin showed a concentration and time-dependent fibrinogenolytic activity. This enzyme completely degraded fibrinogen α-chain with a concentration of 1.25 μg with 15 min of incubation. Rhombeobin degraded fibrinogen β-chain at high concentrations and long incubation times; on the other hand, the enzyme did not show any activity over fibrinogen γ-chain (Figures 3(a) and 3(b)).

The protein digestion (Rhombeobin) with trypsin and protease SV-8, followed by LC/MS/MS, identified nine (T1–T9) and seven peptides (S1–S7), respectively. The deduced sequence and the measured masses of alkylated peptides are summarized in Tables 1 and 2, covering approximately 94% of the protein sequence. Each peptide was submitted separately to the SNAKE database using the protein search program BLAST-p. Using the position matches of the "*de novo*" sequenced peptides with homologous proteins present in the database, it was possible to deduce their original position on the unknown protein. Thus, it was deduced that peptides T-1, S-3, and T-8 contain the catalytic triad histidine, aspartic acid, and serine, respectively. Also, the digested peptides contain the twelve cysteine residues characteristic of the snake venom serineproteinase family. Rhombeobin showed homology (Figure 4) with other thrombin-like SVSPs from *Lachesis m. muta*, *Crotalus durissus terrificus*, *Agkistrodon bilineatus*, *Bothrops jararaca*, *Bothrops jararacussu*, *Bothrops atrox*,

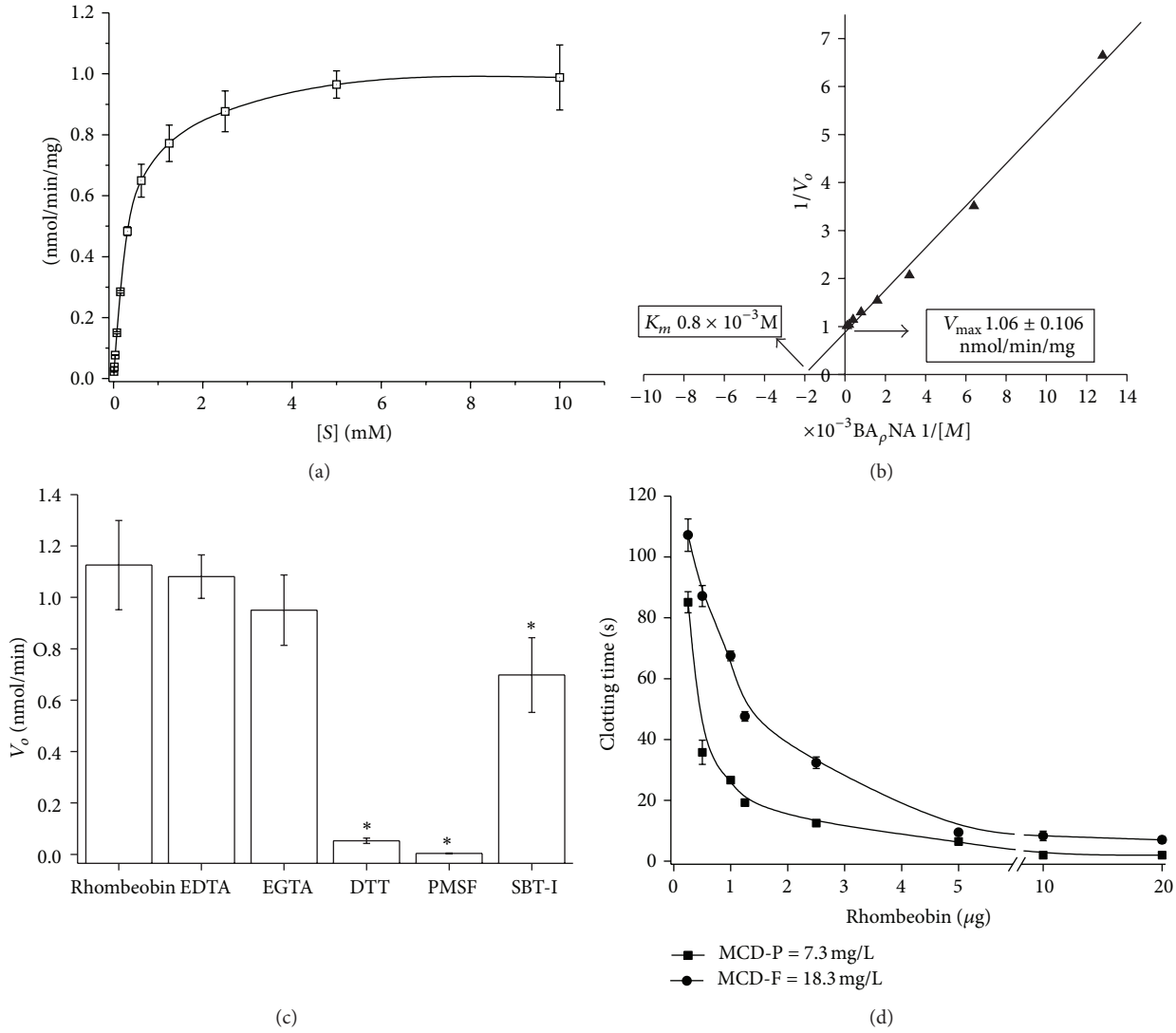


Figure 2: Enzymatic, kinetic properties and thrombin-like activity of Rhombeobin *L. m. rhombeata* venom. (a) The Michaelis-Menten curve. (b) The Lineweaver-Burk (double-reciprocal) plot. (c) Inhibition of the amidolytic activity by chelating agents (EDTA and EGTA), reducing agent (DTT), serineproteinase-specific inhibitors (PMSF), and soybean trypsin inhibitor (SBTI). (d) Minimum coagulant dose of Rhombeobin over citrated plasma (MCD-P) and fibrinogen bovine solution (2 mg/mL, MCD-F). The results of all experiments are the mean ± SEM of three determinations ($P < 0.05$).

