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Isolation, structural and functional characterization of a new Lys49 phospholipase A_2 homologue from *Bothrops neuwiedi urutu* with bactericidal potential

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ABSTRACT

Snake venom is a complex mixture of active compounds consisting of 80–90% proteins and peptides that exhibit a variety of biological actions that are not completely clarified or identified. Of these, phospholipase A_2 is one of the molecules that has shown great biotechnological potential. The objectives of this study were to isolate, biochemically and biologically characterize a Lys49 phospholipase A_2 homologue from the venom of *Bothrops neuwiedi urutu*. The protein was purified after two chromatographic steps, anion exchange and reverse phase. The purity and relative molecular mass were assessed by SDS-PAGE, observing a molecular weight typical of PLA_2s, subsequently confirmed by mass spectrometry obtaining a mass of 13,733 Da. As for phospholipase activity, the PLA_2 proved to be enzymatically inactive. The analyses by Edman degradation and sequencing of the peptide fragments allowed for the identification of 108 amino acid residues; this sequence showed high identity with other phospholipases A_2 from *Bothrops* snake venoms, and identified this molecule as a novel PLA_2 isoform from *B. neuwiedi urutu* venom, called BnuTX-I. In murine models, both BnuTX-I as well as the venom induced edema and myotoxic responses. The cytotoxic effect of BnuTX-I in murine macrophages was observed at concentrations above 12 μg/mL. BnuTX-I also presented antimicrobial activity against gram-positive and negative bacterial strains, having the greatest inhibitory effect on *Pseudomonas aeruginosa*. The results allowed for the identification of a new myotoxin isoform with PLA_2 structure with promising biotechnological applications.

1. Introduction

Snake venoms have become promising sources for new therapeutic candidates (Brahma et al., 2015), since they are composed of a protein fraction (90–95% of the dry weight), responsible for many pathophysiological effects developed during envenoming, such as hemorrhage, coagulopathy, myonecrosis and neurotoxicity (Vital-Brazil; Corbett, 1982; Calvete et al., 2007), and a non-protein fraction consisting of nucleotides, carbohydrates and lipids (Warrell, 2010). Furthermore, some biological activities have been described, such as anti-microbial, parasitic and antitumoral activities (Calderon et al., 2014).

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The phospholipases A_2 (PLA_2s) present in the venom are well-described proteins, because they are associated with activities such as hemolysis, neurotoxicity, cardiotoxicity, platelet aggregation, anticoagulant, edema, myotoxicity (Sousa et al., 2013), hypotension (Andrião-Escarso et al., 2002) and inflammatory processes (Zuliani et al., 2005).

PLA_2s from snake venoms ($sPLA_2$) are classified into two groups according to their primary structures, disulfide bonds and the presence or absence of the elapid loop, as follows: Group I, belonging to the Elapidae family and group II, belonging to the Viperidae family (Dennis, 1994). PLA_2s from the Viperidae family can be subdivided into: i) Asp49 PLA_2s with an aspartate residue (Asp) at position 49 that present enzymatic activity on phospholipids of biological membranes and artificial substrata; and ii) Lys49 PLA_2 homologues with a lysine (Lys) residue at position 49 and little or no catalytic activity on artificial and biological substrates (Kini, 1997). The inactivity in hydrolyzing phospholipids results, as mentioned, in the substitution of an aspartate residue (Asp/D) at position 49 for a lysine residue (Lys/K), a fact that interferes with the binding of calcium (Ca^{2+}), an essential cofactor for the catalytic activity of the enzyme on cell membrane phospholipids (Dennis et al., 2011). However, certain Lys49 PLA_2s-homologues have the ability to hydrolyze phospholipids in special conditions, releasing arachidonic acid in reactions (Soares et al., 2002).

Generally, molecules present in snake venoms act on various processes of change in cell metabolism. Of these, Lys49 PLA_2 homologues are involved, among others, in the venom's pathophysiological effects. In addition, they exert cytotoxic activity against microbial agents. Thus, considering the molecular properties, the pathophysiological and antimicrobial effects, identifying molecules with biotechnological potential like Lys49 $svPLA_2$s may provide significant advances in different areas of health.

The subspecies *Bothrops neuwiedi urutu*, currently grouped according to Carrasco et al. (2012) in the species *B. neuwiedi* (Wagler, 1824), is widely distributed and may represent a source for biotechnological studies with the use of its venom. Given the above, this study aimed to isolate, identify and characterize physicochemical, structural and functional aspects of a new Lys49 phospholipase A_2 homologue with bactericidal potential from *B. neuwiedi* venom.

2. Materials and methods

2.1. Venom and mice

The venom of the snake *B. neuwiedi urutu* was obtained from the serpentarium BioAgents (Batatais-SP, Brazil). The male Swiss mice (18–26 g) from the Oswaldo Cruz Foundation, Rondônia - Brazil, met the Ethical Principles of Animal Experimentation (CONCEA). The study was authorized by the Ethics Committee for Animal Research of Fiocruz Rondônia - FIOCRUZ/RO (CEUA/FIOCRUZ-RO) – No. 2013/08.

2.2. Purification and biochemical characterization

2.2.1. Purification of the Lys49 PLA_2 homologue toxin

The Lys49 PLA_2 was purified from the venom of *B. neuwiedi urutu* using chromatographic techniques in an Akta Purifier system (GE). Replicates of 60 mg of venom were solubilized in 1.0 mL of 20 mM ammonium bicarbonate buffer (AMBIC), pH 8.0 and centrifuged at 11,200*xg* for 5 min. The supernatant obtained was fractionated in: **A**-a DEAE-Sepharose® column (1.0 × 30 cm) with 20 mM AMBIC buffer (Eluent A) and 500 mM AMBIC (Eluent B), both at pH 8.0. The column was previously equilibrated with Eluent A and the sample eluted under a gradient of 0–100% of eluent B. **B**-The fraction of interest, selected according to the presence of proteins with a molecular mass compatible with $svPLA_2$s was solubilized in 0.1% TFA (Solution A), subjected to HPLC in a C18 column (25 × 4.6 mm – Sulpeco) and eluted under a gradient of 0–70% of solution B (99.9% acetonitrile and 0.1% TFA). In both steps, the fractionation was carried out under a flow rate of 1 mL/min, the fractions of the eluate were monitored at 280 nm and collected manually.

