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Article

Antibiofilm Activity of Acidic Phospholipase Isoform Isolated from *Bothrops erythromelas* Snake Venom

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Abstract: Introduction: Bacterial resistance is a worldwide public health problem, requiring new therapeutic options. An alternative approach to this problem is the use of animal toxins, such as phospholipases (PLA_2) isolated from snake venom, which have important biological activities. *Bothrops erythromelas* is one of the snake species in the Northeast of Brazil that attracts great medical-scientific interest. Here we aimed to purify and characterize a PLA_2 from *B. erythromelas*, searching for heterologous activities against bacterial biofilm. Methods: Venom extraction and quantification were followed by RP-HPLC in C18 column, MALDI-ToF mass spectrometry and sequencing by Edman degradation. All experiments were monitored by specific activity using 4-nitro-3 (octanoyloxy) benzoic acid ($4N_3OBA$) substrate. In addition, hemolytic tests and anti-bacterial tests including action against *Escherichia coli*, *Staphylococcus aureus* and *Acinetobacter baumannii,* were carried out. Moreover, tests of antibiofilm action against *A. baumannii* were also performed. Results: PLA_2, after one purification step, presented 31 N-terminal amino acid residues, and molecular weight of 13656.4 Da with enzymatic activity confirmed in 0.06 μM concentration. Antibacterial activity against *S. aureus* (IC_{50} = 30.2 μM) and antibiofilm activity against *A. baumannii* (IC_{50} = 1.1 μM) were observed. Conclusions: This is the first time that PLA_2 purified from *B. erythromelas* venom has appeared as an alternative candidate in studies of new antibacterial medicines.

Keywords: Bacterial resistance; Animal venom; Purification; Antibacterial and antibiofilm activity

Key Contribution: Antibiofilm activity of phospholipase A_2 isolated from snake venom. PLA_2 isolated from *B. erythromelas* venom without hemolytic activity in murine blood.

1. Introduction

With the increase in mortality, morbidity and the rising demand for spending on diagnostic and therapeutic procedures, public health problems require attention from the scientific community.

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Among the main pathologies in Brazil and worldwide, nosocomial infections, such as infections caused by bacteria, have become more potent due to the increase in bacterial resistance. These increases are characterized by natural and evolutionary processes observed in microorganisms as responses to environmental stimuli, which are intensified by the incorrect use of antibiotics, leading to bacterial resistance to the usual drugs. In the next 30 years, the number of deaths related to bacterial infections might reach 10 million people worldwide and 392,000 in Latin America alone [1, 2, 3, 4].

Bacteria can present two forms of life: the first is planktonic, characterized by independent growth that facilitates their proliferation. The second is as biofilms, which form a community involved in an extracellular matrix composed of several biopolymers, such as extracellular polysaccharides, proteins, DNA and lipids, in addition to the association of other microorganisms, such as fungi [5, 6].

The formation and adhesion of surface bacterial biofilm can be reversible or irreversible, depending on the physical-chemical forces present in the environment. It also depends on the mechanisms of regulation of cell density and collective behavior, called quorum sensing, which allow bacteria to synchronize their gene expression for the formation of biofilm [7, 8, 9, 10, 11, 12]. Biofilm formation seems to be related to gene expression and the presence of structures that alters bacterial conformation to the state of biofilm, like flagella and lashes. The absence of a gene or lack of expression may be directly related to the lack of capacity to form biofilms, even within the same species, depending on different strains [6].

Biofilms provide some benefits to bacteria, including the increased tolerance of such microorganisms to extreme environmental conditions. Furthermore, the exopolysaccharides EPS increases protection against bactericidal agents. This mechanism allows the exchange of genetic material between different species of bacteria and between organisms of the same species, thus facilitating the spread of bacterial resistance, a fact that has aroused interest in the scientific community [12, 13, 14, 15].

