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BmajPLA$_2$-II, a basic Lys49-phospholipase A$_2$ homologue from *Bothrops marajoensis* snake venom with parasiticidal potential

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ABSTRACT

Snake venoms contain various proteins, especially phospholipases A$_2$ (PLA$_2$s), which present potential applications in diverse areas of health and medicine. In this study, a new basic PLA$_2$ from *Bothrops marajoensis* with parasiticidal activity was purified and characterized biochemically and biologically. *B. marajoensis* venom was fractionated through cation exchange followed by reverse phase chromatographies. The isolated toxin, BmajPLA$_2$-II, was structurally characterized with MALDI-TOF (Matrix-assisted laser desorption/ionization-time of flight) mass spectrometry, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by two-dimensional electrophoresis, partial amino acid sequencing, an enzymatic activity assay, circular dichroism, and dynamic light scattering assays. These structural characterization tests presented BmajPLA$_2$-II as a basic Lys49 PLA$_2$ homologue, compatible with other basic snake venom PLA$_2$s (svPLA$_2$), with a tendency to form aggregations. The *in vitro* anti-parasitic potential of *B. marajoensis* venom and of BmajPLA$_2$-II was evaluated against *Leishmania infantum* promastigotes and *Trypanosoma cruzi* epimastigotes, showing significant activity at a concentration of 100 μg/mL. The venom and BmajPLA$_2$-II presented IC$_{50}$ of 0.14 ± 0.08 and 6.41 ± 0.64 μg/mL, respectively, against intraerythrocytic forms of *Plasmodium falciparum* with CC$_{50}$ cytotoxicity values against HepG2 cells of 43.64 ± 7.94 and >150 μg/mL, respectively. The biotechnological potential of these substances in relation to leishmaniasis, Chagas disease and malaria should be more deeply investigated.

1. Introduction

Bothropic venoms are biochemically complex, with varying protein components, including serine proteases [1], metalloproteinases [2], L-amino acid oxidases [3], C-type lectins [4], myotoxins [5], disintegrins [6], and group II PLA$_2$s [7], which have proven to be invaluable research tools and have provided leads for development of new therapies [8–12].

Group II PLA$_2$s can display a series of actions that result in toxic effects on victims such as myotoxicity [13], neurotoxicity [14], cytotoxicity [15,16] and genotoxicity [17–19]. Several studies focusing on the biological functions of PLA$_2$s have discovered essential information of their implication in diseases such as rheumatoid arthritis, inflammation and bone erosion [20], cancer [21,22], and neurological disorders such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and epilepsy [23,24]

There are also several studies investigating the antiparasitic effects of PLA$_2$s against the parasites that cause leishmaniasis, Cha-

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gas disease and malaria. Castillo-Vigil et al. [25] proposed that the mechanism of action of the trypanocidal activity of *B. asper* venom occurred through a combination of necrosis and apoptosis, probably mainly caused by the actions of the venom's PLA_2s due to the cell membrane's integrity being compromised.

Adade et al. [26] demonstrated that the use of *para*-bromophenacyl bromide (*p*-BPB), a PLA_2 inhibitor, caused partial inhibition of the trypanocidal activity of *C. v. viridis* venom. In three studies, PLA_2s were isolated from *B. brazili, B. mattagrossensis* and *B. moojeni* venoms and their antiparasitic effects were checked against several different species of *Leishmania* [27–29]. Additionally, a non-catalytic PLA_2 isolated from *Bothrops pauloensis* venom, BnSP-7 inhibited the proliferation of *L. amazonensis* promastigotes and amastigotes and also caused morphological changes in the parasites [30].

Among the results published against the parasite that causes malaria, a PLA_2 isolated from *Crotalus adamanteus* snake venom blocked the *in vitro* development of ookinetes, forms of the parasites *Plasmodium falciparum* and *P. gallinaceum* in the mosquito intestine [31]. Guillaume et al. [32] showed the antimalarial effects of seven PLA_2s, some from snake venoms, against intraerythrocytic forms of *P. falciparum*. These studies are corroborated by other studies with snake venoms and their PLA_2s, as well as bee venoms and their PLA_2s against *Plasmodium* spp. [33–35].

The proteins from *B. marajoensis* venom are still poorly studied. These studies include the biological characterization of the venom [36]; an investigation of the antibacterial and antiparasitic effects of a Lys49 PLA_2 isolated from the venom, called Bmar [37]; the biological characterization of an Asp49 PLA_2, Bmaj-9 [38]; the neurotoxic, myotoxic and cytolytic activity of the Asp49 PLA_2s BmjeTX-I and BmjeTX-II [39]; and the renal and cardiovascular effects of the venom and an isolated PLA_2 [40].

Based on the information presented, this study aimed to biochemically and structurally characterize a new basic phospholipase A_2 from *Bothrops marajoensis* venom and evaluate its antiparasitic activity against the protozoa that cause Leishmaniasis, Chagas disease and malaria.

2. Material and methods

2.1. Venom and authorization

Bothrops marajoensis venom was obtained from a pool of adult specimens, acquired from the BioAgents Serpentarium (Batatais – SP) and donated by FMRP-USP, Brazil. The dehydrated venom was maintained refrigerated (4 °C) at the Amazonian Venom Bank at the Center for Studies of Biomolecules Applied to Health, CEBio-UNIR/FIOCRUZ-RO; authorization: CGEN/CNPq 010627/2011-1.

2.2. Biochemical characterization

2.2.1. Ion exchange chromatography

A 400 mg sample of *B. marajoensis* venom was solubilized in 2 mL of 50 mM ammonium bicarbonate (AMBIC), pH 8.0 (Solution A) and centrifuged at 7000 *xg* for 5 min. The supernatant was fractionated on a CM-Sepharose column (90 cm × 1.5 cm) equilibrated with AMBIC. A linear gradient of 0–100% 500 mM AMBIC, pH 8.0 (Solution B) was used for 600 min under a flow of 2.5 mL/minute, in an Akta Purifier (GE) chromatography system. Elution was monitored using 215 and 280 nm filters and the fractions were collected manually. The samples were lyophilized and stored in refrigerators at −20 °C [41].

2.2.2. Reverse phase chromatography

Fraction 11 from the ion exchange chromatography was diluted in 0.1% trifluoroacetic acid (TFA) (Solution A) and subjected to reverse phase chromatographic fractionation in a C18 column (25 cm × 0.45 cm – Discovery) previously equilibrated with solution A and eluted under a 0–70% gradient of ACN 99.9% (v/v) and 0.1% TFA (v/v) (Solution B) for 5 column volumes (one column volume contains 3.98 mL) under a flow rate of 1 mL/minute. Elution of the sample was monitored at 280 nm. Samples were manually collected, lyophilized and stored at −20 °C [41].