2.2.2. Monodimensional electrophoresis in a 12.5% polyacrylamide gel (SDS–PAGE)

The 12.5% SDS - PAGE (m/v) was performed according to the descriptions of Laemmli (1970) and under reducing conditions, in the BIO-RAD brand MiniProtean system. Protein bands were evident with 0.25% Coomassie Blue R250 solution (m/v). Images of the gels were obtained by Image scanner® (GE Healthcare Lifesc.) and the relative molecular mass (Mr) was determined by the program IQTL® (GE Healthcare Lifesc®).

2.2.3. Two-dimensional electrophoresis

For isoelectric focusing the isolated toxin was solubilized in a solution containing 7 M urea, 2 M thiourea, 2.0% CHAPS (m/v), 0.5% immobilized pH gradient buffer (IPG buffer®) (v/v), and 1.0% bromophenol blue (m/v), applied on a polyacrylamide strip (7 cm) with a pH gradient from 3.0 to 10.0 immobilized linearly (Immobiline DryStrip®). The isoelectric focusing, done in an IPGphord III System (GE healthecare Life Scienc.), followed the manufacturer's instructions. Subsequently, the strip was placed on the superior part of the 12.5% SDS-PAGE (m/v) to perform the second dimension (15 mA, 90 min). The gel was fixed (Fixing) and stained with Coomassie Blue G 250. The capture of the gel image, coming from the BIO-RAD brand MiniProtean system, occurred with the help of "Image Scanner III®" (Amersham Bioscience) and was analyzed using IQTL software (GE Healthcare Life Science®).

2.2.4. Evaluation of the molecular mass of the isolated PLA_2

The molecular mass was obtained in MALDI-TOF/TOF Mass Spectrometry (AXIMA TOF2, Shimadzu Biotech), using a saturated solution of sinapinic acid in a ratio of 3:1 (matrix/protein) as an ionizing matrix and after, the co-crystallization on the equipment's plate was introduced into the vacuum chamber and analyzed in linear mode with the average of laser pulses.

2.2.5. Sequencing of the protein

The N-terminal sequencing was performed using Edman degradation (Edman, 1950) from 05 μg of isolated protein, using a PPSQ-33A automatic sequencer (Shimadzu, Kyoto, Japan). In the complementary sequence, the protein was reduced by treatment with a solution of 5 mM DTT (Dithiothreitol) for 25 min at 56 °C and alkylated with a solution of 14 mM Iodoacetamide for 30 min at room temperature protected from light. After this procedure, the sample was digested for 16 h at 37 °C with trypsin or GluC (Sequencing grade modified, Promega) in the proportion of 1:50 enzyme/substrate. To stop the reaction, 0.4% formic acid was added (the pH was monitored and when it was higher than 2.0, more 0.4% formic acid was added) and the sample was centrifuged at 2500 rpm for 10 min. The pellet was discarded and the supernatant dried in a vacuum centrifuge.

The dried peptides obtained from the digestion procedure were re-suspended in 1% formic acid for LC-MS/MS analysis. The resulting peptides were separated by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nL/min. Bound peptides were eluted from the column with the following gradient:

0–90% acetonitrile in 0.1% formic acid over 20 min. Before performing a tandem mass spectrum, an ESI/MS mass spectrum (TOF MS mode) was acquired for each HPLC fraction over the mass range of 100–2000 *m/z*, in order to select the ion of interest, where these ions were subsequently fragmented in the collision cell (TOF MS/MS mode). Raw data files from LC–MS/MS runs were analyzed using a MassLynx 4.1 SCN662 software package (Waters). A search was run with the search engine Mascot Distiller v.2.3.2.0, 2009 (Matrix Science, Boston, MA) against the SNAKES database. The results have been filtered so only high confidence peptides (FDR≤ 0.01) were considered for identification of the results with a peptide mass tolerance of 0.1 Da, and a fragment mass tolerance of 0.1 Da. The similarity search for the sequenced PLA_2 was done using the BLAST algorithm and multiple alignment with the program Clustal W.

2.2.6. Molecular modeling

To investigate structural characteristics of amino acids, we performed homology modeling of BnuTX-I's monomeric sequence using the crystal structure of Phospholipase A_2 homologue 1 from *Bothrops jararacussu* (PDB code: 3I03) as a template with a resolution of 1.48 Å (Fernandes et al., 2010). For search and retrieval of the template structure Protein Blast (http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1990) and Protein Data Bank (PDB) (http://www.pdb.org) were used. The sequence alignments were carried out in MODELLER v9.10 (Sali; Blundell, 1993) and ClustalW (Thompson et al., 1994) softwares. The model building was carried out in MODELLER v9.10. A total of 1000 models were generated and the final model was selected based on the lowest DOPE scores calculated by MODELLER software. The overall stereochemical quality of the final model for BnuTX-I was assessed by the program PROCHECK (Laskowski et al., 1993). After modeling, we generated a dimeric structure overlapping the monomer BnuTX-I in other phospholipase A_2 of *B. jararacussu* (PDB code: 3I03) (Dos Santos et al., 2011). Interactive visualization, overlapping and comparative analysis of molecular structures were carried out in Swiss-PDB viewer (Guex; Peitsch, 1997) and UCSF Chimera (Goddard et al., 2004).

2.3. Functional characterization

2.3.1. Phospholipase activity

The enzymatic activity of the samples (Venom and PLA_2) was evaluated according to the protocol of Holzer and Mackessy (1996), adapted to a 96-well plate. The absorbance was monitored at 425 nm using an Eon microplate spectrophotometer (Biotek) at intervals of 1 min over 30 min at 37 °C.

2.3.2. Evaluation of the myotoxic activity

The myotoxic activity was evaluated by the quantification of serum levels of creatine kinase (CK) from Swiss male mice (23–26 g) (n = 5). To this end, the animals were inoculated with PBS (negative control), venom or BnuTX-I in their gastrocnemius muscle at concentrations of 25 μg/50 μL diluted in PBS. After 3 h, blood from the orbital plexus was collected, centrifuged and the serum obtained was submitted to analysis using the commercial test CK-NAC (Labtest - Brazil), according to the manufacturer's instructions. The results were expressed as mean ± standard deviation ($P < 0.05$) and statistical analysis was performed by ANOVA and the Tukey test.