On the other hand, the bioprospecting of animal toxin molecules with pharmaceutical application has gained attention, since the variety of these compounds offers alternative candidate sources for the production of new antimicrobial and antitumor drugs for the treatment of viral infections, cancer and parasitic and bacterial infections [16, 17]. Among these sources, snake venoms have a wide variety of components, where about 90% of their dry weight is composed of proteins, among which are phospholipases A_2 (PLA_2), enzymatic proteins that generally have low molecular weight. These are responsible for catalyzing the hydrolysis of the 3-sn-phosphoglyceride-dependent calcium 2-acyl ester bond, obtaining lysophospholipids and fatty acid products. [18, 19, 20].

These enzymes play an important role in the metabolism of lipid molecules and are also related to the production and release of arachidonic acid (AA), a precursor of bioactive lipids, which participates in cellular activities, due to the release of compounds such as prostaglandins, thromboxane and leukotrienes, characterizing a perception of pain and inflammation [20, 21, 22, 23, 24]. Indeed, bites caused by snakes from the genus *Bothrops* show pharmacological effects characteristic of PLA_2 action, such as inflammation, local pain, anticoagulant effects, and edema. The viper *Bothrops erythromelas* is found in the caatinga biome of Northeastern Brazil, and it is of the greatest medical pharmacological interest [25, 26].

The PLA_2 of snake venoms are similar to each other, but they have different toxicological profiles, such as myotoxicity, neurotoxicity, anticoagulant activity, hemolysis, hyperalgesia, inflammation, edema, cytotoxicity, hypotension and antimicrobial activity [20, 27, 28, 29, 30, 31]. In this context, the antibacterial activity already observed for phospholipases has drawn attention to the use of these toxins as an alternative for the production of medicines. Therefore, this work aimed to purify A_2 phospholipases of the venom from *B. erythromelas* and further evaluate their antibacterial and antibiofilm activities.

2. Results

2.1. Purification and characterization of the PLA_2 from B. erythromelas

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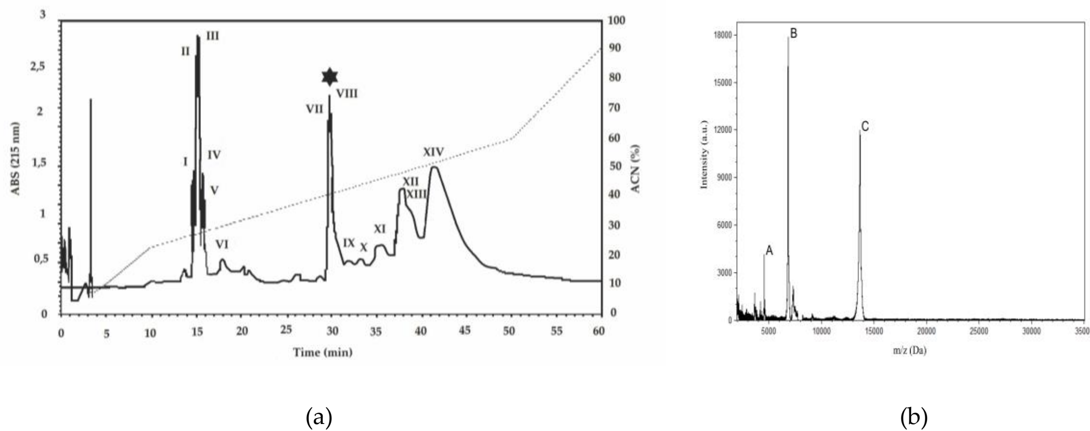
Following venom extraction and lyophilization, the crude venom was applied to a reverse phase chromatograph (RP-HPLC) using a C18 column. The crude venom exhibited a protein profile of 14 peaks, eluted along the gradient of buffer B (**Figure 1a**). Peak 8 showed a retention time of 29.4 and was eluted with ~40% of buffer B, corresponding to the PLA_2 from *B. erythromelas*.