2.2.3. Electrophoresis in SDS-PAGE

SDS-PAGE, 12.5% (m/v), was performed in a discontinuous pH system under reducing conditions with adjustments [42]. 10 μg of toxin was mixed with 4% SDS (m/v), 0.2% Bromophenol Blue (m/v), 20% glycerol (v/v) in 100 mM Tris pH 6.8 and heated for 5 min at 90 °C. 0.2 M Dithiothreitol (DTT) was also added. After electrophoresis, the polyacrylamide gel was fixed in a solution of 40% methanol (v/v) and 7% acetic acid (v/v) for 30 min and then immersed in 0.08% Coomassie Brilliant Blue G-250® (m/v), 8.0% aluminum sulfate (m/v), 1.6% phosphoric acid (m/v) and 20.0% methanol (v/v) for 30 min. Excess dye was removed by soaking in a bleach solution containing 4% ethanol and 7% acetic acid (v/v) in water. Image scanner® (GE Healthcare Lifescience) was used to document the gel.

2.2.4. Molecular mass determination

The molecular mass of the isolated protein was determined by mass spectrometry [43] using MALDI equipment with two TOF analyzers (AXIMA TOF^2 Shimadzu Biotech), previously calibrated using Sigma molecular mass standards. An aliquot containing 1 μg of the sample was solubilized in 0.1% TFA (v/v), co-crystallized with a sinapinic acid saturated solution (ionization matrix) and applied on a metal plate. The instrument was operated in linear mode. Ions were generated by irradiation with a nitrogen laser with fixed wavelength on 337 nm. Signals were captured at 500 MHz and the obtained data was processed, using the software Launchpad.

2.2.5. Isoelectric point determination

The sample was solubilized in a hydration solution containing 7 M urea, 2 M thiourea, 2.0% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (m/v), 0.5% immobilized pH buffer gradient (IPG buffer®) (v/v), and 1.0% bromophenol blue (m/v); and applied on 7 cm polyacrylamide strips with a pH gradient from 3.0 to 10.0 immobilized non-linearly (Immobiline DryStrip®). The isoelectric focusing [44] was performed in an IPGphor III System® (GE Healthcare Life Science) according to the manufacturer's instructions. After isoelectric focusing, the strip containing the protein was placed on the upper portion of the 12.5% SDS-PAGE gel (m/v) to perform one-dimensional electrophoresis as described in 2.2.3. The gel image was obtained with the aid of an Image Scanner III®.

2.2.6. Circular dichroism

Circular dichroism experiments were performed in a Jasco J-815 spectropolarimeter (JASCO Inc., Tokyo, Japan). The solubilized toxin was isolated in 0.5 mg/mL of 50 mM AMBIC, pH 9. Measurements were recorded in the spectral range of 190–260 nm at 20 °C with an optical path length of 0.05 mm, a velocity of 100 nm/minute, a bandwidth of 2 nm and 1 s of response time. Twenty spectra were acquired, averaged and corrected for the buffer solution (baseline) and then normalized to residual molar ellipticity [θ]. Deconvolution of the CD spectra was performed with the Dichroweb on-line server [45] using the CDSSTR algorithm [46] with set Reference [4].

2.2.7. Dynamic light scattering

Dynamic light scattering was performed with the isolated toxin solubilized in 50 mM AMBIC, pH 9 at a concentration of 1 and 2 mg/mL. The sample was filtered using a PVDF membrane with

a pore size of 0.22 μm (Millipore) and centrifuged at 10100 *xg* for 10 min at 0 °C prior to the assay. Data from one hundred measurements were collected at temperatures of 10, 20 and 25 °C at a fixed angle of 90° in Dynapro TITAN equipment (Wyatt Technology). All of the results were analyzed using the software Dynamics v.6.10 [47].

2.2.8. N-terminal sequencing

Sequencing of amino acid residues from the N-terminal region of the isolated protein was done using 10 μg of the protein. A PPSQ-33A automated microsequencer (Shimadzu) was used along with Edman's chemical degradation method [48].

2.2.9. Tryptic digestion and partial sequence determination

Protein fragmentation was performed by trypsinization as described by [49]. Successively, analysis of the fragments was done by LCMS-IT-TOF mass spectrometry (Shimadzu) and using the software Peaks Mass Spectrometry (Bioinformatics Solutions Inc., Canada) as described by [50]. The software PEAKS studio 7.0 was used, coupled with the InChorus multi-algorithmic tool and protein sequences deposited in databanks in order to elucidate the relative position of each trypsinized fragment in the primary sequence of the toxin. A similarity search in UniProtKB and NCBI databases using the BLAST algorithm was performed (http://blast.ncbi.nlm.nih.gov). Multiple alignment using the Clustal W program was performed (http://www.ebi.ac.uk/tools/msa.clustalw2/).

2.3. Functional characterization

2.3.1. Phospholipase activity

The phospholipase activity assay was performed using 4-nitro-3-(octanoyloxy) benzoic acid (4N3OBA) as a colorimetric reagent. First, 200 μg of reagent was solubilized in 2 mL of a solution with 20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 10 mM $CaCl_2$, under agitation. The samples were diluted in Milli-Q water to reach a concentration of 1 μg/μL. In a 96-well Plate 10 μL of sample and 190 μL of 4N3OBA solution, in triplicate, were dispensed. Distilled water and BthTX-II as the negative and the positive controls, respectively, were used. The plate was incubated in a spectrophotometer at 37 °C and the optical density (OD) was measured at 425 nm with kinetic intervals of 30 s over a period of 30 min [51].

2.3.2. In vitro *anti-promastigote activity against* Leishmania infantum, *MCAN/ES/92/BNC83*

Promastigotes were cultured at a concentration of 1.5×10^6 parasites/mL in 96-well plates, using Schneider culture medium supplemented with 10% fetal bovine serum (FBS) and *B. marajoensis* venom or isolated PLA_2 (100, 50, 25, 12.5 and 6.25 μg/mL) or culture medium (negative control). 100 μg/mL of pentamidine was used as a positive control. Thereafter, 20 μL of 3 mM Resazurin (Sigma®) was added. The plate was further subjected to incubation at 26 °C for 4 h and finally the absorbance was determined at 595 and 495 nm. The percentage of dead parasites, and thus the anti-promastigote activity was calculated based on promastigotes cultured only with RPMI [52].

2.3.3. In vitro *anti-epimastigote activity against* Trypanosoma cruzi, *Strain CL-clone B5*

Epimastigote parasites (5×10^4/well) were plated on 96-well plates with 200 μL of liver infusion tryptose culture (LIT) supplemented with 10% FBS and different concentrations of *B. marajoensis* venom or isolated PLA_2 (100, 50, 25, 12.5 and 6.25 μg/mL) or culture medium (negative control). As a positive control, 100 μg/mL of benznidazole was used. The plate was incubated at 28 °C for 72 h. Each concentration was tested in triplicate. Thereafter, 50 μL of 200 μM Chlorophenol red-β-D-galactopyranoside (CPRG) was added; the plate was incubated at 37 °C for 4 h and finally the absorbance was determined at 595 nm. The efficacy of *B. marajoensis* venom or purified protein at different concentrations, and thus the anti-epimastigote activity, was estimated by calculating the percentage of dead parasites [53].