2.3.3. Edema induction

The edema activity, performed according to Andrião-Escarso et al. (2002), was determined after applications (10μg/20 μL) of venom or BnuTX-I in the foot pad of the right paw of the male Swiss mice (23–26 g) (n = 5), as well as for the control group, where the same volume of sterile PBS solution was injected into the contralateral paw. The results were obtained in a plethysmometer (Ugo Basile) evaluating the volumes of both paws and expressed as a percentage of the test paw's edema relative to the control.

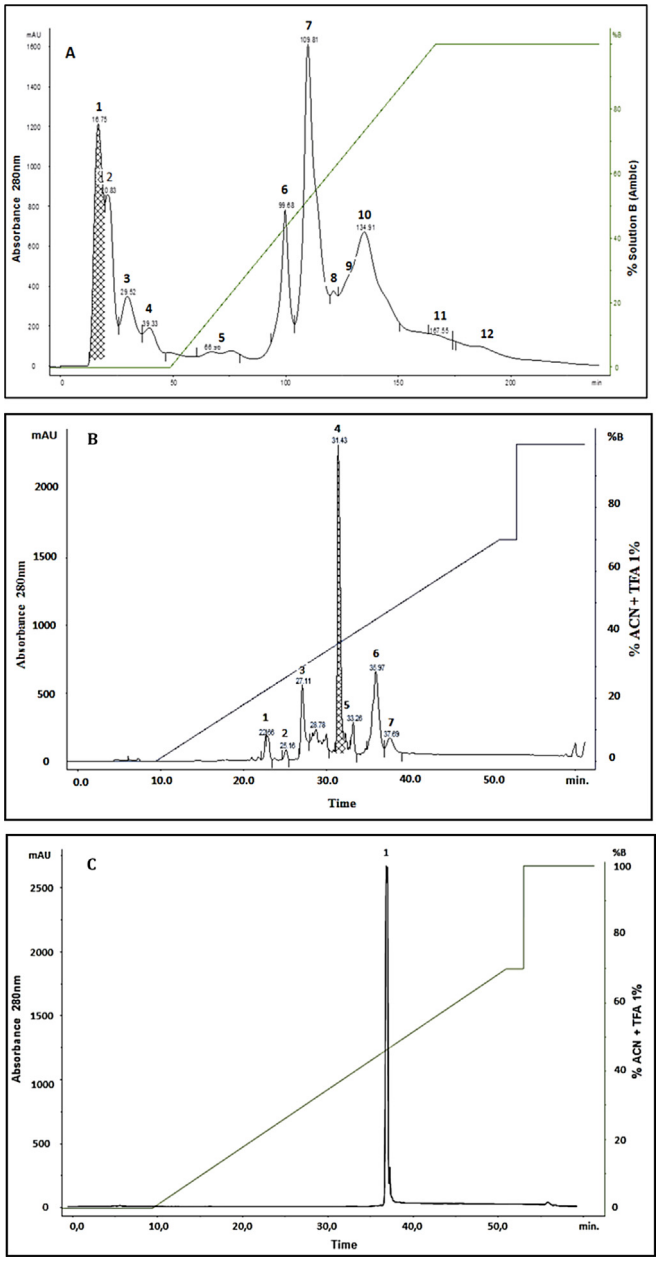

Fig. 1. Chromatographic profile of the venom and isolation of the toxin from *B. neuwiedi urutu* **venom.** Figure A shows the chromatographic fractionation profile in a DEAE-Sepharose 1.0 × 30 cm column previously equilibrated in solvent (A) (20 mM ammonium bicarbonate) and eluted under a gradient from 0 to 100% of solution (B) (500 mM ammonium bicarbonate), pH 8.0 obtaining 12 fractions (1–12). The fraction of interest (as shown) was subjected to 12.5% SDS-PAGE. Figures B and C show the chromatographic profiles obtained on a reverse phase C-18 column (25 × 4.6 mm, 5 μm, Discovery) equilibrated with 0.1% TFA and eluted under a gradient from 0 to 70% of solvent B – 99.9% ACN + 0.1% TFA. Figure B shows the profile in which 7 fractions are obtained and Figure C, the isolation profile. All the chromatographic fractionation steps were monitored at an absorbance of 280 nm and flow rate of 1.0 mL/min.

2.3.4. Determination of cellular viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

2.3.4.1. Harvesting of macrophages. Macrophages were obtained

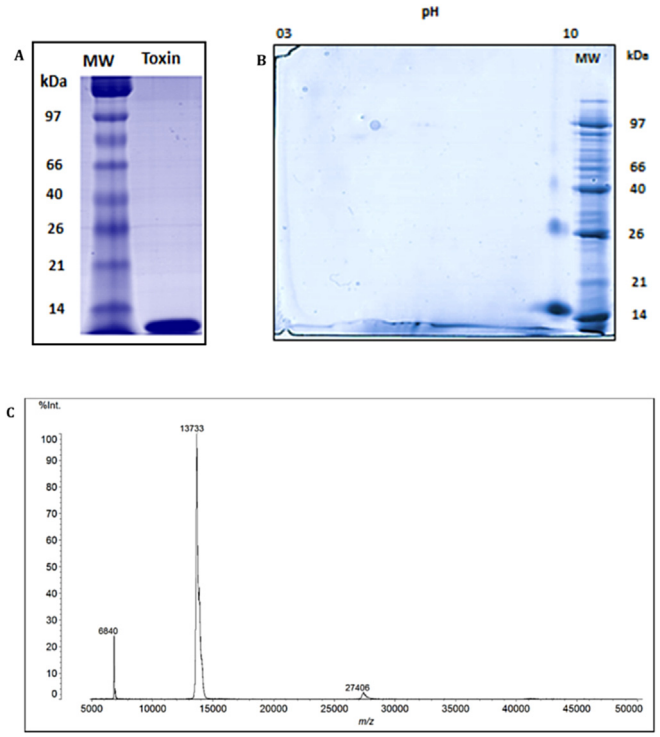

Fig. 2. Molecular mass and pI of the isolated toxin. The figure shows a 12.5% SDS-PAGE gel under reducing conditions (Fig. A). B: 2D SDS-PAGE showing the toxin with a pI of 9.5, the presence of a dimeric mass of about 26 kDa. The 2D SDS-PAGE was done on a 07 cm strip from pH 3 to 10 (linear), with 15 μg/10 μL of toxin and stained with Coomassie Blue G 250. On the side, one observes the molecular mass markers (kDa) and on the upper edge, the range of pH values. In C there is the mass spectrum image obtained in MALDI TOF^2 showing a value of 13733 Da for BnuTX-I, 6840 Da for the double charge, and the dimer with 27406 Da. The spectrum represents the average laser pulses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

