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Synergism of *in vitro* plasmodicidal activity of phospholipase A2 isoforms isolated from panamanian *Bothrops asper* venom

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ABSTRACT

Bothrops asper is one of the most important snake species in Central America, mainly because of its medical importance in countries like Ecuador, Panama and Costa Rica, where this species causes a high number of snakebite accidents. Several basic phospholipases A_2 (PLA_2s) have been previously characterized from *B. asper* venom, but few studies have been carried out with its acidic isoforms. In addition, since snake venom is a rich source of bioactive substances, it is necessary to investigate the biotechnological potential of its components. In this context, this study aimed to carry out the biochemical characterization of PLA_2 isoforms isolated from *B. asper* venom and to evaluate the antiparasitic potential of these toxins. The venom and key fractions were subjected to different chromatographic steps, obtaining nine PLA_2s, four acidic ones (BaspAc-I, BaspAc-II, BaspAc-III and BaspAc-IV) and five basic ones (BaspB-I, BaspB-II, BaspB-III, BaspB-IV and BaspB-V). The isoelectric points of the acidic PLA_2s were also determined, which presented values ranging between 4.5 and 5. The findings indicated the isolation of five unpublished isoforms, four Asp49-PLA, corresponding to the group of acidic isoforms, and one Lys49-PLA_2-like. Acidic PLA_2s catalyzed the degradation of all substrates evaluated; however, for the basic PLA_2s, there was a preference for phosphatidylglycerol and phosphatidic acid. The antiparasitic potential of the toxins was evaluated, and the acidic PLA_2s demonstrated action against the epimastigote forms of *T. cruzi* and promastigote forms of *L. infantum*, while the basic PLA_2s BaspB-II and BaspB-IV showed activity against *P. falciparum*. The results indicated an increase of up to 10 times in antiplasmodial activity, when the Asp49-PLA_2 and Lys49-PLA_2 were associated with one another, denoting synergistic action between these PLA_2 isoforms. These findings correspond to the first report of synergistic antiplasmodial action for svPLA_2s, demonstrating that these molecules may be important targets in the search for new antiparasitic agents.

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1. Introduction

The *Bothrops asper* snake (commonly called "*terciopelo*") is responsible for the majority of cases of snake envenoming in Central America [1,2]. As is characteristic of bothropic venoms, that of *B. asper* consists of a mixture of enzymes with proteolytic activity (metalloproteases and serinoproteases), phospholipases A_2 (svPLA$_2$s), L-amino acid oxidases (LAAOs) and other proteins with no catalytic properties, such as Type C lectins, disintegrins and cysteine-rich secreted proteins (CRISPs) [3].

One of the most abundant toxins in *B. asper* venom are PLA$_2$s [4]. This group of enzymes are responsible for the hydrolysis of phospholipids at the *sn*-2 position, which triggers the release of lysophospholipids and fatty acids as arachidonic acid [5].

One characteristic of snake venoms, including those of the *Bothrops* genus, is the presence of several isoforms of PLA$_2$s [6], and in this sense, PLA$_2$s with basic isoelectric points have been widely studied [7–9]. It is important to mention that the group of basic svPLA$_2$s includes PLA$_2$s with enzymatic properties (Ca^{+2}-dependent), which have a catalytic aspartic acid residue at position 49 (Asp49-PLA$_2$), as well its inactive isoform which lacks enzymatic activity due to the substitution of Asp49 for a Lys49 (Lys49-PLA$_2$-like) [10,11].

As has been reported by several authors, basic svPLA$_2$s are responsible for a wide spectrum of pharmacological effects, such as myotoxicity, edematogenic activity, neurotoxicity and anticoagulant action and play a fundamental role in the damage caused by envenoming [12–14].

On the other hand, acidic isoforms of svPLA$_2$s, which in their entirety have enzymatic activity, are still poorly studied. Although the relationship between these proteins and the pathophysiology of snakebite envenoming has not yet been fully elucidated, several physical-chemical, structural and functional characteristics of these enzymes have been reported [15–17].

Regarding *B. asper*, it is worth mentioning that the venom of this species has been widely studied, which has allowed for the elucidation of biochemical and functional aspects of the toxins present in the venom of this snake, using them as models to understand their participation in the clinical manifestations observed in snake envenoming [18–20]. In this context, mainly basic PLA$_2$s have been the subject of numerous studies, while PLA$_2$s with acidic characteristics that make up *B. asper* venom have not been addressed in comprehensive studies like those carried out with its basic isoforms. Understanding the structural and functional relationship of snake venom toxins can help reveal the role that these proteins play in clinical manifestations in cases of envenoming [21,22]; thus, the characterization of most components of venoms, including acidic PLA$_2$s, becomes relevant.

On the other hand, one aspect frequently addressed in recent decades is the identification of components of snake venoms with microbicidal potential [23–26]. With emphasis on the antiparasitic [27], bactericidal [28] and antiviral [29] action of components of the venoms, it is evident that these molecules, including svPLA$_2$s, may be considered promising candidates with biotechnological potential.