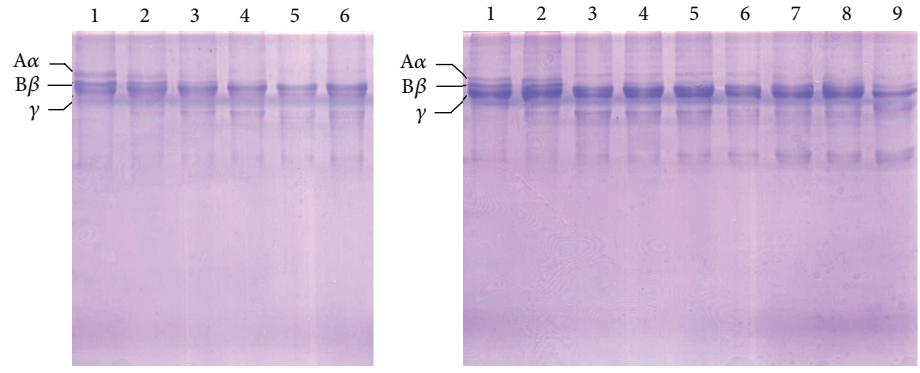


Figure 3: (a) Fibrinogenolytic activity of Rhombeobin at different concentrations: (1) bovine fibrinogen; (2) fibrinogen + Rhombeobin (0,625 μg); (3) 1.25 μg; (4) 2.5 μg; (5) 5 μg; (6) 10 μg. (b) Fibrinogenolytic activity of Rhombeobin (2.5 μg) at different incubation times: (1) bovine fibrinogen; (2) fibrinogen + Rhombeobin (5 min); (3) 15 min; (4) 30 min; (5) 1 h; (6) 3 h; (7) 6 h; (8) 12 h; (9) 24 h. The fibrinogenolytic activity was analyzed by SDS-PAGE (12.5%).

Table 1: Measured molecular masses and deduced amino acid sequences obtained by ESI-Q-Tof-MS/MS based on the alkylated tryptic peptides of Rhombeobin.

Peptide	Position	Amino acid sequence	Measured mass (Da)
T-1	1–13	VI/LGGDEC**N**I/LNEH**R**	1497.6263
T-2	60–71	VPNEDEQT**K**YP**K**	1474.7011
T-3	72–80	E**K**YFFRCPN**KK**	1463.7229
T-4	85–93	WDKDI/LMI/LI/L**R**	1188.6088
T-5	94–121	I/LDSPVSNSEHI/LAPI/LSI/LPSNPPSVGSVC**R**	2915.3930
T-6	122–132	I/LMGWGQTI/LTSP**K**	1317.6720
T-7	160–183	VI/LCAGVI/LEGGI/LDTCN**R**	1732.7533
T-8	184–217	DSGGPI/LI/LCNGQFQGI/LASWGPDPCAQPD**K**PAVYT**K**	3630.5924
T-9	218–239	VFDYTDWI/LQNI/LI/LAGNTDATCPP	2496.0740

The peptides were separated by RP-HPLC and were sequenced by mass spectrometry. *C* = alkylated cysteine; lysine and arginine residues shown in bold were deduced on the cleavage and missed cleavage by trypsin and SV-8. All molecular masses are reported as monoisotopic.

Table 2: Measured molecular masses and deduced amino acid sequences obtained by ESI-Q-Tof-MS/MS based on the alkylated SV-8 peptides of Rhombeobin.

Peptide	Position	Amino acid sequence	Measured mass (Da)
S-1	1–11	VI/LGGDECNIN**E**	1218.5202
S-2	12–36	HRFI/LVAI/LYDGI/LSGTFI/LCGGTI/LI/LNQ**E**	2780.3842
S-3	37–48	WVI/LTAAHCDS**E**	1287.5534
S-4	66–72	QTRYPK**E**	920.4763
S-5	73–82	KYFFRCPNKKND**E**	1744.8213
S-6	134–150	TI/LPDVPHCANI/LNI/LI/LDY**E**	1982.8704
S-7	151–174	VCRAAYAGI/LPATSRVI/LCAGVI/L**E**	2333.1693

The peptides were separated by RP-HPLC and were sequenced by mass spectrometry. *C* = alkylated cysteine; glutamic acid residues shown in bold were deduced on the cleavage *Streptoccocus aureus* SV-8. All molecular masses are reported as monoisotopic.

Agkistrodon acutus, and *Agkistrodon rhodostoma*, sharing high-sequence identity with them, especially with LM-TL from *Lachesis m. muta* (75.6%).

Rhombeobin induced "gyroxin syndrome" when injected into the mice tail vein at dose of 0.2 μg/kg of bodyweight. The treated animals were progressively hypoactive over a period of 1–3 min, followed by a loss of the righting reflex, opisthotonos, and rotations around the long axis, lasting up to 30 minutes. All treated animals lost the ability to right themselves. Two hours following Rhombeobin injection, the animal's behavior returned to normal. Gyroxin syndrome was not observed with lower doses, in which the main effects were flaccid paralysis (mainly of inferior extremities) and hypoactivity.

In vitro, Rhombeobin showed a strong procoagulant activity with a dose-dependent effect on citrated mice plasma, shortening significantly both APPT and PT tests (Figures 5(a) and 5(b)).

Interestingly, *ex vivo*, Rhombeobin treatment produced both anticoagulant and procoagulant effects. The i.v. injection, with 1 μg of protein, renders unclottable blood after 30 minutes of exposure. This effect was sustained through the whole time of the experiment (Figures 5(c) and 5(d)). On the other hand, Rhombeobin shortened the APTT and PT tests in mice treated with a dose ten times lower; this was a rapid and sustained effect on APTT test (Figure 5(c)); although on PT test the shortening effect was rapid, one hour after injection this effect was not observed (Figure 5(d)).

Mice plasma fibrinogen levels were determined after Rhombeobin i.v. injection. At 1 μg dose, the protein showed a strong defibrinogenating effect, with no detectable plasma fibrinogen levels after thirty minutes after injection (Figure 6(a)). On the other hand, with a dose ten times lower, only partial reduction of plasma fibrinogen levels was observed (Figure 6(a)). These results corroborate with the fibrinogenolytic activity on bovine fibrinogen analyzed by SDS-PAGE. While 0.1 μg of the protein lightly degraded fibrinogen α-chain (Figure 6(b)), 1 μg, after 90 minutes of incubation, almost completely degraded fibrinogen α-chain, producing fibrinopeptide A (Figure 6(c)).

When mice were treated with Rhombeobin i.v. at 0.1/animal and after one hour platelet suspensions were obtained, a significant reduction (P = 0,03063) of the platelet aggregating response to ADP as agonist was observed. However, no effect over the platelet aggregating response was observed when Thrombin was used as agonist (Figures 7(a) and 7(b)).