2.3.4. In vitro *antimalarial activity against* Plasmodium falciparum

Roswell Park Memorial Institute (RPMI) culture medium (180 μL/well) containing 0.05% parasitemia and 1.5% hematocrit was added to 96-well plates. Different serial concentrations of *B. marajoensis* venom (3-0.093 μg/mL) and isolated PLA_2 (10-0.3125 μg/mL) were also added, in triplicate. Infected erythrocytes were added as a negative control and artemisinin (Sigma®) (50-0.7813 ng/mL) as a positive control. The plate was incubated for 72 h at 37 °C and then frozen at −70 °C and thawed twice in order to lyse the erythrocytes. Subsequently, an anti-HRPII enzyme immunoassay was performed as described by Noedl et al. [54]. Growth inhibition of 50% of the parasites was determined by dose-response curves based on nonlinear regression using the program Origin (OriginLab Corporation, Northampton, MA, USA).

2.3.5. Cytotoxic activity against hepatic cells, HepG2

The HepG2 cell line (derived from a human hepatoma) was maintained in RPMI medium containing 10% fetal bovine serum and 40 mg/L of gentamycin in an incubator with 5% CO_2, 95% humidity at 37 °C. In a 96-well plate, 180 μL hepatic cells (1×10^4 cells) were dispensed and maintened for 18 h in humidified atmosphere with 5% CO_2 at 37 °C. After, this period the cells were incubated with 20 μL of RPMI (control) or *B. marajoensis* venom (100-1.56 μg/mL) and isolated PLA_2 at concentrations of −150-4.68 μg/mL under humidified atmosphere for 72 h at the same conditions. After that, 10 μL of a solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5 mg/mL in phosphate buffered saline solution) were added to each well and incubated for 4 h at 36 °C as described by Teles et al. [55]. Absorbance was read in a spectrophotometer at 540 nm. Percent of cytotoxicity was determined and the 50% cytotoxicity concentration (CC_{50}) was estimated from dose-response curves created using the program OriginLab Corporation (Northampton, MA, USA).

2.3.6. Statistical analyses

Statistical analyses were performed using Graph Pad Prism version 6.0 software. The results were expressed as mean ± standard deviation (SD). The significance of differences observed was determined by the ANOVA test followed by the Tukey post test with $p \leq 0.05$ considered significant.

3. Results

In order to isolate a basic PLA_2 from *Bothrops marajoensis* two chromatographic steps were used. The first step was cation exchange, which allowed the separation of 11 major fractions (1–11) (Fig. 1A). Based on its basic character and the presence of a component with a relative molecular mass of 14 kDa, the fraction 11 was selected and submited to reverse phase chromatography. The apparent molecular mass in a 12.5% SDS-PAGE was seen (data not shown). The reverse phase chromatographic profile revealed one major peak of a protein that was named BmajPLA$_2$-II (Fig. 1B).

The isolated toxin found in monomeric, dimeric, trimeric, tetrameric, and pentameric forms was analyzed using 12.5% SDS-PAGE under reducing conditions (Fig. 2A). The isoelectric point (pI) equal to 9.68 of BmajPLA$_2$-II was determined by two-dimensional electrophoresis (Fig. 2B). The oligomerization