(a) (b)

Figure 1. Phospholipase A_2 **(**PLA_2) purification from *B. erythromelas* venom. (a) Reverse-phase chromatographic profile, fractions 1 to 14, on a C18 column equilibrated with solvent A (0.1% TFA in water) and eluted with 5-95% solvent B [acetonitrile: solvent A, 9:1, v:v] and a flow rate of 2 $mL.min^{-1}$. (b) Fraction 8 (VIII) (*) analyzed by mass spectrometry; ion mass-to-charge ratios are indicated, demonstrating single (C) $[M + H]^+$ 13.656,4 Da, double (B) $[M + 2H]^{2+}$ 6.826,5 Da and triple (A) $[M + 3H]^{3+}$ 4549,9 Da charge states for the same analyte.

In order to confirm the purity of the collected PLA_2, the sample was subjected to analysis in a mass spectrometer (**Figure 1b**), which generated a spectrum with a mass of 13.656,4 Da and the presence of double $[M + 2H]^{2+}$ (6,826.5 Da) and triple $[M + 3H]^{3+}$ (4,549.9 Da) charge, confirming the purity of fraction collected from RP-HPLC.

Figure 2 demonstrates that the PLA_2 isoform of *B. erythromelas* venom in a concentration of 0.06 µM showed enzymatic activity that was three times more powerful than commercial phospholipase (bovine pancreas phospholipase A_2 - P9913 Sigma) and the crude venom of the snake, compared to the synthetic substrate acid 4-nitro-3 (octanoyloxy) benzoic acid ($4N_3OBA$).

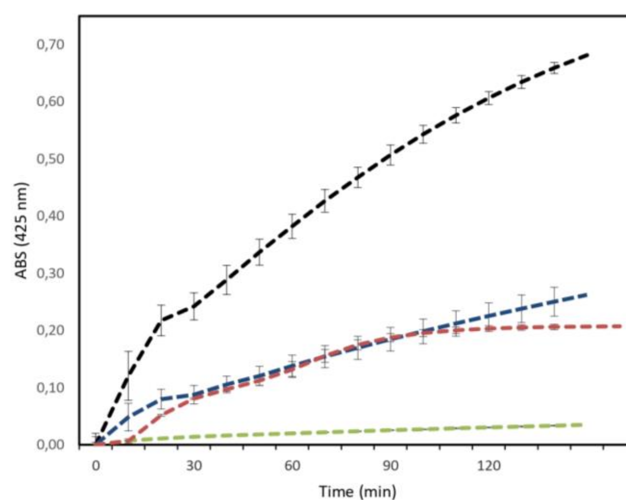


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Figure 2. Comparison between phospholipase activity of the crude venom, the purified fraction of the venom of *B. erythromelas*, a commercial phospholipase and the BSA of the substrate $4N_3OBA$ in concentration of 0.06 μM. Legend colors: black: PLA_2 isoform; blue: PLA_2 commercial; red: crude venom; green: BSA.

Edman's degradation provided an amino acid sequence with 31 N-terminal amino acid residues, with 13 hydrophobic residues and no charge. Subsequently, the sequence was submitted to BLAST, where 96% homology was observed for three acidic PLA_2: bpPLA_2-TXI from *B. pauloensis*, sPLA_2-II from *B. diporus* and BE-I- PLA_2 from *B. erythromelas*. To compare the sequences, alignment was performed using ClustalW, where it was possible to observe that only the amino acids Trp^1 and Asp^{25} in the sequence of our sample are different from the compared sequences, and the PLA_2 isoform has one hydrophobic residue more than other sequences, configuring a more hydrophobic property for the isoform PLA_2 (**Table 1**) [32, 33, 34].

Table 1. Sequence alignment of the phospholipase A_2 isoform with phospholipase activity with BpPLA_2-TXI, sPLA_2-II and BE-I-PLA_2, using the ClustalW tool. Legend: asterisk = identity.