In this study the identification of several PLA$_2$ isoforms, both acidic and basic, from *B. asper* venom was possible. As a result, the biochemical and pharmaceutical potential of these PLA$_2$ isoforms were determined and evaluated regarding their antiparasitic potential through a synergistic plasmodicidal effect of Asp49-PLA$_2$ with PLA$_2$-like.

2. Materials and methods

2.1. Bothrops asper snake venom and reagents

Bothrops asper venom was provided by the Researcher Aristides Quintero of the Universidad Autónoma de Chiriquí - UNACHI, Panama and was maintained in refrigerated conditions (8 °C) at the Bank of Amazonian Venoms at the Center for the Study of Biomolecules Applied to Health, CEBio-UNIR- FIOCRUZ-RO (authorization: CGEN/CNPq 010627/2011–1 and IBAMA 27131–2). All the reagents used were of sequencing or analytical grade.

2.2. Isolation of PLA2s from B. asper venom

2.2.1. Ion exchange chromatography (IEC)

50 mg of *B. asper* venom was dissolved in 1 mL of ammonium bicarbonate (NH_4HCO_3 - AMBIC) 50 mM pH 8.0 (buffer A) and centrifuged at 5000×*g* (10 min) to remove insoluble material. The supernatant was fractionated in a CM-Sepharose FF® column (1 × 30 cm - GE Healthcare Life Science), previously equilibrated with buffer A. The fractions (CM1 to CM11) were eluted under a 0–100 % gradient of AMBIC 500 mM pH 8.0, in 5 column volumes, under a flow of 1 mL/min, in an Akta Purifier 10 chromatography system (GE Healthcare Life Science). Elution was monitored at 280 nm and the manually collected fractions were lyophilized and stored at −20 °C.

2.2.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page-12.5 %)

The relative molecular mass of the proteins was determined as established by Laemmli [30]. The samples diluted in the electrophoresis sample buffer were incubated for 5 min at 95 °C and then applied to the gel. Once the procedure was completed, the gel was washed with deionized water and fixed in an aqueous solution of 50 % ethanol and 12 % acetic acid for 30 min. Then, the gel was stained for 30 min under slight agitation in a PhastGel ™ Blue R solution (GE Healthcare Life Science) and the excess dye was removed by immersion in a bleach solution containing 20 % ethanol and 3 % acetic acid. The gel was documented using a LabScan® scanner (GE Healthcare Life Science).

2.2.3. Hydrophobic interaction chromatography (HIC)

This procedure was performed as described by Sobrinho and collaborators [17]. The CM2 fraction, diluted in buffer A (AMBIC 20 mM pH 8.0 + 4 M NaCl), was applied to an *n*-butyl-Sepharose-HP® column (1 × 15 cm GE Healthcare Life Science). The elution was performed under a segmented gradient, with decreasing concentrations of NaCl in buffer B (AMBIC 20 mM) and a final step using deionized water, under a flow of 1 mL/min in an Akta Purifier 10 chromatography system (GE Healthcare Life Science). Elution was monitored at 280 nm and the manually collected fractions were lyophilized and stored at −20 °C. The relative molecular masses of the eluted proteins were determined using 12.5 % SDS-PAGE, following the same procedures mentioned in item 2.2.2.

2.2.4. Reverse-phase chromatography (RPC)

Fractions CM7, CM8, CM9, CM10 and CM11, from IEC, as well as fraction *n*-bS6 from HIC, were submitted to RPC in a C18 column (25 × 4.6 mm - Kinetex), previously equilibrated with solution A (0.1 % Trifluoroacetic acid (TFA)) and eluted under a gradient of 0–70 % of solution B (99.9 % Acetonitrile and 0.1 % TFA) in 10 column volumes, at a flow rate of 1 mL/min. Elution was monitored at 280 nm and the manually collected fractions were lyophilized in a Speed Vac and stored at −20 °C. *PLA$_2$* activity was measured for all fractions. The apparent molecular mass of the eluted proteins was determined using 12.5 % SDS-PAGE, following the same procedures mentioned in item 2.2.2. Fractions were rechromatographed under the same conditions described in item 2.2.4 to guarantee the highest degree of purity.

2.3. Biochemical characterization

2.3.1. Phospholipase activity with 4-nitro-3 (octanoyloxy) benzoic acid (4N3OBA)

In this procedure, the method described by Holzer and Mackessy [31] was used, adapted for a 96-well plate. 190 μL of chromogenic substrate 4N3OBA 0.25 mM solubilized in buffer (10 mM $CaCl_2$, 150 mM NaCl and Tris-Base pH 8.0) was used, along with 5 μL of the samples (venom, chromatographic fractions and isolated toxins) solubilized at a

concentration of 1 μg/μL. Deionized water was used as a negative control. The mixture was incubated at 37 °C and the optical density was determined at a wavelength of 425 nm immediately after mixing at 30-s intervals, for 30 min. Statistical analysis was performed according to item 2.5.