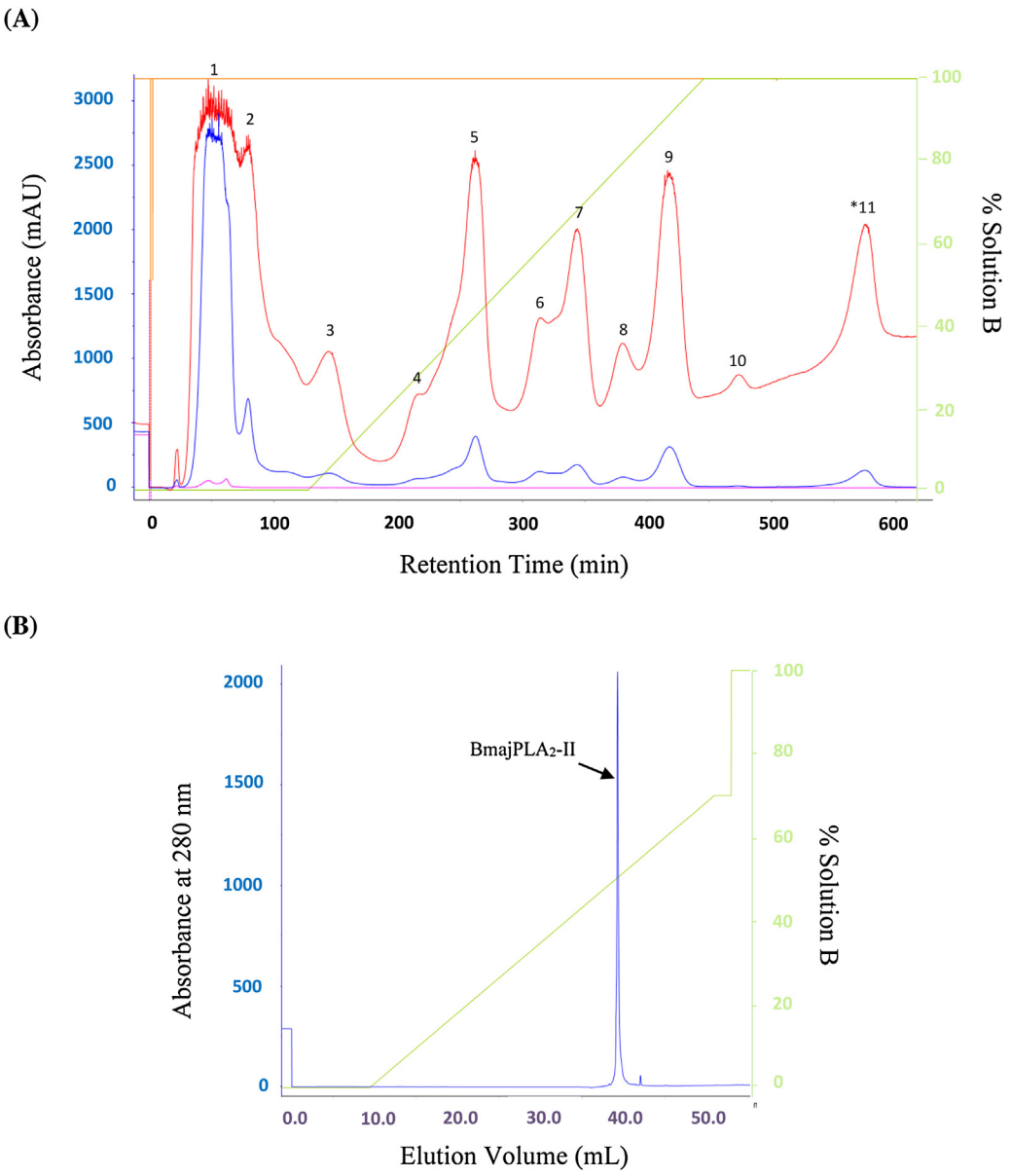

Fig. 1. (A): Chromatographic profile of *Bothrops marajoensis* venom using a cation exchange resin. Chromatographic profile of *Bothrops marajoensis* venom using cation exchange chromatography in a CM-Sepharose column (90 × 1.5 cm) previously equilibrated with Solution A (50 mM AMBIC pH 8.0). The fractions were eluted under a 0–100% gradient of Solution B (500 mM AMBIC pH 8.0) at a constant flow rate of 2.5 mL/minute. *Indicates fraction 11 (F11) which was selected for further fractionation steps. Protein elution was monitored by absorbance at 215 nm (—) and 280 nm (—). The linear gradient of Solution B is indicated by the green line (—). (B): Reverse phase chromatographic profile. Chromatographic profile of fraction 11 from the ion exchange chromatography in a reverse phase column (Discovery C18) previously equilibrated with 0.1% TFA (Solution A) and eluted with 99% ACN + 0.1% TFA (Solution B) at a gradient of 0–100%, under a constant flow of 1 mL/minute. Protein elution was monitored by absorbance at 280 nm (—). The linear gradient of Solution B is indicated by the green line (—). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed in one-dimensional electrophoresis under reducing conditions was confirmed in two-dimensional gel electrophoresis.

The mass spectrometry analysis of the isolated PLA_2 showed a molecular mass of 13,956.00 Da in its monomeric form. Also, the analyis showed peaks at molecular masses of the molecule in the double charge (7,000.53 Da), dimer (27,864.79 Da), trimer (41,818.30 Da), tetramer (55,828.82 Da) and pentamer (69,543.33 Da) forms are apparent (Fig. 3A). A circular dichroism analysis showed about 45% of BmajPLA$_2$-II's secondary structures as alpha helices and 15% as beta sheets. This observation along with the graph's elliptical pattern show characteristics typical of phospholipases [56] (Fig. 3B).

BmajPLA$_2$-II presents great aggregation capacity at pHs close to its isoelectric point, confirmed by dynamic light scattering assays. In situations with low polydispersity (Pd) (near 15%), BmajPLA$_2$-II appears as dimers and tetramers (Tables 1 and 2).

Approximately 80% of the complete primary structure of the isolated protein, 94 amino acid residues, were determined. Its similarity with other Lys49 PLA$_2$s (Fig. 4) reinforces the kinetic evaluation of phospholipase activity (Fig. 5) where BmajPLA$_2$-II was shown to be enzymatically inactive.

The *B. marajoensis* venom demonstrated anti-leishmania activity between 57% and 78%, and activity against *T. cruzi* between 42% and 61% in concentrations from 6.25 to 100 μg/mL. BmajPLA$_2$-II demonstrated anti-leishmania activity between 7% and 29% and activity against *T. cruzi* between 3% and 31% at the same concentrations (Fig. 6A and B).

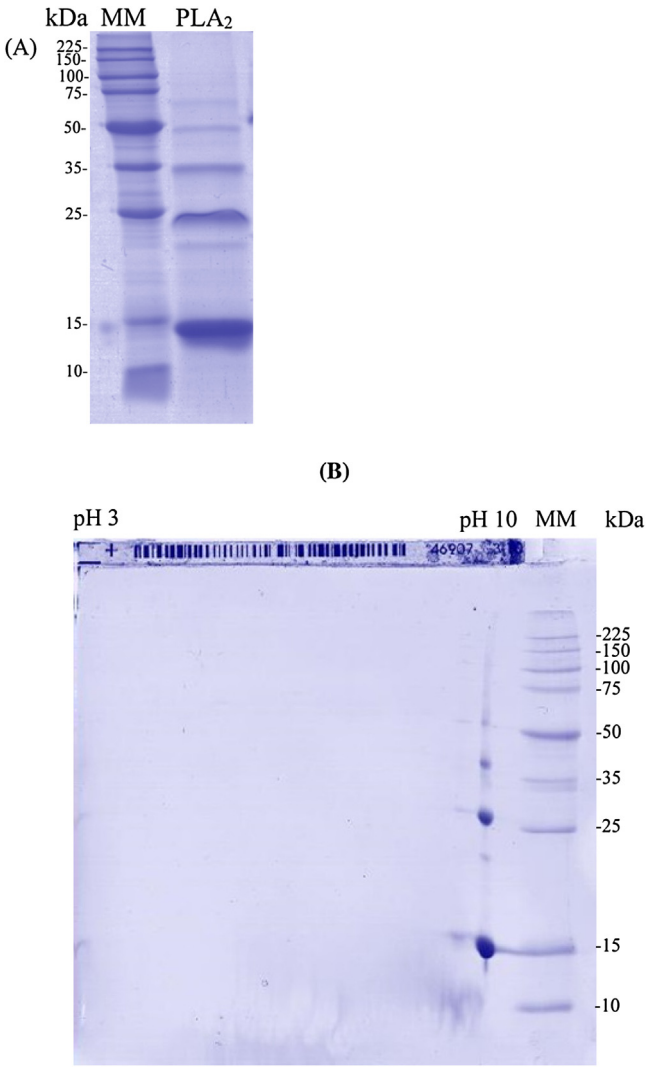

Fig. 2. One-dimensional electrophoresis of the PLA_2 isolated from *B. marajoensis* venom. One-dimensional polyacrylamide gel (12.5%) electrophoresis in (A): Reducing conditions with DTT showing the molecular mass standard from 10 to 225 kDa (lane 1), PLA_2 (line 2). Bands can be seen with approximate molecular masses of 14, 28, 42, 56, and 70 kDa in the lane containing the PLA_2. (B): Two-dimensional (2D) electrophoresis of the isolated PLA_2. (C)**:** Bidimensional (2D) electrophoresis of the basic PLA_2 isolated from *B. marajoensis* venom using a 7 cm polyacrylamide strip with a pH gradient from 3.0 to 10.0. Bands can be seen at a pI of 9.68 with approximate molecular masses of 14, 28, 42, 56 and 70 kDa.

Table 1
BmajPLA$_2$-II in its dimeric form in 50 mM AmBic, pH 9 (1 mg/mL after centrifugation and filtration).