Species	Access number	PLA_2	Alignment	Homology (%)	Charge
B. erythromelas	-	PLA_2 Isoform	WLVQFETLIMKIAGRSGVWYYGSYDCYCGSG	-	0
B. pauloensis	D0UGJ0.1	BpPLA_2-TXI	NLVQFETLIMKIAGRSGVWYYGSYGCYCGSG	96	+1
B. diporus	AFJ79208.1	sPLA_2-II	NLVQFETLIMKIAGRSGVWYYGSYGCYCGSG	96	+1
B. erythromelas	Q2HZ28.1	BE-I-PLA_2	SLVQFETLIMKIAGRSGVWYYGSYGCYCGSG	96	+1
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The similarity observed in the purified fraction with the phospholipases BpPLA_2-TXI, sPLA_2-II and BE-I-PLA_2 offers reliable indications of an acidic characteristic in our sample.

2.2. Hemolytic Activity Assays

Once purified, we investigated the hemolytic activity of PLA_2 from *B. erythromelas* against murine blood, since the absence of hemolysis is a prerequisite for further biochemical and pharmacological assays. The PLA_2 from *B. erythromelas* showed no hemolysis when incubated, even at the maximum concentration assayed, from 1.17 to 37.5 μM. This result shows the feasibility for carrying out biological tests with the purified fraction.

2.3. Antibacterial and Antibiofilm activity

The tests showed that the purified PLA_2 isoform exerts activity in Gram-positive strains. In the first tests with *Staphylococcus aureus* ATCC 7133623, there was activity at all concentrations tested, with the best concentration being 37.49 μM, representing 62 ± 17% of activity, whereas for *Escherichia Coli* ATCC 25922, low activity was observed at the concentration of 37.49 μM, representing only 12 ± 2% of activity (**Table 2**).

Table 2. Antibacterial and antibiofilm activity and IC_{50} *in vitro* evaluation for PLA_2 against *E. coli* ATCC 25922, *A. baumannii* 00332126 and *S. aureus* ATCC 7133623.

Bacteria	Concentration (μM)	Activity (%)	IC_{50} (μM)
E. coli ATCC 25922	37.4	12 ± 20	-
S. aureus ATCC 7133623	37.4	62 ± 17	30.2
A. baumannii 00332126	37.4	37 ± 10	-
Biofilm	**Concentration (μM)**	**Activity (%)**	**IC_{50} (μM)**

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A. baumannii 00332126	1.17	53 ± 11	1.1

The isolated clinical strain of *Acinetobacter baumannii* 00332126 was then tested. Although it showed greater growth, it also showed better anti-bacterial activity at concentrations of 37.49 µM, representing 37 ± 10% of activity. As *S. aureus ATCC 7133623* strain did not present biofilm growth, antibiofilm activity was also tested in *A. baumannii* 00332126. The test showed activity at all concentrations through the PLA_2 isoform. The best concentration was 1.17 µM, with 53 ± 11% of activity (**Table 2**). In all strains, the antibiotic inhibited the growth of the bacteria at all concentrations tested.

3. Discussion

Our results present a PLA_2 with acidic characteristics that showed a homology of 96% with the only PLA_2 already described, so far, for *B. erythromelas* venom (BE-I-PLA_2). The main difference in the purification of our PLA_2 was in the steps used, because in our work we sought to optimize time by applying only one chromatographic step, RP-HPLC, in a C18 column. In this way, a PLA_2 was obtained with a molecular mass of 13,656.4 Da, whereas for the purification of BE-I-PLA_2, four steps were applied with different buffers for elution, and column C4 in RP-HPLC, obtaining a PLA_2 with a molecular mass of 13,649.57 Da [34].

The use of several steps during the purification of PLA_2 from snake venom has been common for a long time. Some studies report the use of at least two stages, such as PLA_2 isolated from the venom of *B. alternatus*, *B. asper* and *B. neuwiedi*, using two chromatographic stages, where in the RP-HPLC stages, like us, they used column C18 [35, 36, 37].

Other reports show the use of up to three stages, such as studies involving the species *B. atrox* and *B. jararaca*, which were submitted to different stages and buffers. The other purifications mentioned were the use of a C18 column in the RP-HPLC stage, differing only from BE-I-PLA_2 isolated from *B. erythromelas* [34, 38, 39].