96 h after an intraperitoneal (i.p.) injection of 1 mL sterile solution of 3% sodium thioglycollate in male Swiss mice (18–22 g) (n = 5) according to Setúbal et al. (2013). The animals were euthanazied by cervical dislocation and exsanguined. Then, peritoneal lavage was performed, after a gentle massage of the abdominal wall, using 3 mL of cold sterile PBS, pH 7.2. Cells were quantified in a Neubauer hemocytometer, classified according to morphology and staining and maintained in RPMI medium (Roswell Park Memorial Institute) supplemented with 10% Fetal Bovine Serum and antibiotics.

2.3.4.2. Evaluation of cellular viability by MTT. The viability of peritoneal macrophages (2×10^5 cells/100 μL) was determined by the method of reduction of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolina bromide (MTT assay) described by Mosmann (1983). The evaluation of the degree of reduction of MTT to formazan was quantified by measuring the optical density (O.D.) at 540 nm in a spectrophotometer after 24 h of incubation of macrophages with different concentrations (1.5, 3.0, 6.0, 12 and 25 μg/mL) of Lys49 PLA_2 at 37 °C and 5% CO_2. As the negative control, cells grown in supplemented RPMI medium were used. The results were expressed as absorbance units measured at 540 nm.

2.3.5. Cytokine concentration

Cytokine IL-1β and TNF-α concentrations were quantified in the supernatant of peritoneal macrophages (2×10^5 cells/100 μL) cultured with different concentrations (1.5; 3.0, 6.0, 12 and 25 μg/mL) of BnuTX-I or RPMI (control group), for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. The presence of cytokines was assessed quantitatively using the commercial test Quantikine ELISA kit (R & D Systems - USA) according to the manufacturer's instructions.

2.3.6. Antimicrobial activity

The antimicrobial activity was determined according to the recommendations of Cockerill, and the Clinical and Laboratory Standards Institute (2012), in 96-well plates. Bacterial strains *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 13883) were grown in LB broth (Luria Bertani) medium for 24 h with the samples (toxins or venom). As a positive control, Chloramphenicol was used (125 μg/mL). The results were evaluated by spectrophotometry at an absorbance of 630 nm.

2.3.7. Statistical analysis

The results were presented as mean and standard deviation or error. In comparisons between the groups, the statistical significance was measured through the parametric test derived from the analysis of variance (ANOVA – One–way analysis of variance) and through the Tukey test considering the parameter of significance to be $p < 0.05$.

3. Results

3.1. Purification and biochemical characterization of BnuTX-I

BnuTX-I was purified by two chromatographic steps: ion exchange (anionic) and reversed-phase (Fig. 1A), the fractions of which were analyzed by 12.5% SDS-PAGE. Fraction 01 showed bands with masses between 14 and 60 kDa and was selected for containing proteins with characteristics consistent with svPLA$_2$s. Subsequently, the fraction was rechromatographed on a reverse phase column and analyzed by 12.5% SDS PAGE. Of the 7 fractions obtained, fraction 4 showed a single protein band with an apparent molecular mass of approximately 14 kDa (Fig. 2A).

Bidimensional electrophoresis (2D) allowed for the confirmation of the purity of the protein and the isoelectric point (pI 9.5), as well as showed dimerisations with masses around 26 and 40 kDa (Fig. 2B). Analysis by mass spectrometry in MALDI TOF^2 showed a molecular mass of 13,733 Da (monomer) and showed a structure with a double charge (6840 Da) and a dimer (27,408 Da) (Fig. 2C).

Subsequently, the toxin was sequenced obtaining 108 amino acid residues, 21 of which were obtained by Edman degradation, and the other 87 by tryptic digestion followed by liquid chromatography LC-MS/MS mass spectrometry ESI/MS (TOF MS mode), and comparison with the database of Mascot Distiller, 2009 (Table 1).

The multiple alignment showed that the isolated protein presents high identity with Lys49 PLA_2 homologues of snake venoms of the genus *Bothrops*, with 92.3% for *Bothrops pirajai* and *B. jararacussu*; the protein was then called BnuTX-I.

Homology modeling is usually the method of choice when a clear relationship of homology between the sequence of the target protein and at least one known structure is found. This approach would give reasonable results based on the assumption that the tertiary structures of two proteins will be similar if their sequences are related (Kroemer et al., 1996). To evaluate the validity of 3I03 as a structural template for modeling BnuTX-I, we have examined the sequences' homology. The alignment of BnuTX-I yielded 99.15% identity with 3I03. The percentage of residues lying in the favored regions of a Ramachandran plot (Ramachandran et al., 1963) is one of the best guides to check the stereochemical quality of a protein model based on the assumption that a good one should have more

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Bothrops n. urutu          ----------------SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCC 44
SP|P82287|B. pirajai       ----------------SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCC 44
SP|Q90249|B. jararacussu MRTLWIMAVLLVGVEGSLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCC 60
SP|Q9IAT9|B. pauloensis    ----------------SFELGKMILQETGKNPAKSYGAYGCNCGVLGRGQPKDATDRCC 43
SP|P86975|B. leucurus      ----------------SLFELGKMILQETGKNSVKSYGVYGCNCGVGGRGKPKDATDRCC 44
SP|P24605|B. asper         MRTLWIMAVLLVGVEGSLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCC 60
TR|I6L8L6|B. brazili       ----------------SLVELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCC 44
SP|Q9I834|B. moojeni       ----------------SLFELGKMILQETGKNPAKSYGVYGCNCGVGGRGKPKDATDRCC 44
SP|P86453|B. alternatus    ----------------SLFELGKMILQETGKNPAKSYGAYYCYCGWGGQGQPKDATDRCC 44
TR|I2DAL6|B. diporus       MRTLWIMAVLLVGVEGNLWQLGKMILLETGKIPAKSYAAYGCNCGLGGRGKPKDATDRCC 60
                                                :****** ****  .***..* * **  *:*:*********