Temperature (°C)	R_H (nm)	Pd (%)	MM (kDa)	Intensity (%)	Mass (%)
10	2.0	17.5	24	8.4	93.0
20	-	-	Aggregates	83.0	100
25	-	-	Aggregates	100	100

R_H = Hydrodynamic Radius; Pd = Polydispersity; MM = Molecular Mass.

Table 2
BmajPLA$_2$-II in its tetrameric form in 50 mM AmBic, pH 9 (2 mg/mL after centrifugation).

Temperature (°C)	R_H (nm)	Pd (%)	MM (kDa)	Intensity (%)	Mass (%)
10	3.4	14.0	54	19.5	97.0
20	-	-	Aggregates	100	100
25	-	-	Aggregates	100	100

R_H = Hydrodynamic Radius; Pd = Polydispersity; MM = Molecular Mass.

In tests against intraerythrocytic forms of *Plasmodium falciparum*, 50% of the maximal inhibitory concentration (IC_{50}) of the venom was 0.14 μg/mL. BmajPLA$_2$-II showed an IC_{50} of 6.41 μg/mL (Fig. 7 and Table 3). The venom had a cytotoxic effect against HepG2 hepatic cells presented as a CC_{50} of 43.64 μg/mL with a selectivity index of 312 while BmajPLA$_2$-II's CC_{50} against liver cells was more than 150 μg/mL with a selectivity index greater than 23.4 (Fig. 8 and Table 3).

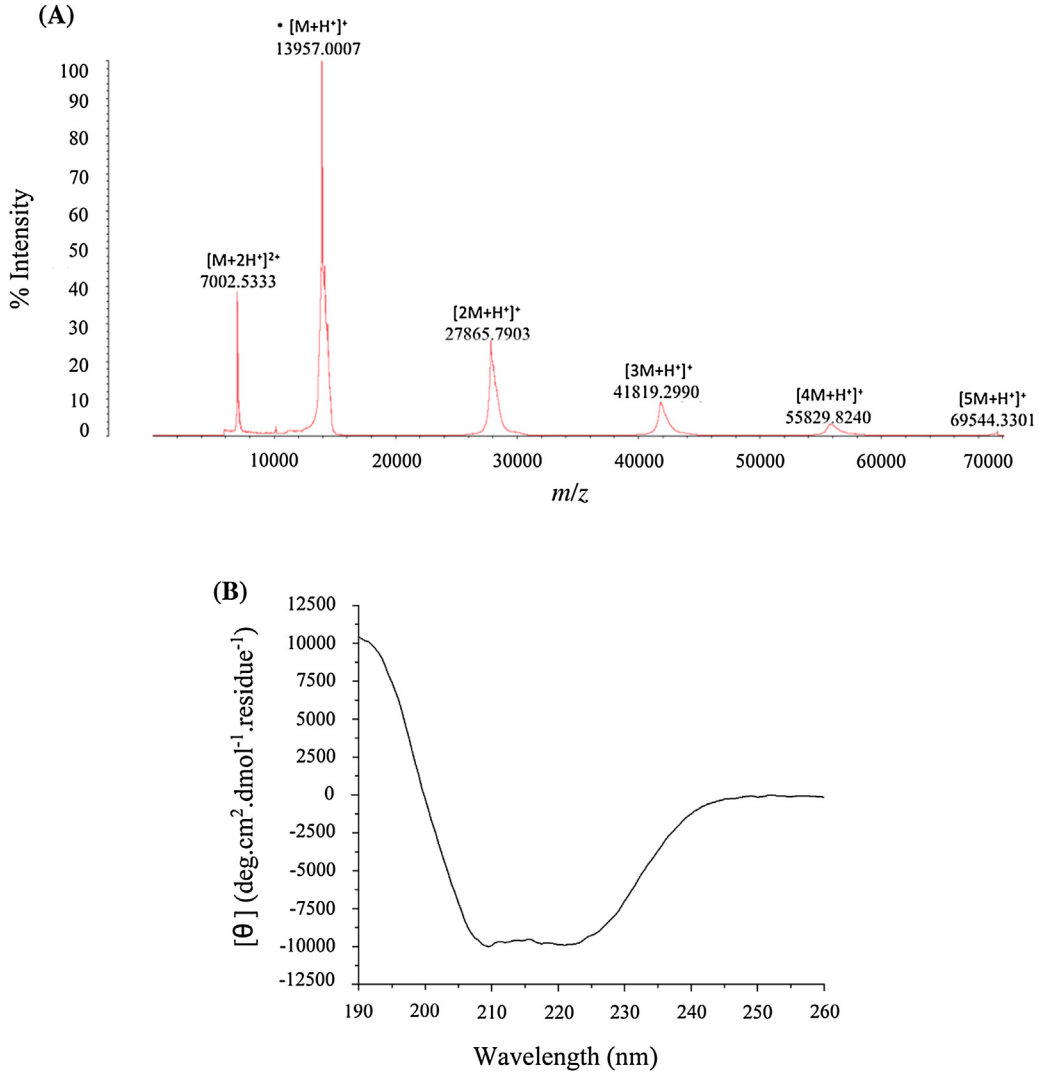

Fig. 3. (A): MALDI-TOF mass spectrum of the isolated PLA_2. Mass spectrum of the PLA_2 obtained in AXIMA TOF2 equipment. The spectrum shows the mass to charge ratio of the enzyme in the form of a monomer, *13957.00. Other forms that also appear include the double charge (m/z = 7002.53), dimer (m/z = 27866.79), trimer (m/z = 41819.30), tetramer (m/z = 55830.82) and pentamer (m/z = 69544.33). (B): Circular dichroism of BmajPLA$_2$-II. Graph showing the results of the circular dichroism experiments using BmajPLA$_2$-II. The ellipticity of the graph and the peaks representing a large amount of alpha-helices and fewer beta sheets are typical characteristics of secondary structures contained in phospholipases A_2.