2.3.2. Phospholipase activity with fluorescent substrate

Phospholipase activity was evaluated by the hydrolysis of synthetic fluorescent phospholipids [NBD-PA (Phosphatidic acid), NBD-PC (Phosphatidylcholine) and NBD-PG (Phosphatidyl Glycerol)]. For the experiment, 20 μg of the samples were added to a solution containing substrate (8 mM) in a solution of $CaCl_2$, 100 μM of 20 mM Tris-HCl pH 7.5. The experiment was carried out on a Shimadzu spectrofluorometer, RF-5301PC and using RFPC software with excitation and emission wavelengths at 460 and 534 nm, respectively. The fluorescence intensity was evaluated after an interval of 5 min and expressed as RFU (Relative Fluorescence Units). Statistical analysis was performed according to item 2.5.

2.3.3. Isoelectric point determination

The PLA_2s were solubilized in a rehydration solution (7 M urea, 2 M thiourea, 2.0 % CHAPS (m/v), immobilized pH gradient buffer (IPG buffer® (GE Healthcare Life Science) 0.5 % (v/v) and 1.0 % bromophenol blue (w/v)) and applied over 7 cm polyacrylamide strips with a pH gradient from 3 to 10 immobilized in a linear manner (Immobiline DryStrip® - GE Healthcare Life Science). Isoelectric focusing was performed on an IPGphor III System (GE Healthcare Life Science) according to the manufacturer's instructions. Then, the strip was transferred to a 12.5 % SDS-PAGE system, and the procedure was performed as reported in item 2.2.2.

2.3.4. Mass spectrometry

Mass spectrometry was performed in MALDI equipment (matrix-assisted laser desorption/ionization), (AXIMA TOF2 Shimadzu Biotech), operating in linear mode. A mixture of 1 μL (1 mg/mL) of each PLA_2 (from RPC) solubilized in 0.1 % TFA was co-crystallized with 1 μL of a saturated solution of synapinic acid in 50 % acetonitrile and 0.05 % TFA (ionization matrix) on the metal plate. The data obtained were analyzed using the software Launchpad.

2.3.5. N-terminal Sequencing and multiple alignment

N-terminal sequencing of each PLA_2 isolated from *B. asper* was performed in a PPSQ-33A® automatic sequencer (Shimadzu Biotech), which uses the chemical process of N-terminal cleavage sequencing, derived from the method developed by Edman [32]. After obtaining the partial sequences of the nine isolated PLA_2s, a search for similarity was performed using the BLAST algorithm and multiple alignment was carried out using the program Clustal Omega, available at http://www.uniprot.org/align/.

2.4. Antiparasitic activity of PLA_2s isolated from B. asper snake venom

2.4.1. In vitro trypanocidal activity

The methodology was described by Vega and collaborators [33]. Epimastigote forms of *Trypanosoma cruzi* from Strain CL clone B5 were grown at a concentration of 2.5×10^5 parasites/mL in 96-well plates, using Liver Infusion Tryptose (LIT) culture medium supplemented with 10 % fetal bovine serum (FBS). The plate was incubated for 72 h at 26 °C, with different serial concentrations (100–6.25 μg/mL) of *B. asper* venom and acidic PLA_2s. Each concentration was tested in triplicate. Subsequently, a solution of Chlorophenol Red-β-D-galactopyranoside (CPRG) was added, at a final concentration of 200 μM. The plate was again subjected to incubation at 37 °C for 4 h and finally, absorbance was monitored at 575 nm using a Synergy H1 multimodal spectrophotometer (Biotek). Parasites incubated in the LIT culture medium were used as a growth control, and the reference drug Benznidazole was used as a positive control. The antiparasitic activity of the venom and PLA_2s was estimated by calculating the percentage of *anti*-epimastigote activity. Finally, the concentration required to achieve 50 % of trypanocidal activity was calculated using the program GraphPad Prism 6.

2.4.2. In vitro leishmanicidal activity

For this test, promastigote forms of *Leishmania infantum* (MCAN/ES/92/BCN 83) were cultivated at a concentration of 1.5×10^6 parasites/mL in 96-well plates, using Schneider culture medium, supplemented with 10 % FBS. The experiment was carried out based on the protocol of Rolón and collaborators [34], with modifications. The plate was incubated at 26 °C for 48 h with different serial concentrations (100–6.25 μg/mL) of *B. asper* venom and acidic PLA_2s. Each concentration was tested in triplicate. Subsequently, a 3 mM Resazurin solution was added. The plate was again incubated at 26 °C for 4 h and the absorbance was determined at 570 and 600 nm using a Synergy H1 multimodal spectrophotometer (Biotek). Parasites incubated in Schneider culture medium were used as a growth control and the reference drug Pentamidine was used as a positive control. The antiparasitic activity of the venom and PLA_2s was estimated by calculating the percentage of *anti*-promastigote activity. Finally, the concentration required to achieve 50 % of leishmanicidal activity was calculated using the program GraphPad Prism 6.