More recent studies point to a reduction in the chromatographic steps during the purification of PLA_2, as in our work. Using the same methodology applied in the present work, a study involving the species *B. pauloensis* showed the purification of a PLA_2 (BpPLA_2-TXI), with 96% homology with our isoform. Confirming that the use of only one step is satisfactory during purification, another study was done with the species *B. cotiara*, which used a methodology similar to ours and managed to purify a basic PLA_2 with a mass of 13.716 ± 3Da [32, 40].

Therefore, the type of solvent involved in the dilution of the lyophilized sample, as well as the methodology applied, in relation to the linear gradient and the separation column, proved to be important factors for the possible purity of the sample in just one chromatographic step. Thus, it is important to establish the best method of purification, optimizing the time spent on research.

Once purified, we submitted the PLA_2 from *B. erythromelas* to enzymatic assays. The enzymatic profile observed in **Figure 2** demonstrated that our purification process yielded a catalytically active PLA_2, since a high consumption of the substrate by the purified fraction was observed, indicating a possible Asp^{49} residue, as in the isoform BE-I-PLA_2 reported earlier [34]. Comparing the enzymatic activity of two PLA_2 isolated from *B. jararacussu* (BthTx-I and BjVIII) with a commercial PLA_2, and using $4N_3OBA$ as substrate, enzymatic activity was observed. This is a basic characteristic of this type of PLA_2, determining a PLA_2 Lys^{49}. Likewise, the enzymatic activity of a PLA_2 from *B. neuwiedi urutu*, which contains Lys^{49}, was absent when the synthetic substrate $4N_3OBA$ was used [41, 37]. Similarly, studies using the substrate $4N_3OBA$ compared the catalytic activity of a PLA_2 (Bmaj-9) isolated from the venom of the snake *B. marajoensis* with the crude venom of the same snake. They observed that Bmaj-9 also showed catalytic activity at a concentration of 1.46 µM, higher than the snake's crude venom [42].

The N-terminal amino acid sequencing for phospholipase showed two different amino acids when compared with another PLA_2 (BpPLA_2-TXI; sPLA_2-II and BE-I-PLA_2). However, the similarity among N-terminal sequences was maintained at 96%. The presence of a Trp^1 indicates the greater hydrophobicity of the sample, since this amino acid has aromatic characteristics with a relatively

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non-polar side chain, which also facilitates the absorption of light.The presence of an Asp^{25} indicates an increase in the acidic characteristic of our sample, since this amino acid is between the two amino acids that have the negatively charged group R at pH 7.0, thus giving it an acidic property. Asp^{25} also justifies the presence of a null charge in the isolated sequence, since the presence of this amino acid increases the positive charges, making them equal to the negative charges, which are consequently annulled [32, 33, 34, 43].

In our study, no hemolytic activity was observed for PLA_2 from *B. erythromelas*. The lack of hemolytic activity for a PLA_2 is unusual, but studies speculate that some actions of PLA_2 are still not well described, based on the absence of toxicity for some prey. Furthermore, the actions may be related to the evolution of this enzyme, which can be present in the venom gland, but not developing its expected toxic activity. Studies with an acidic PLA_2 ($BmooPLA_2$) isolated from *B. moojeni* showed a presence of hemolytic activity at 0.07 µM [44, 45, 46].

A further study carried out with an acidic PLA_2 isolated from *Porthidium nasutum* ($PnPLA_2$), displayed hemolytic activity from 0.47 µM. Indirect hemolytic activities in sheep blood were also reported for PLA_2 from *B. alternatus* at 47.26 µM [47, 35]. On the other hand, two PLA_2 from *Micrurus fulvius* showed absence of intravascular hemolysis in a mouse model [48].

Our findings show that the isoform purified from the *B. erythromelas* venom showed IC_{50} for the Gram-positive strain, whereas for the Gram-negative strains no IC_{50} was reached at the assayed concentration. These data are accordance with studies where basic PLA_2 isolated from *B. marajoensis* venom showed loss of inhibitory activity in all tested strains [49].