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BmajPLA2-II  NLWQLGKMILLETGKIPAKSYGAYGCNCGVLGRXXXXXXXXXCCYVHKXXXXKLTGCDPK 60
Myo-II       NLWQLGKMILLETGKIPAKSYAAYGCNCGLGGRGKPKDATDRCCYMHKCCYKKLTGCDPK 60
MTX-II       SLVELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTDCDPK 60
MtxII        SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCNPK 60
PrTX-I       SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCNPK 60
PrTX-II      SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCNPK 60
BthTX-I      SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGKPKDATDRCCYVHKCCYKKLTGCDPK 60
BnSP-7       SLFELGKMILQETGKNPAKSYGAYGCNCGVLGRGQPKDATDRCCYVHKCCYKKLTGCDPK 60
             .* :****** **** *****.*******: **         ***:**    *** *:**

BmajPLA2-II  XXXYSYSWKXXTIVCGENNSCLKELCECDKAVAICLRENLDTYNKXXXYNYLKPF 115
Myo-II       KDRYSYSWKDKTIVCRENNSCLKELCECDKAVAICLRENLDTYNKKYRYNYLKPF 115
MTX-II       KDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLDTYNKKYRNNHLKPF 115
MtxII        KDRYSYSWKDKTIVCGENNSCLKELCECDKAVAICLRENLNTYNKKYRY-YLKPL 114
PrTX-I       KDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYNKLYRY-HLKPF 114
PrTX-II      KDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYNKKYRY-HLKPF 114
BthTX-I      KDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYNKKYRY-HLKPF 114
BnSP-7       KDRYSYSWKDKTIVCGENNPCLKELCECDKAVAICLRENLGTYNKKYRY-HLKPF 114
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Fig. 4. Partial sequence of BmajPLA$_2$-II and its multiple alignment. Multiple alignment of the partial sequence of BmajPLA$_2$-II. Myo-II: Myotoxin II from *Bothrops diporus* venom; MTX-II: Myotoxin II from *B. brazili* venom; MtxII: Myotoxin II from *B. asper* venom; PrTX-I: Piratoxin-I from *B. pirajai* venom; PrTX-II: Piratoxin-II from *B. pirajai* venom; BthTX-I: Bothropstoxin I from *B. jararacussu* venom; BnSP-7: a PLA_2 from *B. pauloensis* venom. All are Lys49 PLA_2s. *Represents conserved residues among all sequences. :Indicates conservation between groups with strongly similar properties = >0.5 in Gonnet PAM 250 matrix. .Indicates conservation between groups with weakly similar properties = <0.5 in Gonnet PAM 250 matrix.

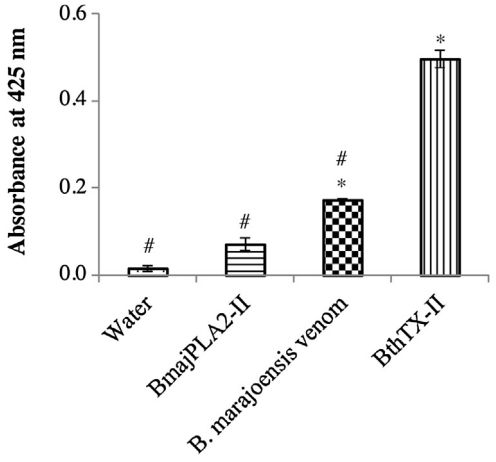

Fig. 5. Phospholipase activity of *B. marajoensis* venom and Bmaj-II. *In vitro* phospholipase activity of the venom and PLA_2 from *B. marajoensis*. Absorbance readings were done at 425 nm every 30 s for 30 min in triplicate. Distilled water was used as the negative control. The Asp49 PLA_2 BthTX-II from *B. jararacussu* snake venom was used as the positive control. *Statistically different from the negative control. #Statistically different from the positive control (BthTX-II).

4. Discussion

BmajPLA$_2$-II showed biochemical characteristics, such as molecular mass and pI, similar to other svPLA$_2$s, specifically from members of the family Viperidae and even other phospholipase A$_2$s described from *B. marajoensis* venom [37–39,57,58]. Bothropic PLA$_2$s' basic character may be associated with various attributes of these proteins. For example, the interaction between basic residues in the binding surface with anionic vesicles plays an important role in the interfacial bonding with anionic phospholipid membranes [59]. Also, it is proposed that basic residues on the surface of PLA$_2$s are responsible for their antibacterial activity [60].

It is currently known that svPLA$_2$s occur as monomers or dimers in physiological conditions. However, BmajPLA$_2$-II presents itself in dimeric and tetrameric forms, as seen by dynamic light scattering assays (Tables 1 and 2), which is an uncommon behavior for snake venom PLA$_2$s (svPLA$_2$s). Similar aggregates were observed for MjTX-I from *B. moojeni*, showing monomeric, dimeric, trimeric, tetrameric and pentameric forms in gel electrophoresis depending on the presence or absence of reducing agents [61,62]. Oligomerization can occur in high concentrations of the toxin, when the PLA$_2$ is interacting with a phospholipid substrate, achieving a more efficient enzymatic action, or in the presence of certain ions [63–66]. Other studies suggest that this oligomerization may be attributed to the inactivation of the PLA$_2$ for venom storage purposes, to generate a new function for it, or to create a more lethal complex of the molecule [67–69].

The multiple alignment, based on 80% of the primary sequence of BmajPLA$_2$-II identified, revealed that BmajPLA$_2$-II is a distinct protein from those previously described from *B. marajoensis* venom. It showed 95% similarity with the myotoxin Myo-II from *B. diporus*