2.4.3. Evaluation of antiplasmodial activity

2.4.3.1. Plasmodium falciparum culture and synchronization. CQ-resistant *P. falciparum* (W2 strain) was maintained in complete culture medium RPMI 1640 (Sigma) supplemented with 50 mg/mL of albumax (Gibco) in addition to previously washed fresh human erythrocytes (O+ blood) (2 % hematocrit) (approval number CEP/CEPEM 1.233171). The parasite culture was maintained at 37 °C in an atmosphere of 5 % O_2 and balanced N_2. The development of parasites was monitored daily through a blood smear stained with panotic and observed under an optical microscope (1000 X). For the chemotherapy tests, the parasites were synchronized with sorbitol to obtain a predominance of ring forms [35]. The hematocrit was adjusted to 1.5 % for the tests, in which the parasitemia was 0.05 % (young trophozoites) in order to obtain a predominance of ring forms. The hematocrit was adjusted to 1.5 % for the tests, in which the parasitemia was 0.05 % (young trophozoites).

2.4.3.2. In vitro antiplasmodial activity assay procedure to test toxins. Synchronized *P. falciparum* cultures were distributed in 96-well microplates. The toxins to be tested (BaspB-II and BaspB-IV) were added to the test plate, in triplicate, and at different serial concentrations [BaspB-II (40–0.625 μM) and Basp-B-IV (0.2–0.0031 μM)]. The negative control consisted of infected erythrocytes without treatment and the positive control consisted of serial dilutions of artemisinin from 0.177 to 0.001 μM. The activity was evaluated for 48 h at 37 °C. Antiplasmodial activity was performed using SYBR Green I [36]. After 48 h of treatment with toxins, infected erythrocytes were washed with 1 × PBS and centrifuged at 700×*g* (10 min). Then, the supernatant was discarded and subsequently, the red blood cell sedimentation was diluted in 100 μL of SYBR Green I-containing lysis buffer (0.001 % v/v in lysis buffer - 20 mM Tris, pH 7.5; 5 mM EDTA; 0.08 % w/v Triton X-100; 0.008 % w/v saponin in 1 × PBS, pH 7.5). The contents were transferred to microplates containing 100 μL of 1 × PBS. The plates were incubated for 30 min at room temperature. Fluorescence data was obtained using a microplate reader spectrophotometer (Synergy, Biotek) at an excitation of 485 nm and an emission of 535 nm. All experiments were performed at least twice, and each sample was tested in triplicate. The inhibition of 50 % of parasite growth (IC_{50}) was obtained by nonlinear curve fitting of the serial concentrations computed by the software Origin (OriginLab Corporation, Northampton, MA, USA).

2.4.3.3. Interaction between BaspB-II and BaspB-IV for experimental antiplasmodial chemotherapy. Chou and Talalay's [37] model was applied to evaluate the effect of the combination of BaspB-II and BaspB-IV on an *in vitro* culture of *P. falciparum* (W2). IC_{50} values obtained for each toxin were utilized to determine the initial concentration of BaspB-II and BaspB-IV which were adjusted in the combination experiment to ensure that the IC_{50} of each was close to the fourth dilution in a series of 8 dilutions (Table 1). Thus, initial concentrations of BaspB-II and BaspB-IV were defined based on the IC_{50} and were then used to prepare the mixture for both toxins. Following dilution of the toxins, alone and in combination (Table 1), an antiplasmodial assay was performed using the SYBR Green I method as described previously.

After calculating the IC_{50} for monotherapy and combination therapy, the fractional inhibitory concentration (FIC) was used to classify the nature of the interaction between BaspB-II and BaspB-IV *in vitro*. FICs and $\sum$FICs were calculated as follows:

BaspB-II FIC = IC_{50} of BaspB-II in mixture/IC_{50} of BaspB-II in monotherapy.

BaspB-IV FIC = IC_{50} of BaspB-IV in mixture/IC_{50} of BaspB-IV in monotherapy.

Following the FIC calculation for each toxin, the $\sum$FICs was obtained as follows:

$\sum$FICs = BaspB-II FIC + BaspB-IV FIC.

$\sum$FICs $\leq$1 indicates synergism, which is a positive interaction between the two substances, meaning the effect of the combination is greater than that obtained for each substance alone; 1 < $\sum$FICs $\leq$2 indicates additivity, when there is no interaction, because the effect of the combination is similar to that observed with the use of each substance in monotherapy; and >2 indicates antagonism, meaning the mixture of the compounds results in a lesser effect than that expected [37].

2.4.4. Hemolysis assay

BaspB-II and BaspB-IV were diluted in 1 × PBS, being tested in serial concentrations ranging from 40 to 0.0031 μM. After dilution of the proteins, 20 μL of the samples were added to 180 μL of a red cell suspension (human), to a 1 % hematocrit in 96-well microplates with a "U" bottom. A saponin solution (Sigma-Aldrich) at 0.05 % w/v in 1 × PBS was used as a positive control of hemolysis. After preparation, the microplate was incubated at a temperature of 37 °C (30 min) with constant shaking every 5 min; then, the plate was centrifuged at 1500 rpm (10 min) and the supernatant was transferred to another 96-well microplate with a flat bottom. The absorbance reading was performed at 540 nm in a microplate spectrophotometer (Biochrom model: Expert plus).