The PLA_2 Lys^{49} from *Lachesis muta* venom, also belonging to the Viperidae family, showed antimicrobial activity against *S. aureus* ATCC 29213 at 0.9 µM. Similarly, studies of a basic PLA_2 isolated from *Daboia russelii* (Viperidae), showed better antimicrobial effects for Gram-positive bacteria in comparison with Gram-negative bacteria. These data corroborate our findings [50, 51].

It is believed that the antimicrobial activity of PLA_2, especially those with basic properties, is related to disturbances of bacteria membrane integrity [37, 52, 53]. As Gram-negative bacteria have a cell wall with an outer membrane made up of asymmetric lipids, followed by a layer of peptidoglycans, and an inner membrane made up of phospholipids, it is well established that this conformation makes it difficult for some drugs to enter. This can also be seen in the activity of PLA_2, since the outer membrane is naturally resistant to the action of PLA_2 [16].

On the other hand, Gram-positive bacteria have only one layer of peptidoglycans followed by an internal cell membrane, showing that they are more susceptible to the action of a PLA_2. Thus, the low bactericidal activity of some phospholipases in Gram-negative bacteria compared to the activity observed in Gram-positive bacteria is probably related to the structure of their cell wall, which makes Gram-negative bacteria more resistant to the action of toxic compounds [16, 54, 55].

Commercial polypeptide antibiotics, such as bacithramycin, act on Gram-positive bacteria, inhibiting the synthesis of the bacterial cell wall, preventing the addition of amino acids and nucleotides to the cell wall. Based on the mechanism of action observed in polypeptide antibiotics, it is believed that such proteins should act similarly to these antibiotics in the tested bacteria [56, 57].

Research involving the participation of bioactive molecules from several organisms, such as microalgae, plants and animals against biofilm, is ongoing. These molecules have several pharmacological and toxicological actions that can be used as an alternative for production of drugs that help in the treatment of infections caused by microorganisms, an emerging problem in the human population, also caused by biofilm formation [58, 59].

There are several molecular mechanisms involved in the formation of biofilms between species and between strains of bacteria. It is known that a determining factor for the formation of biofilm is the presence of a disturbance or stress caused by bacteria, as well as the presence of proteins or genes that provide for the formation of these matrices. This is observed in *S. aureus* strains, which have as a determinant for the formation of biofilm the presence of Operon ICA, or even the formation of a biopolymer essential for the formation of biofilm in this species, such as N-acetyl glucosamine [6, 60].

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In our experiments, however, we observed that the *S. aureus* ATCC 7133623 strain is not capable of forming biofilm. For this is reason, antibiofilm assays were carried out only with *A. baumannii* 00332126. Studies involving an antimicrobial peptide isolated from *Naja atra* (NA-CATH) showed a 50% reduction in the biofilm formation of the bacterium *Burkholderia thailandensis* at a concentration of 0.22 μM, indicating the proven pharmacological potential of snake venoms organic molecules, corroborating the findings, since they have identified a relevant reduction in the biofilm formation of *A. baumannii* 00332126 [61].

The antimicrobial peptide Cath-A, purified from *Bungarus fasciatus* (Elapidae), also reduced *A. baumannii* biofilm at ≥ 2.2 μM. At a higher concentration (≥ 17.6 μM), Cath-A destroys almost all cells adhering to the biofilm. Further studies with synthetic antimicrobial peptides showed antibiofilm activity against *Pseudomonas aeruginosa* and *A. baumannii* with IC_{50} and IC_{90} 4 and 8 μM, respectively [62, 63].

In antibiofilm tests with C-type lectins, isolated from *B. jararacussu* venom, an IC_{50} at a concentration of 6.67 μM was observed for *S. aureus* and *S. epidermidis*, but the protein was not able to interfere in bacterial growth. Similarly, a study involving *B. moojeni* isolated molecules showing a reduction in biofilm formation, without influencing bacterial growth [64, 65].