4. Discussion

This work reports an efficient and simple procedure for the purification of LMR-47: a thrombin-like/gyroxin-like enzyme from *Lachesis muta rhombeata* venom, which we renamed as Rhombeobin. In our approach, using a simple chromatographic step based on RP-HPLC showed that the *L. m. rhombeata* venom can be separate in twenty-three main

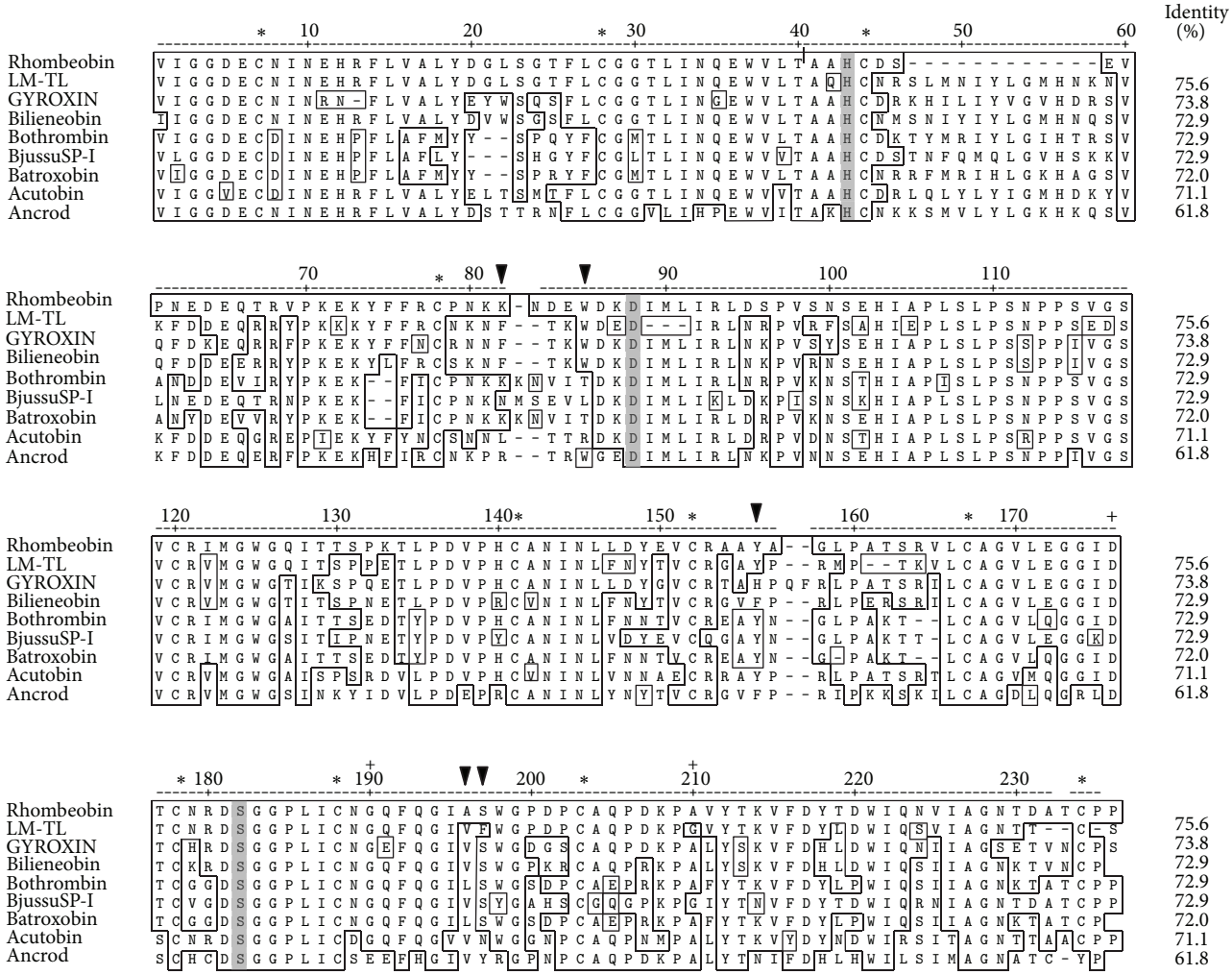


Figure 4: The amino acid sequence alignment of Rhombeobin with selected serine proteases sequences obtained from the BLAST protein data bank (PubMed/Medline). LM-TL, *Lachesis muta muta* [11]; gyroxin, *Crotalus durissus terrificus* [12]; bilineobin, *Agkistrodon bilineatus* [13]; bothrombin, *Bothrops jararaca* [14]; bjussuSP-I, *B. jararacusu* [15]; batroxobin, *B. atrox* [16]; acutobin, *Agkistrodon acutus* [17]; ancrod, *Agkistrodon rhodostoma* [18]. Numbering is according to ANCROD. Catalytic triad residues are shown in grey, and conserved cysteinve residues are shown by "∗". Specificity sites and the residues forming the hydrophobic site are shown by "+" and "▼", respectively, according to Castro et al. [19].

fractions. From all of them, only fraction sixteen presents amidolytic and thrombin-like activities. The purification of thrombin-like enzymes from the venom is usually made using methods based on molecular-size exclusion followed by either ion-exchange or affinity-binding (benzamidine- or arginine-Sepharose) chromatography [8], but this approach requires several steps, considerable time, and loss of material by the dialysis procedures prior to obtaining a lyophilized sample. Asingle chromatographic step using HPLC systems provided more purified protein samples than other conventional methods reducing time and material loss. de-Simone et al. [14] reported the purification of LMR-47 (Rhombeobin) with a single affinity chromatography step on HPLC system using two columns in tandem with the same ligand; although the authors were able to obtain a purified protein, the purification method was rather complicated by the time require and did not improve the protein recovery observed with conventional methods [9]. In comparison, with the purification procedure described here, we were able to recover near 5% of the purified protein (data not shown), twice more efficient in terms of protein recovery.

The molecular homogeneity of Rhombeobin was assessed by RP-HPLC (Figure 1(b)) showing a single and symmetric peak with elution and retention time typical for the snake venom serineproteinase family [28–30]. The SDS-PAGE analysis (Figure 1(b), insert) showed a single band with an identical electrophoretic mobility and relative molecular mass of ~45 kDa shown, by LMR-47 [9]. ESI-MS analysis showed that the molecular mass of Rhombeobin was 47035.49 Da.