2.5. Statistical analyses

Statistical analysis was performed using the software Graph Pad Prism, version 6.1. The results were expressed as the mean ± standard deviation (SD). The significance of the observed differences was determined using the ANOVA test and Tukey's post-test with $p \leq 0.05$ considered to be significant.

Table 1
BaspB-II and BaspB-IV concentrations used in the antiplasmodial *in vitro* combination experiment. Gray box indicates that IC_{50} values for both toxins (monotherapy) are in the third or fourth dilution range.

Mixture (BaspB-II μM + BaspB-IV μM)	Monotherapy BaspB-II (μM)	Monotherapy BaspB-IV (μM)
22 + 0.0436	22	0.0436
11 + 0.0218	11	0.0218
5.5 + 0.0109	5.5	0.0109
2.75 + 0.00545	2.75	0.00545
1.37 + 0.002725	1.37	0.002725
0.68 + 0.0013625	0.68	0.0013625
0.34 + 0.00068125	0.34	0.00068125
0.17 + 0.000340625	0.17	0.000340625

3. Results

3.1. Isolation and biochemical characterization of PLA_2s from *B. asper* venom

In the present study, *B. asper* venom was fractionated in order to purify nine PLA_2s using three chromatographic techniques: cation exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and reverse phase chromatography (RPC). In the first step performed on a CM-Sepharose resin, 11 main fractions were eluted (Fig. 1A). Based on their apparent molecular masses, fractions CM2, CM7, CM8, CM9, CM10 and CM11 were selected for further experiments (Fig. 1B).

Fraction CM2, submitted to HIC (Fig. 2A), gave rise to six fractions (*n*-bS1 to *n*-bS6). After this process and based on its apparent molecular mass, fraction *n*-bS6 was selected (Fig. 2B) and subsequently subjected to RP-HPLC, a process in which the elution of 4 acidic PLA_2s was observed (Fig. 3A), which were called BaspAc-I, BaspAc-II, BaspAc-III and BaspAc-IV. The eluted PLA_2s were rechromatographed to guarantee the purity of the isolated proteins (Fig. 3B–E). In Fig. 3F, it is observed that the isolated proteins have a relative molecular mass of 14 kDa.

Basic PLA_2s from *B. asper* venom were also isolated. For this, fractions CM7, CM8, CM9, CM10 and CM11 were submitted to RPC (Fig. 4A–E). This procedure allowed for the purification of 5 basic PLA_2s, which were called BaspB-I, BaspB-II, BaspB-III, BaspB-IV and BaspB-V. In Fig. 4F, the electrophoretic profile of the isolated PLA_2s is observed.

3.2. Biochemical characterization of the isolated PLA_2s

As expected, the four acidic PLA_2s showed enzymatic properties, catalytically degrading the substrate 4N3OBA (Fig. 5A). In addition, two basic PLA_2s (BaspB-I and Basp-IV) showed enzymatic activity. The basic PLA_2s BaspB-II, III and V were considered Lys49-PLA_2-like isoforms based on their molecular weights and absence of catalytic activity (Fig. 5B).

All the isolated Asp49-PLA_2s hydrolyzed phospholipids like phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidic acids (PA). As for the acidic phospholipases A_2 (BaspAc-I, BaspAc-II, BaspAc-III and BaspAc-IV), there was no significant difference in their specificity for any phospholipid. On the other hand, when analyzing the basic Asp49-PLA_2s (BaspB-I and Basp-IV), a greater specificity against phosphatidic acid (PA) and phosphatidylglycerol (PG) was observed compared to low action against phosphatidylcholine (PC) (Fig. 5C).

The isoelectric points of the acidic isoforms were determined using two-dimensional electrophoresis. In Fig. 6, it can be seen that the pIs of the four acidic PLA_2s have values between 4.5 and 5, as is characteristic of this group of isoforms. Table 2 shows the pI values of the acidic PLA_2s and the molecular weights of the 9 isolated isoforms. The mass/charge ratio (*m/z*) of PLA_2s, both for the acidic isoforms (Fig. 7) and for the basic ones (Fig. 8) was determined using mass spectrometry analysis.

Through Edman's chemical degradation, it was possible to identify 50 amino acid residues in the N-terminal region (Fig. 9). It is worth mentioning that a database search, even considering the first 50 amino acid residues alone, showed that the four acidic isoforms and one basic PLA_2 (BaspB-II) isolated in this study have sequences not yet previously described (multiple alignments of $svPLA_2$ can be observed in Figs. S1 and S2 of the supplementary material S1). As is characteristic for this group of enzymatically active PLA_2s, an aspartic acid residue can be seen at position 49, while in Lys49-PLA_2-like isoforms, a lysine residue is present.