In our studies, growth reduction of the biofilm was obtained from the lowest concentration tested. We observed that the achieved antibiofilm activity was about 20% more concentrated in the enzyme activity of the molecule, indicating a strong interaction between the enzyme and its specific substrate, in view of its low concentration. The reported activity, however, is unusual for acidic PLA_2, since antibacterial activity is often present in basic PLA_2, as previously reported. This may explain the activity in biofilm and bacteria at concentrations starting at 20% higher than the enzyme activity of the molecule [49].

This is the first report of an isoform of PLA_2 that exhibits anti-biofilm activity in the literature, demonstrating how molecules from iological sources can contribute to research regarding bacterial infections, acting as an important source of molecules capable of reducing or eradicating biofilms. The PLA_2 from *B. erythromelas*, purified by our group, is safe for further biological assays, since no hemolytic activity was noticed against murine erythrocytes. These findings emphasize the importance of bioprospection studies with molecules from animal toxins, especially snakes, to control bacterial biofilms, contributing to advances in the control of infections caused by these microorganisms.

4. Conclusions

The purification of the PLA_2 isoform from *B. erythromelas* venom using a single chromatographic step was reported, resulting in protein with 13,656.4 Da. The amino-terminal portion of the PLA_2 isoform showed 96% of identity with another PLA_2 previously described. Beyond the high enzymatic activity, no hemolytic activity was observed against murine erythrocytes. Notable antibiofilm activity was seen against *A. baumannii* clinical isolates at a low concentration. These findings confirm that purified molecules from snake venoms possess several biological and pharmacological properties. It is therefore necessary to develop basic research around these components, aiming to develop new drugs for the treatment of various diseases that affect human health.

5. Material and Methods5.1. Ethics Committee

All experiments developed in this research, using blood and molecules from animals, were submitted to the ethics committee and approved. Venom samples are registered with the Genetic Heritage Management Council (SisGen) under registration: A883C5B. *Mus musculus* erythrocytes were approved by the ethics committee of Universidade Católica Dom Bosco (UCDB) under registration n°. 014/2018.

5.2 B. erythromelas venom extraction

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B. erythromelas venom was collected from 5 adult specimens in captivity at the Zoo for Reptiles of the Caatinga, located in the municipality of Puxinanã, metropolitan region of Campina Grande, state of Paraiba. After lyophilization, the venom was kept at -20°C until use.

5.3 Quantification of venom proteins

After diluting the lyophilized sample in ultrapure water, the Bradford method (1976), was carried out to quantify the proteins present in the purified fraction. Serial dilutions of the sample were used. As standard for these concentrations, Bovine Serum Albumin (BSA) was used in the same concentration as the purified sample. All samples were tested in triplicate [66].

5.4 Purification of venom proteins

The crude venom was subjected to high performance liquid chromatography (Waters and 2695 Separations Module) in a C18 column (Xterra MS 5 µm - 4.6 x 250 mm column). The solvent system was composed of 0.1% trifluoroacetic acid (TFA) in H_2O (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) in a flow of 2 $mL.min^{-1}$ and a linear gradient of 5-95% acetonitrile, for 60 min. Protein peaks were monitored at 216 and 280 nm. The fractions presented in the graphical representation were collected and lyophilized. Subsequently, the fraction with phospholipase activity was selected to be subjected to the mass spectrometer.

5.5 Phospholipase activity

To analyze the phospholipase activity of *B. erythromelas* venom, the methodology described by Holzer and Mackessy (1996) was used, with changes made by Serino-Silva et. al. (2014) [67, 68].

The substrate for reaction, 4-nitro-3-octanoyloxybenzoic acid ($4N_3OBA$, Enzy Life Science, USA) was used. As a positive control, a commercial phospholipase with a concentration of 0.06 µM (1$mg.mL^{-1}$) (bovine pancreas phospholipase A2 - P9913 Sigma) was prepared and BSA was used as negative protein control, at the same concentration.