Rhombeobin showed a high amidolytic activity upon BApNA with a Michaelian enzymatic behavior (Figure 2(a)). The calculated values of K_m, V_{max}, and k_{cat} (Figure 2(b)) were similar to those exhibited for LMR-47 over the same substrate [9]. As with other snake venom serine proteinases

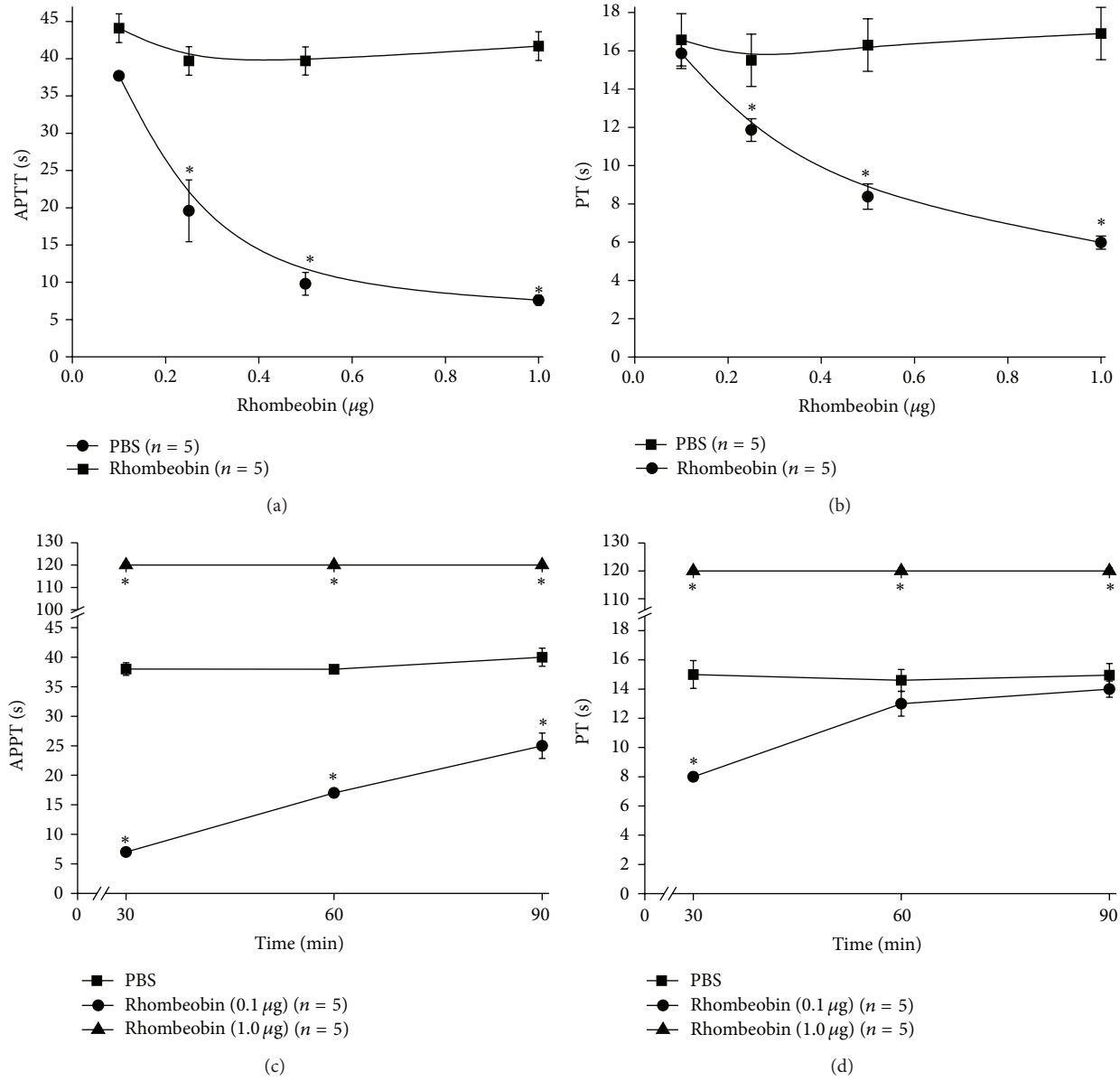


Figure 5: "*In vitro*" (a) activated partial thromboplastin time (APPT) and (b) prothrombin time (PT) blood tests of PBS (■) or different quantities of Rhombeobin (•) on mice citrated platelet poor plasma. *In vivo* time course effect on (c) APPT and (d) PT blood tests of male adult mice after i.v. injection of Rhombeobin (•) control mice received i.v injection of PBS (■) only. Results are expressed in seconds. Error bars indicate mean plus or minus standard deviation: "*in vitro*" ($n = 3$ for each group) and "*ex vivo*" ($n = 5$ for each group). Error bars indicate mean plus or minus SME ($n = 5$); $*$ means a significant difference ($P < 0.05$) compared with the control.

[31–33], Rhombeobin loses all enzymatic activity at high temperatures. Interestingly, this enzyme showed a broad range of optimum pH; the maximal enzymatic activity remained unaltered along of neutral and mild basic conditions (pH 7–10). The high enzymatic activity observed for Rhombeobin at the range of pH has been described for other snake venom serine proteinases with thrombin-like activity like bothrops protease A (*Bothrops jararaca*) [34]. PMSF completely inhibited enzymatic activity of Rhombeobin. On the other hand, the partially inhibitory effect of Kunitz-type inhibitor SBT-I over the enzymatic activity of this enzyme has been described for other snake venom serine proteinases, although complete inhibitions were achieved, in some cases, at high concentrations [8]. As a thrombin-like enzyme, Rhombeobin showed a high clotting activity upon bovine fibrinogen, and its activity was higher over citrated plasma (Figure 2(d)). This enzyme showed an α-fibrigenonolytic behavior when incubated with bovine fibrinogen, and it degraded fibrinogen β-chain at higher doses and prolonged incubation times (Figures 3(a) and 3(b)). The thrombin-like activity and the pattern found on the fibrinogenolytic activity of Rhombeobin were similarly shown by SVSP LMR-47 [9].