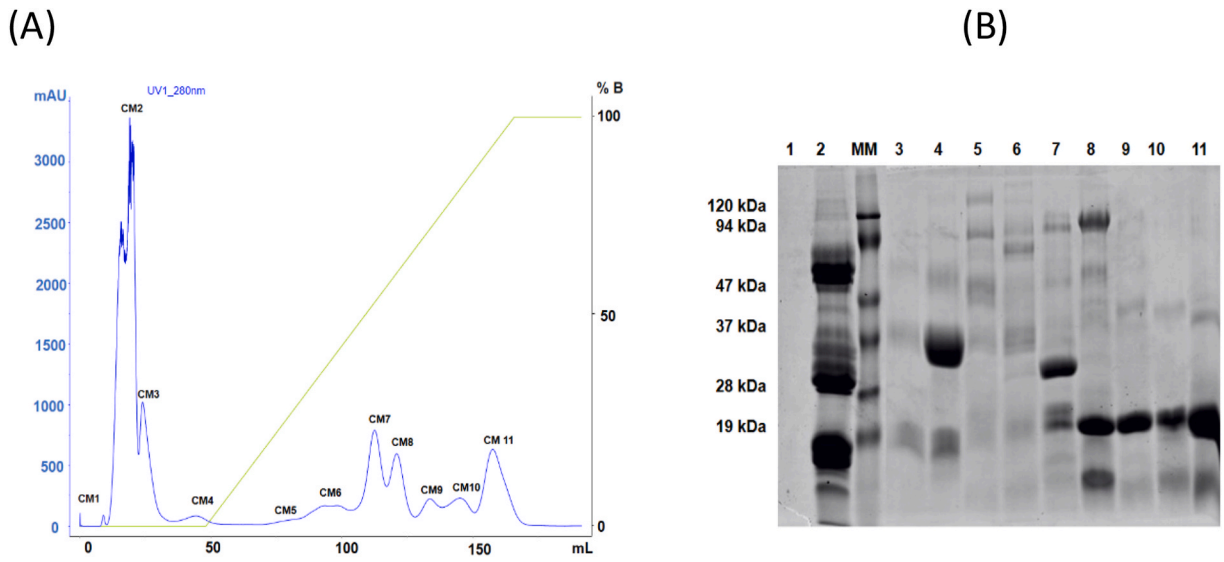

Fig. 1. Fractionation of *Bothrops asper* venom in IEC (CM-Sepharose): the elution of 11 fractions (A) was observed, which were named CM1 to CM11. In the electrophoretic profile (B), the relative molecular mass of the proteins present in the eluted fractions can be observed. The CM2 fraction and the CM7, CM8, CM9, CM10 and CM11 fractions were selected for further experiments.

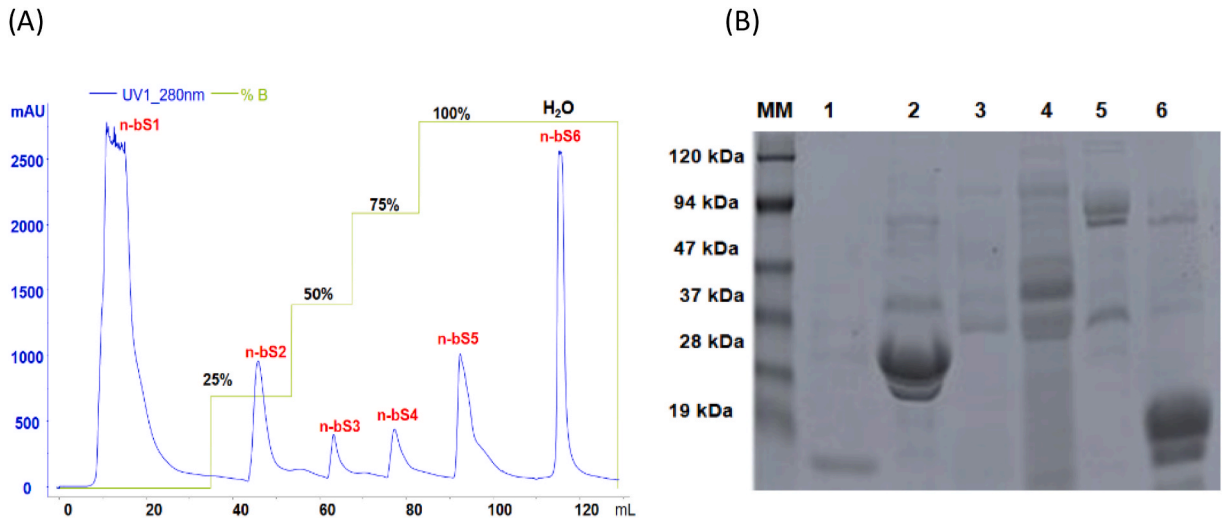

Fig. 2. Fractionation of CM2 in HIC: Six fractions, named *n*-bS1 to *n*-bS6, were eluted (A). In the electrophoretic profile (B), the relative molecular mass of the proteins present in the eluted fractions can be observed. Fraction *n*-bS6, which eluted with water, presented proteins with molecular masses compatible with those of PLA_2s. This fraction was selected for the next step.