Bothrops n. urutu          YVHKCCYKXXXXXXXXXXDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYN 95
SP|P82287|B. pirajai       YVHKCCYKKLTGCNPKKDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYN 104
SP|Q90249|B. jararacussu YVHKCCYKKLTGCDPKKDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYN 120
SP|Q9IAT9|B. pauloensis  YVHKCCYKKLTGCDPKKDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYN 103
SP|P86975|B. leucurus      YVHKCCYKKLTGCDPKKDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYN 104
SP|P24605|B. asper         YVHKCCYKKLTGCNPKKDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLNTYN 120
TR|I6L8L6|B. brazili       YVHKCCYKKLTDCDPKKDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLDTYN 104
SP|Q9I834|B. moojeni       YVHKCCYKKLTGCDPKKDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLDTYN 104
SP|P86453|B. alternatus    YVHKCCYKKLTGCNPKKDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLNTYN 104
TR|I2DAL6|B. diporus       YMHKCCYKKLTGCDPKKDRYSYSWKDKTIVCRENNSCLKELCECDKAVAICLRENLDTYN 120
                           *:******          ************** *** ******************** ***

Bothrops n. urutu          KKYRY-HLKPFCKKXXXX 108          Identity (%)
SP|P82287|B. pirajai       KKYRYH-LKPFCKKADDC 121             92.3%
SP|Q90249|B. jararacussu KKYRYH-LKPFCKKADPC 137               92.3%
SP|Q9IAT9 B. pauloensis  KKYRYH-LKPFCKKADPC 120               91.3%
SP|P86975|B. leucurus      KKYRYH-LKPFCKKADPC 121             88.9%
SP|P24605|B. asper         KKYRYY-LKPLCKKADAC 137             88.9%
TR|I6L8L6|B. brazili       KKYRNNHLKPFCKKADPC 122             88.1%
SP|Q9I834|B. moojeni       KKYRYNYLKPFCKKADPC 122             87.3%
SP|P86453|B. alternatus    KKYRYY-LKPLCKKADAC 121             83.8%
TR|I2DAL6|B. diporus       KKYRYNYLKPFCKKADPC 138             80.5%
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Fig. 3. Multiple alignment of BnuTX-I. The data shows identity with other Lys49 PLA_2 homologues: 92.3% with *B. pirajai* and *B. jaracussu*; 91% with *B. n. pauloensis*; 88.9% with *B. leucurus* and *B. asper*; 88.1% with *B. brazili*; 87.3% with *B. moojeni*; 83.8% with *B. alternatus* and 80.5% with *B. diporus*. The sequence was compared with data contained in the NCBI (National Center for Biotechnology Information) system evaluated by the BLAST® program (Basic Local Alignment Search Tool). The sequences in table (X) correspond to unidentified amino acid residues. The similarities and differences are represented between the phospholipases and isoforms. The L and Q amino acids at positions 5 and 11, respectively, are conserved and evidenced. The asterisk (*), period (.) and colon (:) symbols (presented in the bottom line of the alignment) indicate maximum identity among all the sequences, alteration of one amino acid residue or alteration of two amino acids, respectively. The absence of these symbols in the bottom line in each set indicates the presence of at least three different amino acids between the sequences analyzed.

Table 1
Mass-to-charge ratio in ppm (*m*/*z*) of the fragments obtained in BnuTX-I sequencing.

No. Frag.	Sequenced fragment	*Monoizotopic* (*m*/*z*)		Accuracy (ppm)
		Measured	Theoretical	
TSC1	SLFELGK	792.36	792.44	103.73
TSC2	MILQETGKNPAK	1328.68	1328.71	25.81
[a]TSC3	SYGAYGCNCGVLGR	1532.55	1532.65	66.43
TSC4	GKPKDATDR	986.51	986.51	0.71
[a]TSC5	DATDRCCYVHK	1423.50	1423.59	66.47
[a]TSC6	CCYVHKCCYK	1476.57	1476.58	5.78
TSC7	DRYSYSWK	1103.46	1103.50	37.70
TSC8	YSYSWKDK	1075.46	1075.50	35.52
[a]TSC9	DKTIVCGENNPCLK	1646.71	1646.77	40.39
[a]TSC10	ELCECDKAVAICLR	1735.72	1735.80	49.36
TSC11	ENLGTYNKK	1065.48	1065.55	66.82
[a]TSC12	YRYHLKPFCK	1410.63	1410.72	61.93
[a]TSC13	YHLKPFCKK	1219.58	1219.65	62.28

[a] Cysteine residues are modified into carboxyamidomethylcysteine.

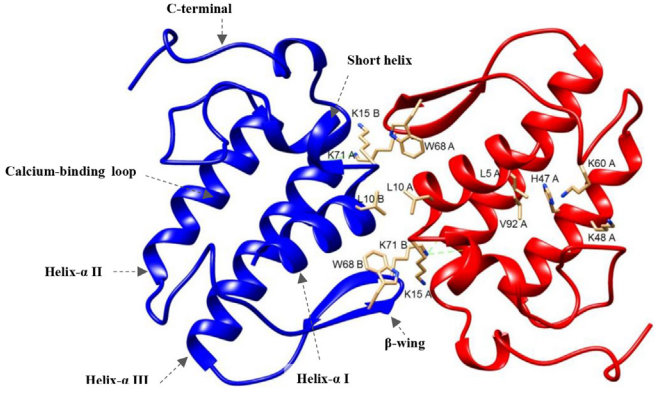

Fig. 4. Dimeric BnuTX-I modeled using homology modeling. The template structure selected was Phospholipase-A_2 homolog 1 from *Bothrops jararacussu* (PDB code: 3I03). Similarity between the template and target is about 99.15%. Ribbon representation performed in UCSF Chimera. Monomer A is in red and B in blue. The regions of the three-dimensional structure (monomer B) and the amino acids of the interface and the active site (monomer A) are highlighted and identified. Hydrogens bonds are in dashed green lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

than 90% of its residues in allowed regions (Laskowski et al., 1993). Analysis of the Ramachandran plot for the structure of BnuTX-I (Fig. 4) showed that 92.0% of the amino acids are in favorable regions. The quality of the model was also assessed by comparing the predicted structure with the template structure via superimposition and the RMS deviation of atomic positions (RMSD) assessment. The RMSD of Cα trace between the homology structure and the template is 0.19 Å supporting that the generated model is reasonably good and quite similar to the template.