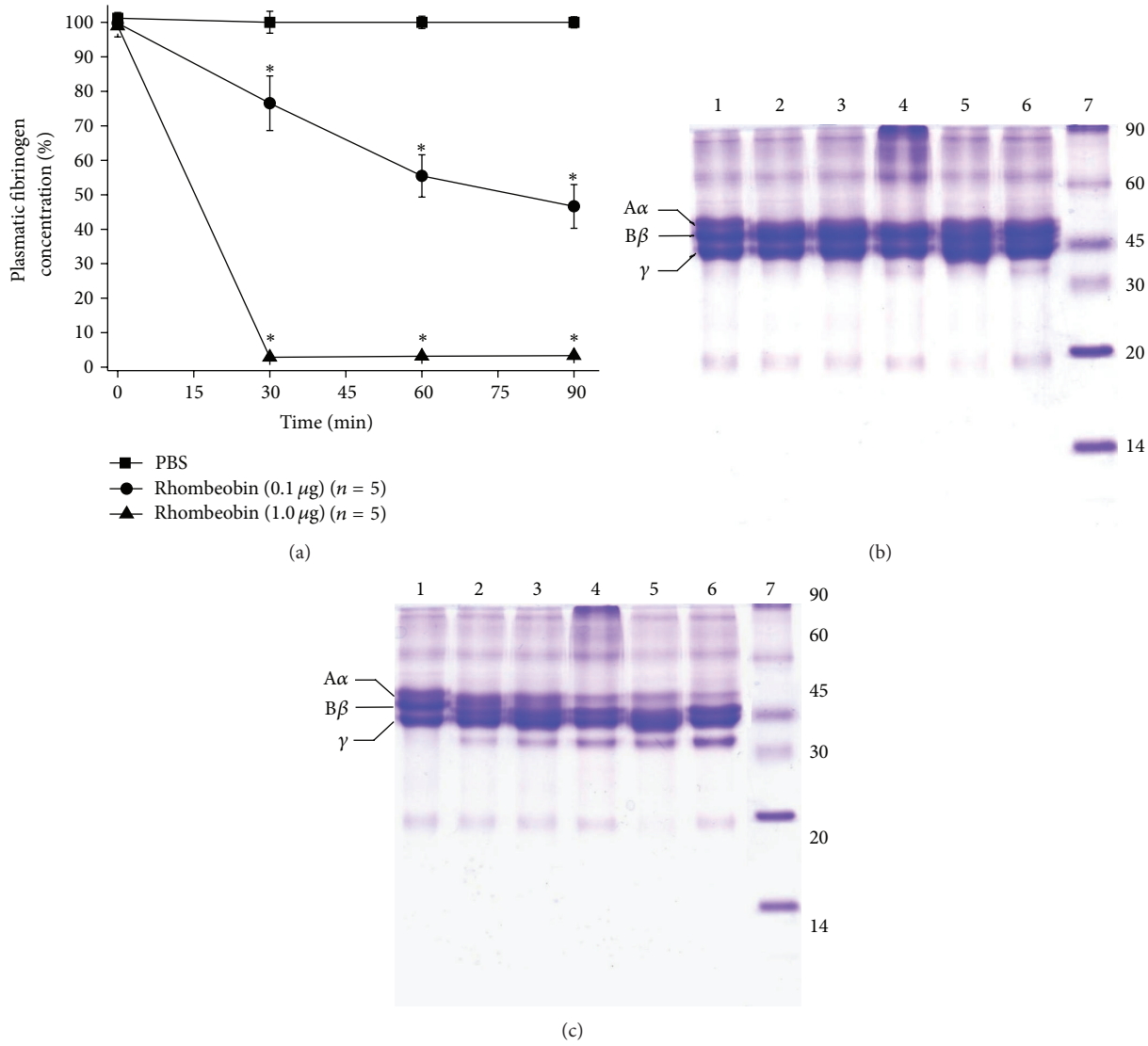
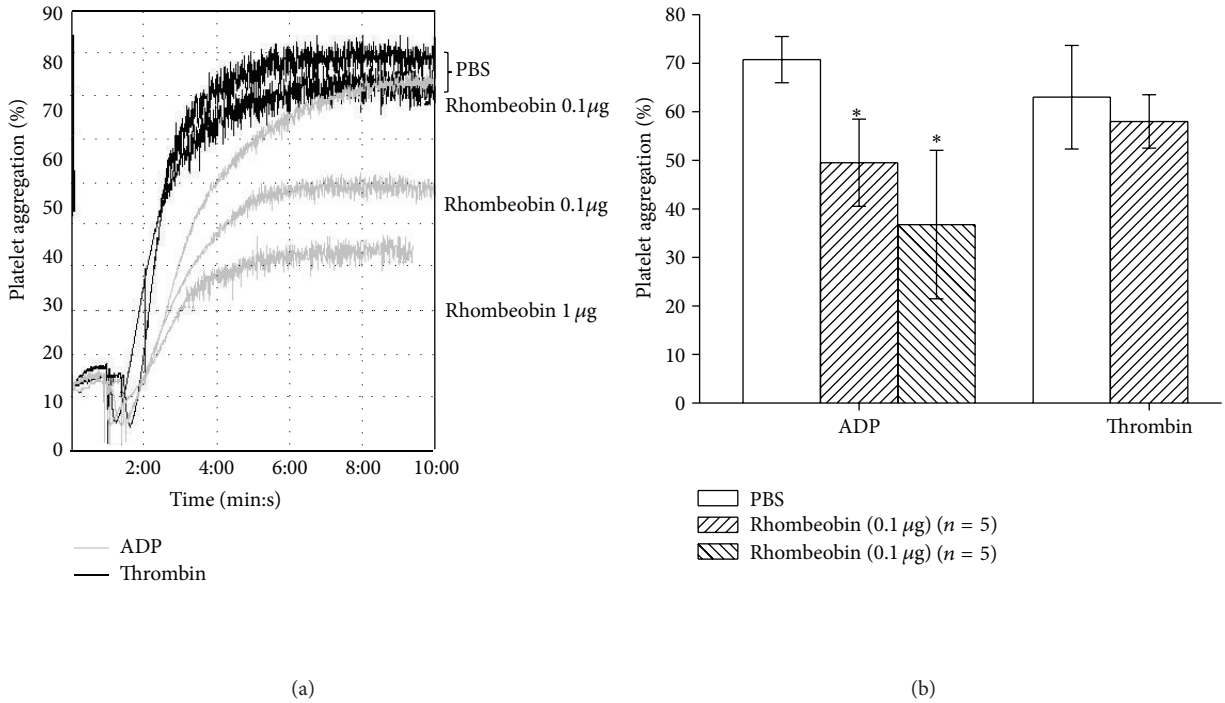


Figure 6: (a) Relative levels of mouse plasma fibrinogen after intravenous injection of PBS (■) or Rhombeobin: 0.1 μg (•) and 1 μg (▲). Values were expressed as % compared with the control. Rhombeobin fibrinogenolytic activity at different incubation times: (1) bovine fibrinogen; (2) fibrinogen + Rhombeobin (5 min); (3) 15 min; (4) 30 min; (5) 1 h; (6) 2 h; 0.1 μg (b) or 1 μg (c) of enzyme mixture with bovine fibrinogen solution (2 mg/mL) and analysis by SDS-PAGE (12.5%); MW: molecular markers. Error bars indicate mean plus or minus SME ($n = 5$); $*$ means significant difference ($P < 0.05$) compared with the control.