3.3. Antiparasitic activity of *B. asper* PLA_2s

B. asper venom had a trypanocidal effect against *T. cruzi* epimastigotes, reaching IC_{50} values of 34.7 μg/mL (Table 3). Of the PLA_2s evaluated, BaspAc-II, BaspAc-III and BasAc-IV showed approximately 30 % antiparasitic activity against epimastigotes (Supplementary material S2, Table S1).

The leishmanicidal potential of *B. asper* venom was also determined, with IC_{50} values of 8.6 μg/mL for the venom (Table 3). Of the PLA_2s evaluated, BaspAc-I and BaspAc-IV were able to achieve approximately 30 % cytotoxic activity against *L. infantum* promastigotes (Supplementary material S2, Table S2).

In the antiplasmodial activity assay, both Lys-49-PLA_2 (BaspB-II) and an Asp49 phospholipase (BaspB-IV), when tested alone (monotherapy), showed activity against the malaria parasite. As can be seen, the IC_{50} for BaspB-II, a Lys49-PLA_2-like was 2.46 μM, and for BaspB-IV, a basic Asp49-PLA_2, the IC_{50} was 0.019 μM. Artemisinin showed an inhibitory concentration of 0.026 μM against the parasite (Fig. 10).

In order to verify whether or not the association between the toxins BaspB-II and BaspB-IV *in vitro* have a synergistic effect rather than an additive effect, the nature of the interaction was evaluated by summing the FIC values (∑FICs) from each monotherapy, as follows:

$$\boldsymbol{FIC\ BaspB-II} = \frac{0,98}{2,46} = 0,398$$

$$\boldsymbol{FIC\ BaspB-IV} = \frac{0,0019}{0,019} = 0,1$$

$$\sum FICs = FIC\ BaspB-II + FIC\ BapsB-IV = 0,398 + 0,1 = 0,498$$

BaspB-II's IC_{50} dropped from 2.46 μM to 0.98 μM, a 2.5-fold increase in antiplasmodial activity. In the case of BaspB-IV, this value dropped from 0.019 μM to 0.0019 μM, a 10-fold increase in antiplasmodial activity (Fig. 11). Both results indicate an increase in antiplasmodial