3.2. Functional characterization

With respect to functional characterization, in the evaluation of phospholipase activity, BnuTX-I, different from the venom of *B. neuwiedi urutu*, was not able to hydrolyze the synthetic substrate

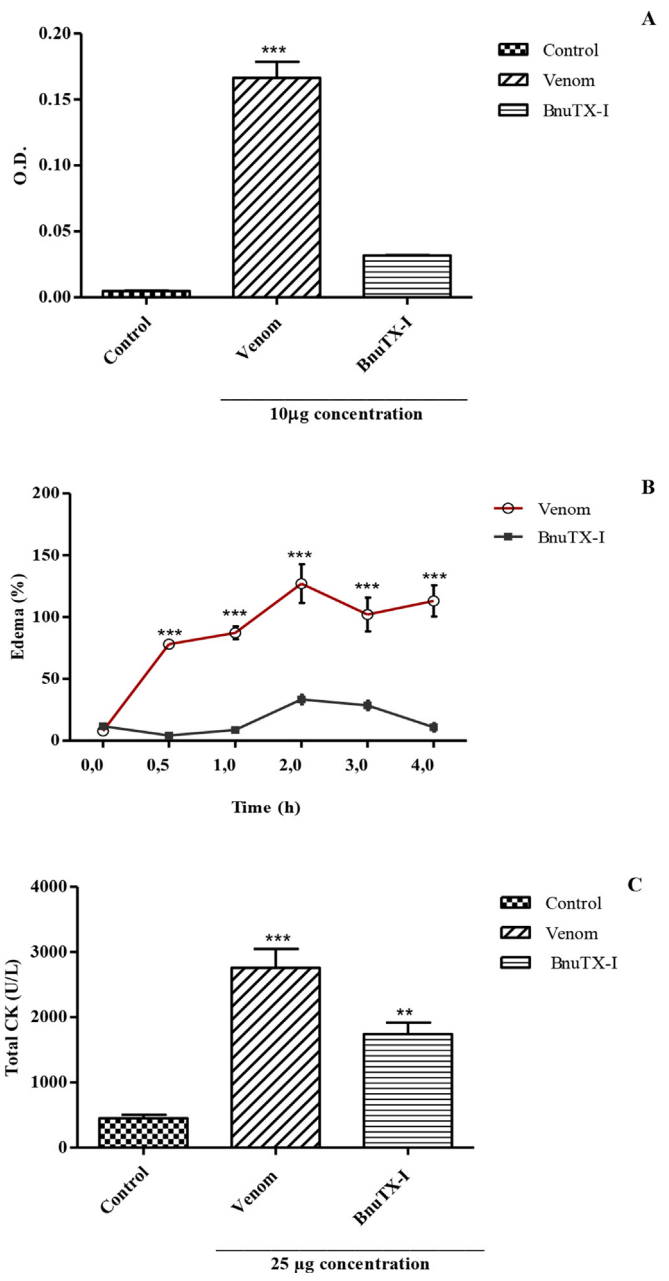

Fig. 5. Evaluation of phospholipase, edema and myotoxic activity. (5A) shows the phospholipase activity profile of BnuTX-I and *B. neuwiedi urutu* venom, on 4N3OBA (190 µL). The substrate was diluted in buffer (10 mM Tris–HCl + 10 mM $CaCl_2$ + 100 mM NaCl), pH 8, 10 µL of samples (the venom or 10 µg/mL of BnuTX-I) or deionized water [negative control]. Statistical analysis was performed by ANOVA and the Tukey test and expressed as mean ± standard deviation ($P < 0.05$). The PLA_2 and the negative control remained near the basal region (inactive), different from the positive control (+) (venom), which showed a higher Optical Density (O.D.). The experiment, conducted in a solution incubated at 37 °C, was analyzed in O.D. and monitored at 425 nm for 30 min. B) shows the evaluation of edematogenic activity performed with injection of the venom or BnuTX-I by s.c. route (10 µg/20 µL) into the footpad, the right hind paw of male Swiss mice (23–26 g) and as a negative control, sterile PBS 1× (20 µL), injected into the left paw. The data were analyzed with 30 min intervals for 4 h. The significance of differences between the samples and the control were evaluated by the ANOVA method $P < 0.05$ and Tukey test. Myotoxic activity (C), assessed based on CK levels was observed in tests with the venom or BnuTX-I, injected at concentrations of 25 µg/50 µL (PBS) in the gastrocnemius muscle. For the negative control 50 µL 1× PBS was injected. After 3 h the levels of enzymatic activity of the creatine kinase protein (CK) were evaluated at 340 nm. Results are presented as mean ± standard deviation ($P < 0.05$) and statistical analysis by ANOVA and the Tukey test. Readings of the tests were performed in a microplate spectrophotometer, Eon (Biotek).

(Fig. 5A). The kinetics of the edema response were statistically different from the negative control after 30 min of inoculation with the venom, and after 1 h of inoculation with BnuTX-I, with maximum activity after 2 h (Fig. 5B). In evaluating the myotoxic activity of the toxin and the venom, CK concentrations present in the plasma increased after administration of the toxin and venom, with values of 424.240 U/L for the negative control; 2759.170 U/L for the venom and 1954.8 U/L for BnuTX-I (Fig. 5C).

The analysis of cell viability of thioglycollate-elicited peritoneal macrophages incubated with BnuTX-I showed that this toxin presented cytotoxic effects at concentrations above 12 µg/mL (Fig. 6A). Interleukin 1β (IL-1β) concentrations in thioglycollate-elicited peritoneal macrophages incubated with BnuTX-I were significantly detected at concentrations of 12 and 25 µg/mL (Fig. 6B) compared to control cells incubated with RPMI. On the other hand, TNF-α was not significantly detected at all BnuTX-I concentrations tested compared to control cells incubated with RPMI (Fig. 6C).

The antimicrobial tests showed different percentages of microbial growth inhibition promoted by the venom and BnuTX-I against strains of *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *S. aureus*, having more significant activity for *P. aeruginosa* in concentrations of 50 and 100 µg/mL (Fig. 7).