The biochemical, enzymatic, and biological data obtained in this work strongly suggest that Rhombeobin is the same protein LMR-47 thrombin-like/gyroxin-analog characterized by Aguiar et al. [9]. According to the Scientific and Standardization Subcommittee of the International Society on Thrombosis and Haemostasis [11], we propose to rename LMR-47 as Rhombeobin, which is the result of the combination of the subspecies name *rhombeata* with the suffix "obin".

The results obtained from the sequences provided several peptides derived of trypsin (Table 1) and SV-8 (Table 2) enzyme digestion; from these peptides, we were able to deduce almost the entire Rhombeobin protein sequence. Furthermore, the peptides named T-1, S-1, and S-2, combined, contain a 46 N-terminal amino acid sequence which showed 100% identity with the 30 N-terminal of LMR-47 [9], confirming that Rhombeobin and LMR-47 are the same protein.

The homology study (Figure 4) showed that Rhombeobin shared a high degree of sequence identity with SVSP with thrombin-like activity [12, 13, 15–18, 28, 35] especially with LM-TL (*Lachesis muta muta*). Detailed analysis showed small but important differences between Rhombeobin and LM-TL. In both enzymes, the primary (D176) and secondary (G199) specificity sites are conserved, while the tertiary specificity site (position 210) in LM-TL is a Gly residue, while in Rhombeobin it is an Ala residue. Another difference is observed in part of the hydrophobic site [19], LM-TL has Phe, Val, and Phe residues in positions 82, 196, and



(a) (b)

Figure 7: "*In vivo*" effect of Rhombeobin on platelet aggregation. (a) Representative recordings showing the platelet aggregation of mice washed platelet suspension with ADP (grey line) or human thrombin (black line) one hour after i.v. injection of the enzyme (0,1 and 1 μg/animal). Control animals received PBS only. (b) Bar representation of platelet aggregation. Error bars indicate mean plus or minus standard deviation (n = 5 for each group); $^*P < 0.05$.

197, respectively, while Rhombeobin shows Lys, Ala, and Ser in the same positions. These substitutions suggest that these proteins could have differences in their affinity for natural and synthetic substrates [36]. Also, there are some modifications in the region called "90 Loop" (position 79 to 85 in our numbering), in which Phe82 and Trp85 are responsible for the resistance of LM-TL to the inhibitory effect of Kunitz-type inhibitors by obstruction of the inhibitor binding region and sterically hindering other residues (Lys80, Arg180, and Ser233) [19]. The substitution of Phe82 by Lys82 in Rhombeobin could have allowed better interaction with the binding site of SBT-I inhibitor as suggested by the results of the inhibitory studies (Figure 2(c)).

In general, snake venom serine proteinases (SVSPs) affect the coagulation cascade by activation or proteolytic degradation of specifically coagulation factors [8, 36]. A number of snake venom serine proteinases with thrombin-like activity showed procoagulant activity "*in vitro*" by direct activation of factor V [37–39], factor VIII [28, 40], and factor X [41]. *In vitro*, Rhombeobin acts as a procoagulant SVSP shortening both APPT and PT tests (Figures 5(a) and 5(b)). These results suggest that this enzyme could act over the common coagulation pathway, likely by activating coagulation factor V and/or factor X. However, the fact that Rhombeobin induces a more marked shortening of APPT than PT test could be an indicator of activation of other specific intrinsic pathway factors.

Venom-induced consumptive coagulopathy or VICC [42] is characterized by activation of the coagulation pathway and consumption of coagulation factors, mainly fibrinogen, resulting in multiple factor deficiencies in snakebitten patients [42–44]. In laboratory, it is characterized by the alteration of basic clotting tests like WBTC, APPT, and PT tests [42, 43]. In this regard, Rhombeobin (0.1 μg/animal) activates both the intrinsic and extrinsic coagulation pathways (Figures 5(c) and 5(d)), but it does not induce defibrinogenation (Figure 6(a)) because, at this concentration, Rhombeobin did not effectively degrade fibrinogen (Figure 6(b)). However, with 1 μg/animal, Rhombeobin rendered mice blood unclottable (Figures 5(c) and 5(d)), by activating the blood coagulation factors and mainly by a rapid and sustained defibrinogenation (Figure 6(a)) due to a proteolytic degradation (Figure 6(c)). Thus, Rhombeobin appears to be a key player in the induction of the severe coagulopathy induced by *L. m. rhombeata* venom [6].

Snake venom serine proteinases also act on platelet-rich plasma or washed platelet suspensions promoting platelet-aggregation and platelet release reactions [8, 36]. This activation is mediated by the proteolytic activation of the membrane receptors PAR1 and PAR4 [45], or by interacting with glycoprotein GPIb or GPIIbIIIa [41].

However, when Rhombeobin was injected i.v. in adult mice, we observed a reduction on platelet aggregation in response to ADP (20 μM, Figures 7(a) and 7(b)) as agonist. This impairment of platelet function was found to be well correlated with a decrease in plasma fibrinogen concentration, an effect previously described for other SVSPs with thrombin-like activities [46]. Interestingly, Rhombeobin did not show

any effect over the activity of thrombin as platelet aggregation agonist.

5. Conclusion

Our results showed that venom components, like Rhombeobin, can evoke a venom-induced consumptive coagulopathy by itself, mainly through procoagulant effect mainly through the intrinsic and common pathway that involves catalytic activation of coagulation factors and the enzymatically hydrolyzing plasma fibrinogen. Thus, Rhombeobin has a main role in the coagulopathies induced by *Lachesis muta rhombeata* snake venom. The mechanisms by which Rhombeobin interacts with platelet membrane receptors *in vivo* are different from those described in the literature, and, therefore, necessary additional studies for better understanding of the "*in vivo*" mechanism of this enzyme that delays the thrombus formations are needed.

Conflict of Interests

The authors do not have any conflict of interests.

Acknowledgments

The authors acknowledge the Mass Spectrometry Laboratory at Brazilian Biosciences National Laboratory, CNPEM-ABTLUS, Campinas, Brazil, for its support with the mass spectrometric analyses. The authors thank Mr. Paulo A. Baldasso for general technical assistance. This work was supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) Process no. 09/02299-8.

References

[1] K. R. Zamudio and H. W. Greene, "Phylogeography of the bushmaster (*Lachesis muta*: Viperidae): implications for neotropical biogeography, systematics, and conservation," *Biological Journal of the Linnean Society*, vol. 62, no. 3, pp. 421–442, 1997.