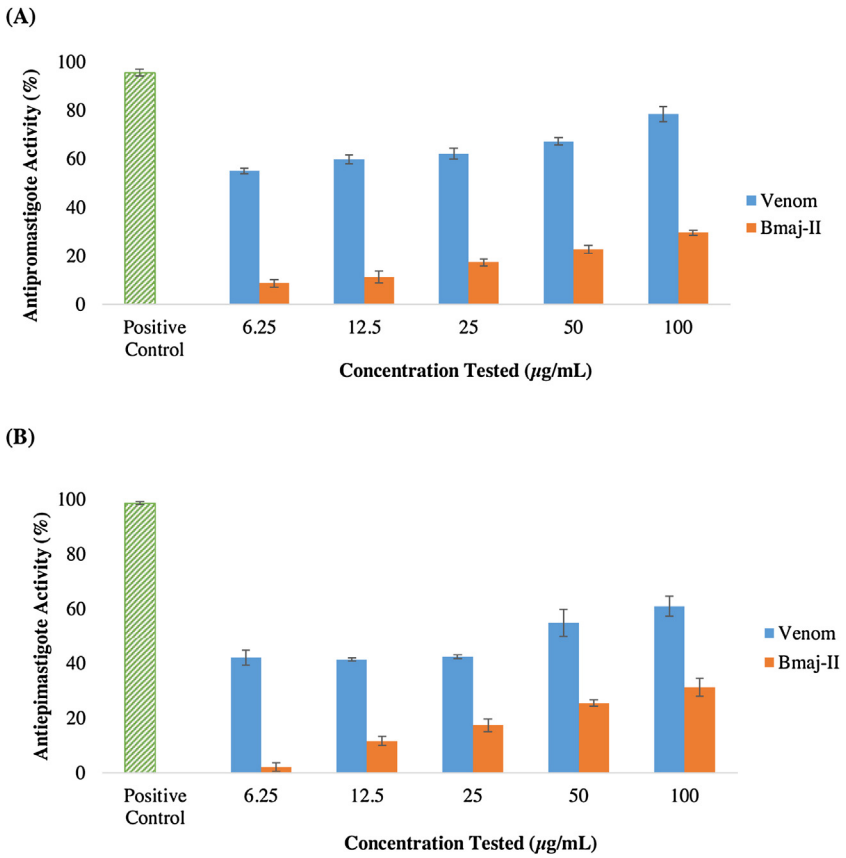

Fig. 6. (A): Activity of the venom and BmajPLA$_2$-II from *B. marajoensis* against *L. infantum*. In *vitro* activity of the venom (blue) and PLA$_2$ (orange) from *B. marajoensis* against *Leishmania infantum* promastigotes, MCAN/ES/92/BNC83 at concentrations of 6.25, 12.5, 25, 50 and 100 μg/mL. Both showed anti-promastigote activity, BmajPLA$_2$-II at a lower intensity. Culture medium was used as a negative control. 100 μg/mL of pentamidine was used as a positive control. (B): Activity of the venom and BmajPLA$_2$-II from *B. marajoensis*against *T. cruzi*. In *vitro* activity of the venom (blue) and PLA$_2$ (orange) from *B. marajoensis* against *Trypanosoma cruzi* epimastigotes, CL-clone B5 strain at concentrations of 6.25, 12.5, 25, 50 and 100 μg/mL. Both exhibited anti-epimastigote activity, BmajPLA$_2$-II at a lower intensity. Culture medium was used as a negative control. 100 μg/mL of benznidazole was used as a positive control. The results were expressed as percentage of anti-parasitic activity compared to the positive control, and are presented as mean ± standard deviation (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

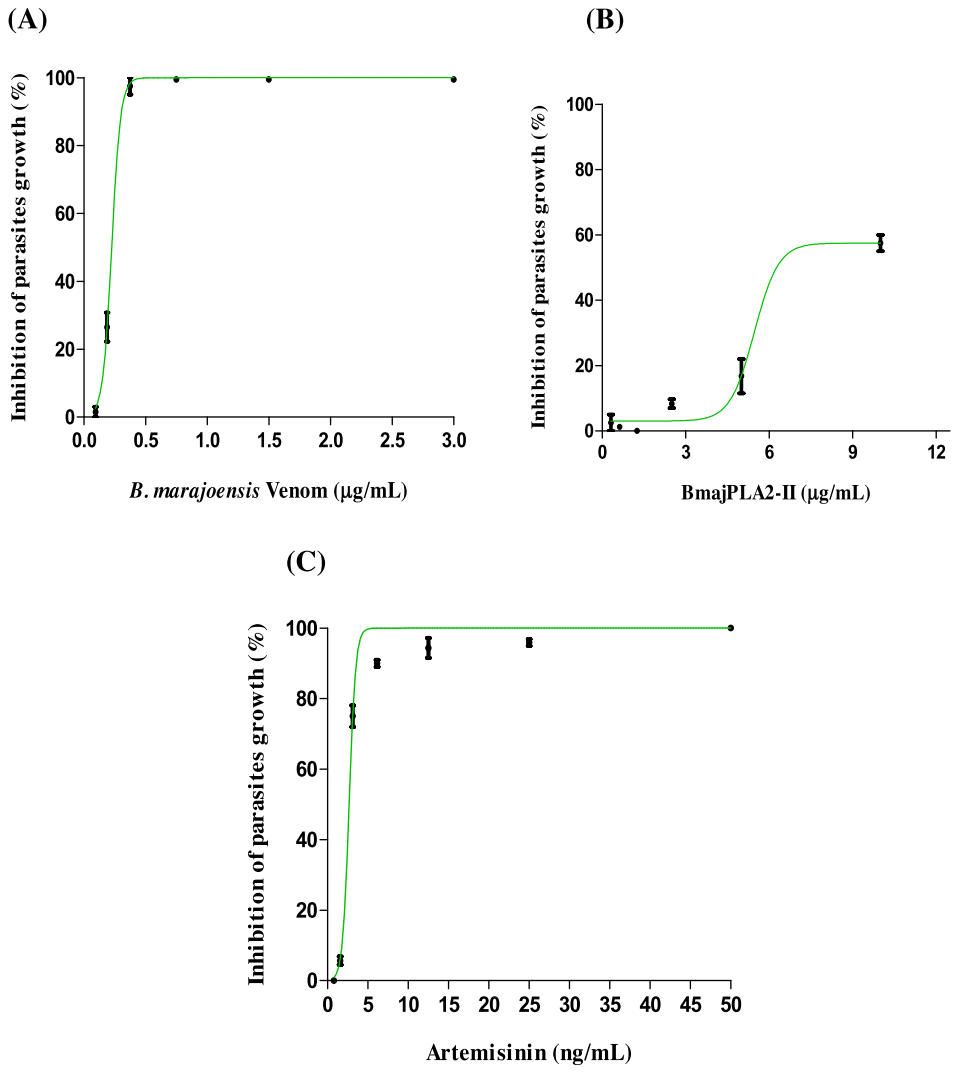

Fig. 7. Antimalarial activity of *B. marajoensis* venom and Bmaj-II. A representative curve of the *in vitro* activity against *P. falciparum* W2 (A) *B. marajoensis* venom at concentrations of 0.093, 0.187, 0.375, 0.75, 1.5, and 3.0 µg/mL, and (B) Bmaj-II at concentrations of 0.312, 0.625, 1.25, 2.5, 5 and 10 µg/mL. Artemisinin (C) was used as a positive control at concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25 and 50 ng/mL.

Table 3
IC_{50}, CC_{50} and selectivity index of *B. marajoensis* venom and BmajPLA$_2$-II against *P. falciparum* and HepG2 cells.

Sample	IC_{50} against intraerythrocytic forms of *P. falciparum* (µg/mL)	R^2	CC_{50} against HepG2 hepatic cells (µg/mL)	R^2	Selectivity Index
B. marajoensis Venom	0.14 ± 0.08	0.99	43.64 ± 7.94	0.99	312
BmajPLA$_2$-II	6.41 ± 0.64	0.97	>150		>23.4
Artemisinin	0.00253 ± 0.00021	1.00	≥ 1000		≥ 1000

IC_{50} = Half maximal (50%) inhibitory concentration; CC_{50} = Half maximal (50%) cytotoxic concentration

snake venom and 89–91% similarity with other bothropic PLA$_2$s (Fig. 4). All these proteins have the amino acid lysine in the 49th position of the sequence being classified as Lys49 PLA$_2$ homologues. Due to its similarity with other Lys49 PLA$_2$s, along with its residual catalytic activity (Fig. 5), we conclude that BmajPLA$_2$-II is also a Lys49 PLA$_2$ homologue.