4. Discussion

In snake venom, molecules are labeled with different biotechnological potential, where the isolation and biochemical characterization represent initial and fundamental steps for the identification of useful compounds in the production of new drugs (Kini, 2002). A new basic toxin called BnuTX-I was isolated from *B. neuwiedi urutu* venom using two chromatographic steps: anion exchange and reverse phase.

Sequencing of BnuTX-I obtained 108 amino acid residues which, after multiple alignment, showed high identity with snake PLA_2 homologues (Lys49), with up to 92.3% identity with BthTX-I and PrTX-I toxins from *B. jararacussu* and *B. pirajai*, respectively. Conserved amino acids such as phenylalanine at position 5 (L5) and a glutamine at position 11 (Q11) were observed (Fig. 3). The amino acid composition in BnuTX-I's sequence showed a high number of Lys (K), Tyr (Y) Gly (G) residues, and lower concentrations of Pro (P), and Cys (C), all characteristic of basic snake venom PLA_2s (Dua and Cho, 1994). In addition, from the comparative analysis with other Lys49 PLA_2s it was possible to identify the residues from the catalytic site (G29; D80 H47; K48; Y51; Y55) in green, those from the calcium binding loop (N27; G29; L31 and K48) in yellow, and those from the hydrophobic site (L2; L5; I8; A18 and V30) in blue. Moreover, the amino acids G29 and K48 act, simultaneously, on calcium binding and catalysis (Fig. 3).

From the three-dimensional structural model of the toxin considering the numbering proposed by Renetseder et al. (1985), it was possible to identify structures similar to those of other PLA_2 Lys49 homologues such as the N-terminal region (residues 1–12) represented by α-helix I; the short helix formed by residues 18–23; the calcium binding loop (residues 25–34); α-helix II consisting of residues 40–55; the antiparallel β sheet (β-wing) (residues 75–77 and 82–84); α-helix III (residues 90–107), which binds to a flexible region, named the C-terminal loop (residues 108–133), the latter being commonly involved in the biological activities of these toxins (Fernandes et al., 2014; Sudharshan and Dhananjaya, 2015).

BnuTX-I showed no phospholipase activity on synthetic substrates, but induced edema and myotoxicity *in vivo*, in a similar way to that described for other Lys49 myotoxins (Gutiérrez; Ownby, 2003; Soares et al., 2004; Stábeli et al., 2012). Several studies have suggested mechanisms involved in the myotoxic activity of Lys49 PLA_2s, such as conformational changes and the exhibition of

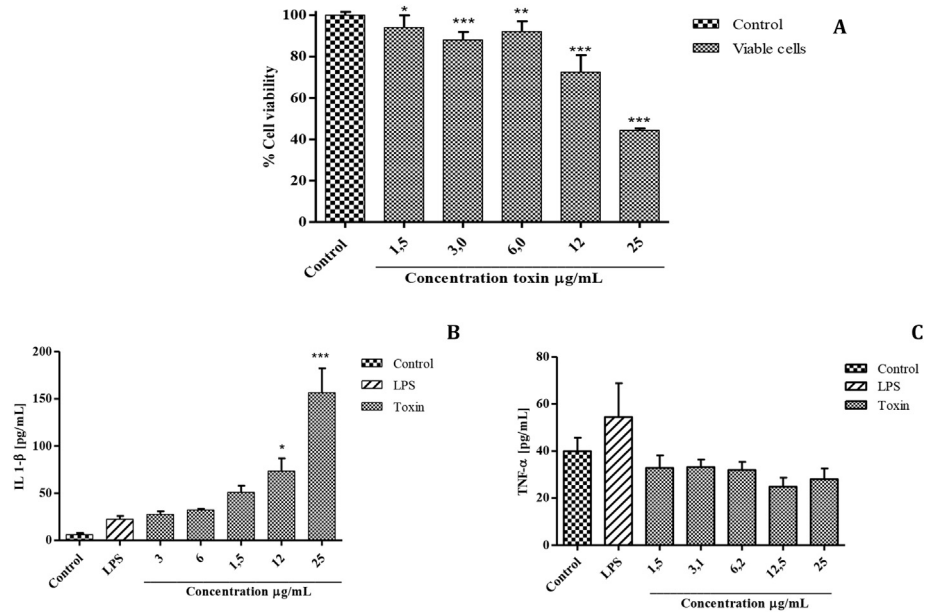

Fig. 6. Effect of BnuTX-I from *B.neuwiedi urutu* on cell viability (**A**) **and cytokine concentrations**. Fig. 5A represents the thioglycollate-elicited peritoneal macrophage viability after 24 h of incubation with different concentrations (1.5, 3.0, 6.0, 12 and 25 µg/mL) of Lys49 PLA_2 (BnuTX-I) at 37 °C and 5% CO_2. As a negative control, cells were incubated with RPMI medium. Statistical analysis was performed using an ANOVA test ($P < 0.05$). B and Fig. 5C show cytokine IL-1β and TNF-α concentrations (pg/mL), respectively, of thioglycollate-elicited peritoneal macrophages after a 12-h incubation with different concentrations (1.5, 3.0, 6.0, 12 and 25 µg/mL) of Lys49 PLA_2 (BnuTX-I) at 37 °C and 5% CO_2. As a negative control, cells were incubated with RPMI medium and for the positive control, cells were incubated with LPS. Statistical analyses were determined by the ANOVA test ($P < 0.05$).