[2] M. T. Jorge, I. S. Sano-Martins, S. C. Tomy et al., "Snakebite by the bushmaster (*Lachesis muta*) in Brazil: case report and review of the literature," *Toxicon*, vol. 35, no. 4, pp. 545–554, 1997.

[3] A. Rucavado, E. Flores-Sanchéz, A. Franceschi, A. Magalhaes, and J. M. Gutiérrez, "Characterization of the local tissue damage induced by LHF-II, a metalloproteinase with weak hemorrhagic activity isolated from *Lachesis muta muta* snake venom," *Toxicon*, vol. 37, no. 9, pp. 1297–1312, 1999.

[4] Ministerio da Saúde do Brasil—Fundação Nacional de Saúde, *Manual de Diagnóstico e Tratamento de Acidentes por Animais Peçonhentos*, Federal District, Brazil, 2001.

[5] D. C. Damico, L. G. Bueno, L. Rodrigues-Simioni, S. Marangoni, M. A. da Cruz-Höfling, and J. C. Novello, "Neurotoxic and myotoxic actions from *Lachesis muta muta* (surucucu) whole venom on the mouse and chick nerve-muscle preparations," *Toxicon*, vol. 46, no. 2, pp. 222–229, 2005.

[6] R. Otero, M. F. Furtado, C. Gonçalves et al., "Comparative study of the venoms of three subspecies of *Lachesis muta* (bushmaster) from Brazil, Colombia and Costa Rica," *Toxicon*, vol. 36, no. 12, pp. 2021–2027, 1998.

[7] J. Barrett and N. D. Rawlings, "Families and clans of serine peptidases," *Archives of Biochemistry and Biophysics*, vol. 318, no. 2, pp. 247–250, 1995.

[8] S. M. Serrano and R. C. Maroun, "Snake venom serine proteinases: sequence homology vs. substrate specificity, a paradox to be solved," *Toxicon*, vol. 45, no. 8, pp. 1115–1132, 2005.

[9] S. Aguiar, C. R. Alves, A. Melgarejo, and S. Giovanni-de-Simone, "Purification and partial characterization of a thrombin-like/gyroxin enzyme from bushmaster (*Lachesis muta rhombeata*) venom," *Toxicon*, vol. 34, no. 5, pp. 555–565, 1996.

[10] S. Giovanni-de-Simone, A. S. Aguiar, A. R. Gimenez, K. Novellino, and R. S. de Moura, "Purification, properties, and N-terminal amino acid sequence of a kallikrein-like enzyme from the venom of *Lachesis muta rhombeata* (bushmaster)," *Journal of Protein Chemistry*, vol. 16, no. 8, pp. 809–818, 1997.

[11] H. Pirkle and N. Marsh, "Nomenclature of exogenous hemostatic factors," *Toxicon*, vol. 30, no. 12, pp. 1513–1514, 1992.

[12] A. Magalhães, B. C. da Fonseca, C. R. Diniz, J. Gilroy, and M. Richardson, "The complete amino acid sequence of a thrombin-like enzyme/gyroxin analogue from venom of the bushmaster snake (*Lachesis muta muta*)," *FEBS Letters*, vol. 29, no. 1-2, pp. 116–120, 1993.

[13] C. M. Yonamine, A. R. Prieto-da-Silva, G. S. Magalhaes et al., "Cloning of serine protease cDNAs from *Crotalus durissus terrificus* venom gland and expression of a functional gyroxin homologue in COS-7 cells," *Toxicon*, vol. 54, no. 2, pp. 110–120, 2009.

[14] S. G. de-Simone, C. Correa-Netto, O. A. Antunes, R. B. de-Alencastro, and F. P. Silva Jr., "Biochemical and molecular modeling analysis of the ability of two p-aminobenzamidine-based sorbents to selectively purify serine proteases (fibrinogenases) from snake venoms," *Journal of Chromatography B*, vol. 822, no. 1-2, pp. 1–9, 2005.

[15] T. Nikai, A. Ohara, Y. Komori, J. W. Fox, and H. Sugihara, "Primary structure of a coagulant enzyme, bilineobin, from Agkistrodon bilineatus venom," *Archives of Biochemistry and Biophysics*, vol. 318, no. 1, pp. 89–96, 1995.

[16] C. D. Sant'Ana, C. P. Bernardes, L. F. M. Izidoro et al., "Molecular characterization of BjussuSP-I, a new thrombin-like enzyme with procoagulant and kallikrein-like activity isolated from *Bothrops jararacussu* snake venom," *Biochimie*, vol. 90, no. 3, pp. 500–507, 2008.

[17] N. Itoh, N. Tanaka, I. Funakoshi, T. Kawasaki, S. Mihashi, and I. Yamashina, "Organization of the gene for batroxobin, a thrombin-like snake venom enzyme. Homology with the trypsin/kallikrein gene family," *The Journal of Biological Chemistry*, vol. 263, no. 16, pp. 7628–7631, 1988.

[18] Y. M. Wang, S. R. Wang, and I. H. Tsai, "Serine protease isoforms of *Deinagkistrodon acutus* venom: cloning, sequencing and phylogenetic analysis," *Biochemical Journal*, vol. 354, no. 1, pp. 161–168, 2001.

[19] H. C. Castro, D. M. Silva, C. Craik, and R. B. Zingali, "Structural features of a snake venom thrombin-like enzyme: thrombin and trypsin on a single catalytic platform?" *Biochimica et Biophysica Acta*, vol. 1547, no. 2, pp. 183–195, 2001.

[20] U. K. Laemmli, "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, 1970.

[21] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[22] B. F. Erlanger, N. Kokowsky, and W. Cohen, "The preparation and properties of two new chromogenic substrates of trypsin," *Archives of Biochemistry and Biophysics*, vol. 95, no. 2, pp. 271–278, 1961.

[23] R. D. Theakston and H. A. Reid, "Development of simple standard assay procedures for the characterization of snake venoms," *Bulletin of the World Health Organization*, vol. 61, no. 6, pp. 949–956, 1983.

[24] D. C. Damico, T. Vassequi-Silva, F. D. Torres-Huaco et al., "LmrTX, a basic PLA_2 (D49) purified from *Lachesis muta rhombeata* snake venom with enzymatic-related antithrombotic and anticoagulant activity," *Toxicon*, vol. 60, pp. 773–781, 2012.

[25] C. Seki, J. C. Vidal, and A. Barrio, "Purification of gyroxin from a South American rattlesnake (*Crotalus durissus terrificus*) venom," *Toxicon*, vol. 18, no. 3, pp. 235–247, 1980.