5.6 Mass spectrometry

To measure the molecular mass of the selected fraction, a matrix-assisted ionization time-of-flight (MALDI-ToF) mass spectrometer (AutoFlex III) Smartbeam (Bruker Daltonics, Bremen, Germany) controlled by Flex Control 3.0 software was used (Bruker Daltonics, Bremen, Germany). A 0.37 µM sample was solubilized in Ultrapure water, mixed (1:1 v:v) in a saturated solution of siapinic acid, as matrix, and applied to the target plate (Bruker Daltonics, Bremen, Germany) to dry at room temperature. The compound had its molecular mass obtained in the positive linear mode after external calibration, with Protein Calibration Standard (Bruker Daltonics, Bremen, Germany). The MALDI-ToF spectra were processed with Flex Analysis 3.0 software (Bruker Daltonics, Bremen, Germany).

5.7 *Amino-terminal sequencing of PLA_2 from B. erythromelas*

The amino-terminal sequencing was obtained through Edman's degradation, using an automatic Shimadzu PPSQ-31B/33B), initially calibrated with the PTH-amino acid mixture standard. A sample of the purified PLA_2 was resuspended in 37% acetonitrile and applied onto a nitrocellulose membrane (PVDF) and dried under nitrogen flow. According to the manufacturer's recommendations, phenyl thiohydantoin amino acids were detected after separation on an RP-HPLC C18 column (4.6 × 250 mm). The resulting sequences were applied to the NCAST protein BLAST search (BLASTP 2.8.0+) and the significant sequences were aligned using ClustalW 1.2.4.

5.8 Hemolysis test

Erythrocytes of *Mus musculus* were used for the tests. The collected blood was stored at 4 ° C until use. The cells were washed three times with 50 mM phosphate buffer, pH 7.4. To the

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erythrocyte suspension was added the fraction of *B. erythromelas* venom referring to phospholipase at a concentration of 0.07 μM, in serial dilution of 1.17 to 37.49 μM in a final volume of 100 mL. The samples were incubated at room temperature for 60 min. After centrifugation at 3000 rpm, hemoglobin release was monitored by reading the absorbance of the supernatant at 425 nm in a Spectramax microplate maker. To control hemolysis, erythrocytes suspended in $5x10^4$ μM phosphate buffer, pH 7.4 were used; as a positive control (100% erythrocyte lysis), a 1% (by volume) solution of triton X-100 dissolved in distilled water was used to replace the venom fraction. The tests were performed in triplicate [69].

5.9 Antibacterial activity

Strains of *E. coli* ATCC 25922, *S. aureus* ATCC 7133623 and *A. baumannii* 00332126 (a resistant clinical isolate) were used. For the antibacterial tests, a purified fraction of the venom of the snake *B. erythromelas* with phospholipase activity was used. The tests to observe the antibacterial activity were performed according to the protocol described by CLSI, using the 96-well microplate dilution method. Three technical replicates were organized on the microplates at a final bacterial concentration of 2.5×10^5 $UFC.mL^{-1}$. The samples were tested in concentrations ranging from 1.17 to 37.49 μM. For positive control, the antibiotic ciprofloxacin was used in the same concentrations as the samples, while the bacterial suspension in MHB was used as a negative control [70].

5.10 Antibiofilm Activity

Basal Medium 2 (BM2) was used to analyze the biofilm formation. Bacterial cultures of *A. baumannii* 00332126, proven to be clinical isolate resistant, were used. As bacterial suspensions, they were inoculated into 96-well round-bottom plates, including samples from serial dilutions from 1.17 to 37.49 μM. As negative control, only bacteria were used in the BM2 medium, and as a positive control, the antibiotic ciprofloxacin was used in the same concentrations as the sample. To analyze the growth of planktonic cells, an absorbance of 600 nm was used [71, 72].

To assess for biofilm formation, performed as described by Naves et al, 2019, the biofilm formation was read at an absorbance of 595 nm. All absorbance readings were performed with the Multiskan Go microplate reader (Thermo Scientific). All tests were performed in triplicates [73].

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