In order to evaluate BmajPLA$_2$-II's parasiticidal potential, we investigated the effects of the purified toxin and *B. marajoensis* total venom against *Leishmania* spp., *T. cruzi*, *Plasmodium* spp., and HepG2 cells. BmajPLA$_2$-II showed lower cytotoxic activity when compared to the total venom, showing 57% leishmanicidal activity against *L. infantum* promastigotes at a concentration of 6.25 µg/mL, reaching 78% at 100 µg/mL, while BmajPLA$_2$-II exhibited 7% and 29% at the same concentrations (Fig. 6a). Against *T. cruzi* epimastigotes, the venom showed 42% trypanocidal activity at concentrations of 6.25 µg/mL, reaching 61% at 100 µg/mL (Fig. 6b). Against the HepG2 cells, *B. marajoensis* total venom showed a CC_{50} value of 43.64 ± 7.94 µg/mL and a CC_{50} greater than 150 µg/mL for BmajPLA$_2$-II (Table 3).

The venom of Viperidae snakes, in general, exhibit more pronounced leishmanicidal activity than that exhibited by *B. marajoensis* venom [35,70,71]. Both enzymatically inactive and active PLA$_2$s from bothropic venoms have shown activity against several species of *Leishmania* similar to the present study [27–30,37]. Studies addressing the trypanocidal activity of svPLA$_2$s are scarce, but other components of these venoms are being investigated. Antipar-

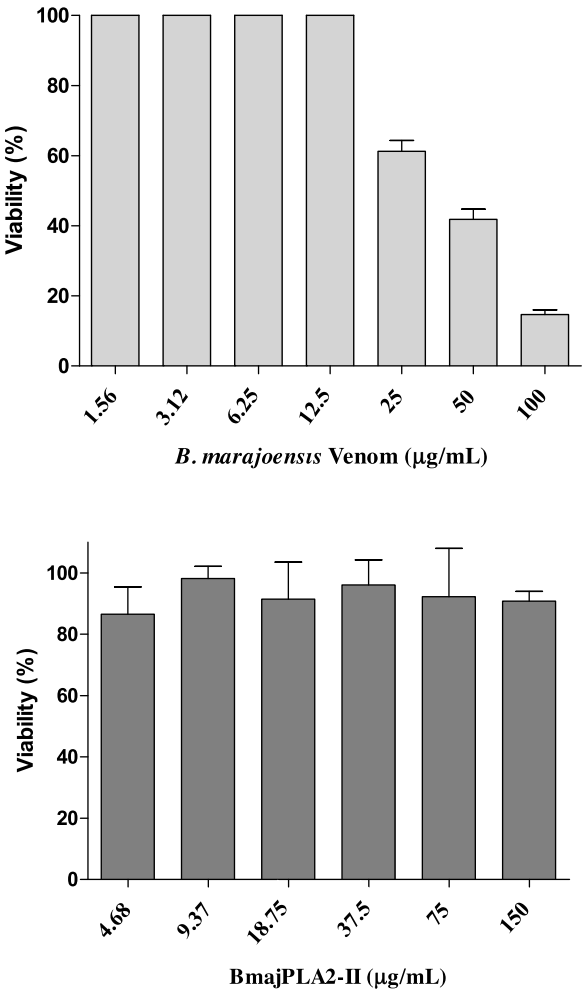

Fig. 8. Cytotoxic activity of *B. marajoensis* venom and Bmaj-II. A representative result of the *in vitro* activity against HepG2 hepatic cells from (A) *B. marajoensis* venom at concentrations of 100-1.56 µg/mL and (B) Bmaj-II PLA_2-II at concentrations of 150-4.68 µg/mL. Artemisinin was used as a positive control (data not shown).

asitic potential against *T. cruzi* has been observed for such snake venom components as snake venom cysteine rich secretory protein and L-amino acid oxidases [26,72–75].

The results of the present study suggest a less distinct leishmanicidal and trypanocidal effect for the isolated PLA_2 when compared with the crude snake venom (Fig. 6). We propose that complete toxicity is not achieved with the isolated toxin because there may be a synergistic or additive effect with other components of the venom.

Intraerythrocytic forms of *P. falciparum* showed higher susceptibility against *B. marajoensis* venom and BmajPLA$_2$-II, showing an IC_{50} of 0.14 ± 0.08 µg/mL for total venon and an IC_{50} of 6.41 ± 0.64 µg/mL for BmajPLA$_2$-II, revealing a selectivity index of 312 and at least 23.4, respectively, against HepG2 cells (Table 3). A value greater than 10 is desired for drugable bioactives [76]. Similar results for antiplasmodial activity were found for PLA_2s from venoms of other snakes of the Viperidae family, including *B. asper*, *C. d. cumanensis* and *Micrurus spixii* [33–35]. Quintana et al. [34] showed the ratio of hemolytic activity and antiplasmodial activity by means of an indirect hemolysis test. In this case, the fraction from *C. d. cumanensis* venom with no hemolytic activity, indicating that it does not have phospholipase activity, showed less anti-malarial activity (IC_{50} 48.36 ± 16.16 µg/mL) and a smaller selectivity index (1.19) compared with other hemolytic fractions from the venom, relative to host mononuclear peripheral blood cells. It was concluded that the ability of the toxins to release free fatty acids and lysophospholipids due to their enzymatic activity might be responsible for their antiplasmodial activity. However, BmajPLA$_2$-II, an enzymatically inactive PLA_2, object of this study showed activity against *Plasmodium* and a selectivity index comparable with some enzymatically active PLA_2s studied by Castillo et al. [33] and Quintana et al. [34].

5. Conclusion

The absence of enzymatic activity, 80% of the primary sequence obtained, and the composition of secondary structures, among other results found in this study, allow us to classify BmajPLA$_2$-II as an unprecedented basic Lys49 PLA_2 with a high degree of similarity with other basic Lys49 PLA_2s from bothropic venoms and a tendency to present multimeric forms. *B. marajoensis* venom and BmajPLA$_2$-II showed antiparasitic activity against *Leishmania infantum* promastigotes, *Trypanosoma cruzi* epimastigotes and intraerythrocytic forms of *Plasmodium falciparum*; BmajPLA$_2$-II contributes in combination with other toxins to achieve the anti-parasitic activity of the venom. Cytotoxicity assays revealed promising selectivity indexes for future more profound studies with *B. marajoensis* venom and BmajPLA$_2$-II.

Further investigations will be required to better elucidate the oligomerization of BmajPLA$_2$-II, such as protein crystallization techniques, X-ray analysis, three dimensional modeling, among others [77]. Such a characterization would help to understand the mechanism of action of BmajPLA$_2$-II and the importance of its oligomerization. It is worth noting that the determination of amino acids involved in the pharmacological actions of the protein is extremely useful for future studies in protein engineering.

Conflict of interest

The author(s) confirm that this article content has no conflict of interest.

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