[26] H. Barrabin, J. L. Martiarena, J. C. Vidal, and A. Barrio, "Isolation and characterization of gyroxin from *Crotalus durissus terriftcus* venom," in *Toxins: Animal, Plant and Microbial*, P. Rosenberg, Ed., p. 133, Pergamon, New York, NY, USA, 1978.

[27] S. L. Maruñak, O. C. Acosta, L. C. Leiva, R. M. Ruiz, M. V. Aguirre, and P. Teibler, "Mice plasma fibrinogen consumption by thrombin-like enzyme present in rattlesnake venom from the North-East region of Argentina," *Medicina B*, vol. 64, no. 6, pp. 509–517, 2004.

[28] S. Nishida, Y. Fujimura, S. Miura et al., "Purification and characterization of bothrombin, a fibrinogen-clotting serine protease from the venom of *Bothrops jararaca*," *Biochemistry*, vol. 33, no. 7, pp. 1843–1849, 1994.

[29] A. Magalhães, H. P. B. Magalhães, M. Richardson et al., "Purification and properties of a coagulant thrombin-like enzyme from the venom of *Bothrops leucurus*," *Comparative Biochemistry and Physiology A*, vol. 146, no. 4, pp. 565–575, 2007.

[30] S. M. Serrano, C. A. Sampaio, R. Mentele, A. C. Camargo, and E. Fink, "A novel fibrinogen-clotting enzyme, TL-BJ, from the venom of the snake *Bothrops jararaca*: purification and characterization," *Thrombosis and Haemostasis*, vol. 83, no. 3, pp. 438–444, 2008.

[31] J. W. Lee, J. H. Seu, I. K. Rhee, I. Jin, Y. Kawamura, and W. Park, "Purification and characterization of brevinase, a heterogeneous two-chain fibrinolytic enzyme from the venom of Korean snake, *Agkistrodon blomhoffii brevicaudus*," *Biochemical and Biophysical Research Communications*, vol. 260, no. 3, pp. 665–670, 1999.

[32] R. K. Bortoleto, M. T. Murakami, L. Watanabe, A. M. Soares, and R. K. Arni, "Purification, characterization and crystallization of Jararacussin-I, a fibrinogen-clotting enzyme isolated from the venom of *Bothrops jararacussu*," *Toxicon*, vol. 40, no. 9, pp. 1307–1312, 2002.

[33] F. P. Silva-Junior, H. L. Guedes, L. C. Garvey et al., "BJ-48, a novel thrombin-like enzyme from the *Bothrops jararacussu* venom with high selectivity for Arg over Lys in P1: role of N-glycosylation in thermostability and active site accessibility," *Toxicon*, vol. 50, no. 1, pp. 18–31, 2007.

[34] N. Murayama, K. Saguchi, R. Mentele et al., "The unusual high molecular mass of bothrops protease A, a trypsin-like serine peptidase from the venom of *Bothrops jararaca*, is due to its high carbohydrate content," *Biochimica et Biophysica Acta*, vol. 1652, no. 1, pp. 1–6, 2003.

[35] W. Burkhart, G. F. Smith, J. L. Su, I. Parikh, and H. LeVine III, "Amino acid sequence determination of ancrod, the thrombin-like α-fibrinogenase from the venom of *Akistrodon rhodostoma*," *FEBS Letters*, vol. 297, no. 3, pp. 297–301, 1992.

[36] H. C. Castro, R. B. Zingali, M. G. Albuquerque, M. Pujol-Luz, and C. R. Rodrigues, "Snake venom thrombin-like enzymes: from reptilase to now," *Cellular and Molecular Life Sciences*, vol. 61, no. 7-8, pp. 843–856, 2004.

[37] W. Kisiel, "Molecular properties of the factor V-activating enzyme from Russell's viper venom," *The Journal of Biological Chemistry*, vol. 254, no. 23, pp. 12230–12234, 1979.

[38] F. Tokunaga, K. Nagasawa, S. Tamura, T. Miyata, S. Iwanaga, and W. Kisiel, "The factor V-activating enzyme (RVV-V) from Russell's viper venom. Identification of isoproteins RVV-V alpha, -V beta, and -V gamma and their complete amino acid sequences," *The Journal of Biological Chemistry*, vol. 263, no. 33, pp. 17471–17481, 1988.

[39] E. Siigur, M. Samel, K. Tonismagi, J. Subbi, T. Reintamm, and J. Siigur, "Isolation, properties and N-terminal amino acid sequence of a factor V activator from *Vipera lebetina* (Levantine viper) snake venom," *Biochimica et Biophysica Acta*, vol. 1429, no. 1, pp. 239–248, 1998.

[40] S. Niewiarowski, E. P. Kirby, T. Brudzynski et al., "Thrombocytin, a serine protease from *Bothrops atrox* venom. 2. Interaction with platelets and plasma-clotting factors," *Biochemistry*, vol. 18, no. 16, pp. 3570–3577, 1979.

[41] N. Marrakchi, R. Barbouche, S. Guermazi, H. Karoui, C. Bon, and M. El Ayeb, "Cerastotin, a serine protease from *Cerastes cerastes* venom, with platelet-aggregating and agglutinating properties," *European Journal of Biochemistry*, vol. 247, no. 1, pp. 121–128, 1997.

[42] G. K. Isbister, "Procoagulant snake toxins: laboratory studies, diagnosis, and understanding snakebite coagulopathy," *Seminars in Thrombosis and Hemostasis*, vol. 35, no. 1, pp. 93–103, 2009.

[43] J. White, "Snake venoms and coagulopathy," *Toxicon*, vol. 45, no. 8, pp. 951–967, 2005.

[44] G. K. Isbister, "Snakebite doesn't cause disseminated intravascular coagulation: coagulopathy and thrombotic microangiopathy in snake envenoming," *Seminars in Thrombosis and Hemostasis*, vol. 36, no. 4, pp. 444–451, 2010.

[45] B. F. Santos, S. M. Serrano, A. Kuliopulos, and S. Niewiarowski, "Interaction of viper venom serine peptidases with thrombin receptors on human platelets," *FEBS Letters*, vol. 477, no. 3, pp. 199–202, 2000.

[46] M. C. Chang and T. F. Huang, "Characterization of a thrombin-like enzyme, grambin, from the venom of *Trimeresurus gramineus* and its *in vivo* antithrombotic effect," *Toxicon*, vol. 33, no. 8, pp. 1087–1098, 1995.