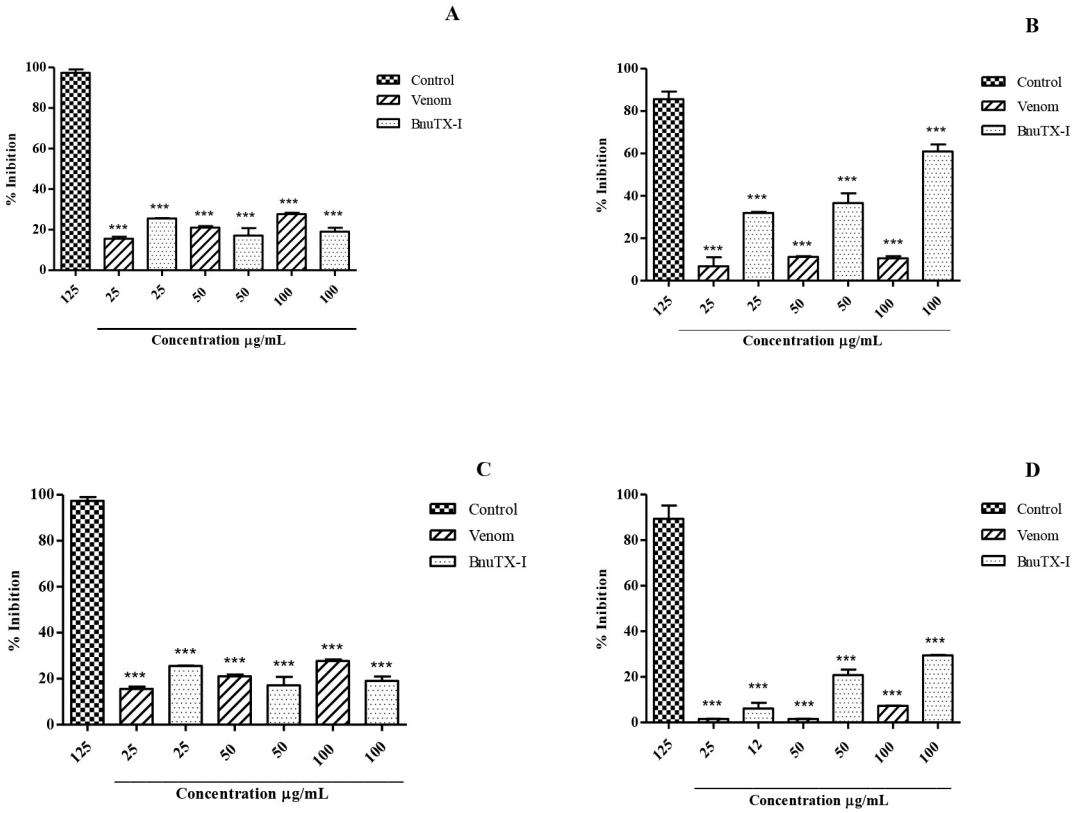

Fig. 7. Antimicrobial activity of the venom (**V**) **and BnuTX-I against strains of Gram-negative and Gram-positive bacteria**. The figures show different percentages of inhibition in bacterial growth at different concentrations (25–100 µg/mL) for the venom or BnuTX-I. As a positive control an LB broth medium with Chloramphenicol (125 µg/mL) was used. 7A shows tests with strains of *Escherichia coli* (ATCC 25922). B shows strains of *P. aeruginosa* (ATCC 28853). C shows *S. aureus* (ATCC 29213) and D, strains of *Klebsiella pneumoniae* (ATCC 13883). The evaluations were spectrophotometric by measuring absorbance at 630 nm and significance tests (*) determined by the ANOVA method ($P < 0.05$).

positively charged amino acids in the C-terminal region, responsible for membrane rupture (Ambrosio et al., 2005), and the release of K^+ and ATP after skeletal muscle injury (Cintra-Francischielli et al., 2010).

In the assessment of cytotoxicity and cytokine production (IL-1β and TNF-α) with murine macrophages, BnuTX-I was cytotoxic to the cells at concentrations above 12 μg/mL. Data also observed the production of IL-1β interleukins after 24 h of exposure. These results are corroborated by studies with Lys49 PLA_2 variants by Zuliani et al. (2005), Setúbal et al. (2013) and Furtado et al. (2014), which reported the low toxicity of these toxins for this cell type. Among the main mediators of inflammation molecules are interleukins 1 (IL-1) and tumor necrosis factor alpha (TNF-α) (Motta et al., 2014). IL-1β has the ability to induce the synthesis of a wide variety of factors, including MMPs, nitric oxide, prostanoids and other cytokines, such as TNF-α, which contributes to inflammatory responses (Kida et al., 2005). Since the increase of both cytokines was observed in macrophages stimulated with BnuTX-I, it is suggested that this toxin can act in any way in the inflammatory effect induced by the venom.

Other studies have shown that Lys49 PLA_2 variants from *Bothrops* snakes can induce the release of cytokines (Lomonte, 1994; Zuliani et al., 2005; Furtado et al., 2014). In addition, they mention the involvement of IL-1 in the synthesis of enzymes related to the production of prostaglandins and in the synthesis of IL-6 and IL-8 by endothelial cells (Lomonte, 1994).

BnuTX-I demonstrated antimicrobial potential, with higher inhibition of bacterial growth for *P. aeruginosa*. Other Lys49 PLA_2 homologues also exhibited antimicrobial effects, such as myotoxin II (*Bothrops asper*) on *S. aureus*, *V. cholerae* and *E. coli* strains (Páramo et al., 1998), and BthTX-I (*B. jararacussu*) on strains of *E. faecalis* and *E. coli* (Silveira, 2013).

Studies report that the activity against bacteria may be associated with interactions of the PLA_2 with the membrane (Buckland and Wilton, 2000; Villalobos et al., 2007), which directly or indirectly, disrupted the cellular integrity of different bacteria (Yan et al., 2000; Soares et al., 2000). Sites with a negative charge in the membrane could serve to anchor the protein, providing a chemical interaction which would disrupt the membrane, inducing cell death (Gutiérrez and Lomonte, 1995). Ward et al. (1998) showed that a loop in the C-terminal region of Lys49 PLA_2s (positively charged residues and aromatic residues) would be related to the pharmacological properties (myotoxic and antimicrobial actions), by mechanisms independent of calcium and phospholipid hydrolysis (Diaz et al., 1991; Chioato et al., 2007). This information corroborates the data from Páramo et al. (1998) where synthetic peptides from the C-terminal region comprising residues 115–129 of myotoxin II reproduced the same effects as the full protein.

5. Conclusions

The present study shows the isolation of a Lys49 phospholipase A_2 homologue isoform called BnuTX-I which showed high identity with other phospholipase homologues from snake venoms of the same genus, demonstrating pro-inflammatory activity as evidenced by the ability to induce edema and interleukin (IL1 and TNFα) production. In addition, there was significant antimicrobial activity against the different bacterial strains tested.

Conflict of interest

The work doesn't show any conflict of interest.

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