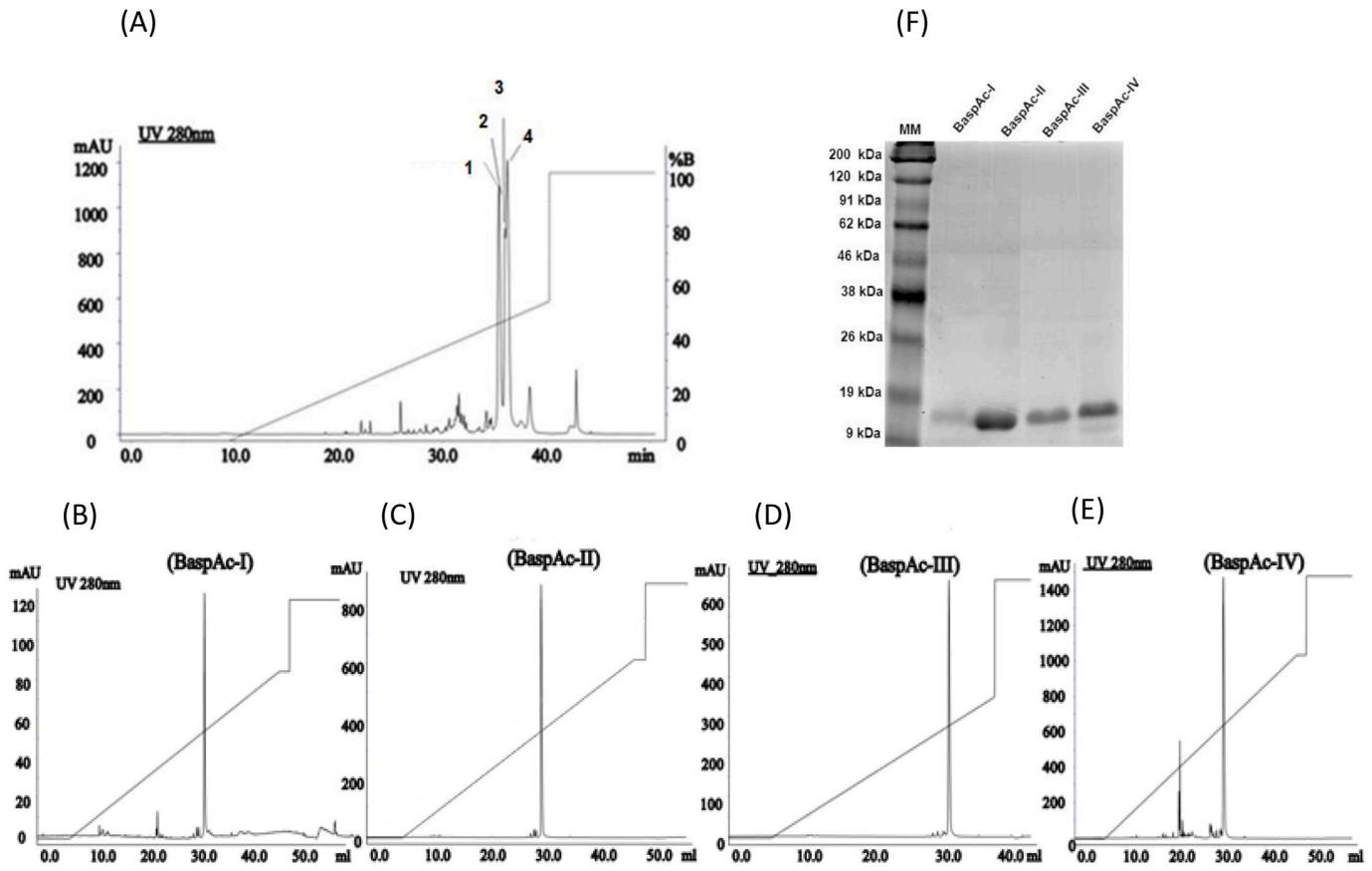

Fig. 3. (A) Isolation of four acidic PLA_2 isoforms: The elution of four PLA_2s was observed. These samples were rechromatographed and later named BaspAc-I, BaspAc-II, BaspAc-III and BaspAc-IV (B–E). The relative molecular mass of the proteins present, the eluted PLA_2s, are compatible with the molecular mass of $svPLA_2$s (F).

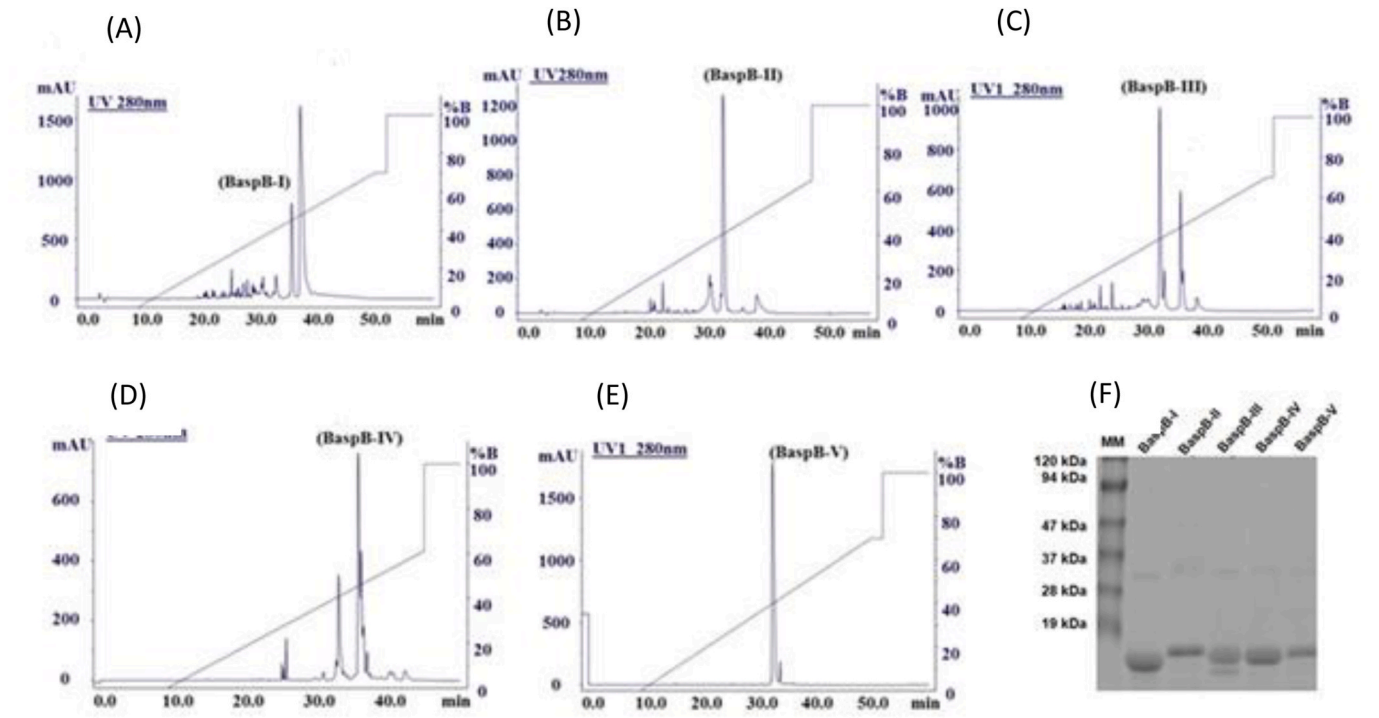

Fig. 4. Isolation of five basic PLA_2 isoforms: RPC of the basic fractions (CM7-CM11) from the IEC permitted the isolation of five basic PLA_2s, called BaspB-I, BaspB-II, BaspB-III, BaspB-IV and BaspB-V (A–E). The electrophoretic profile showed a relative molecular mass of 14 kDa for the purified proteins, compatible with this group of proteins (F).

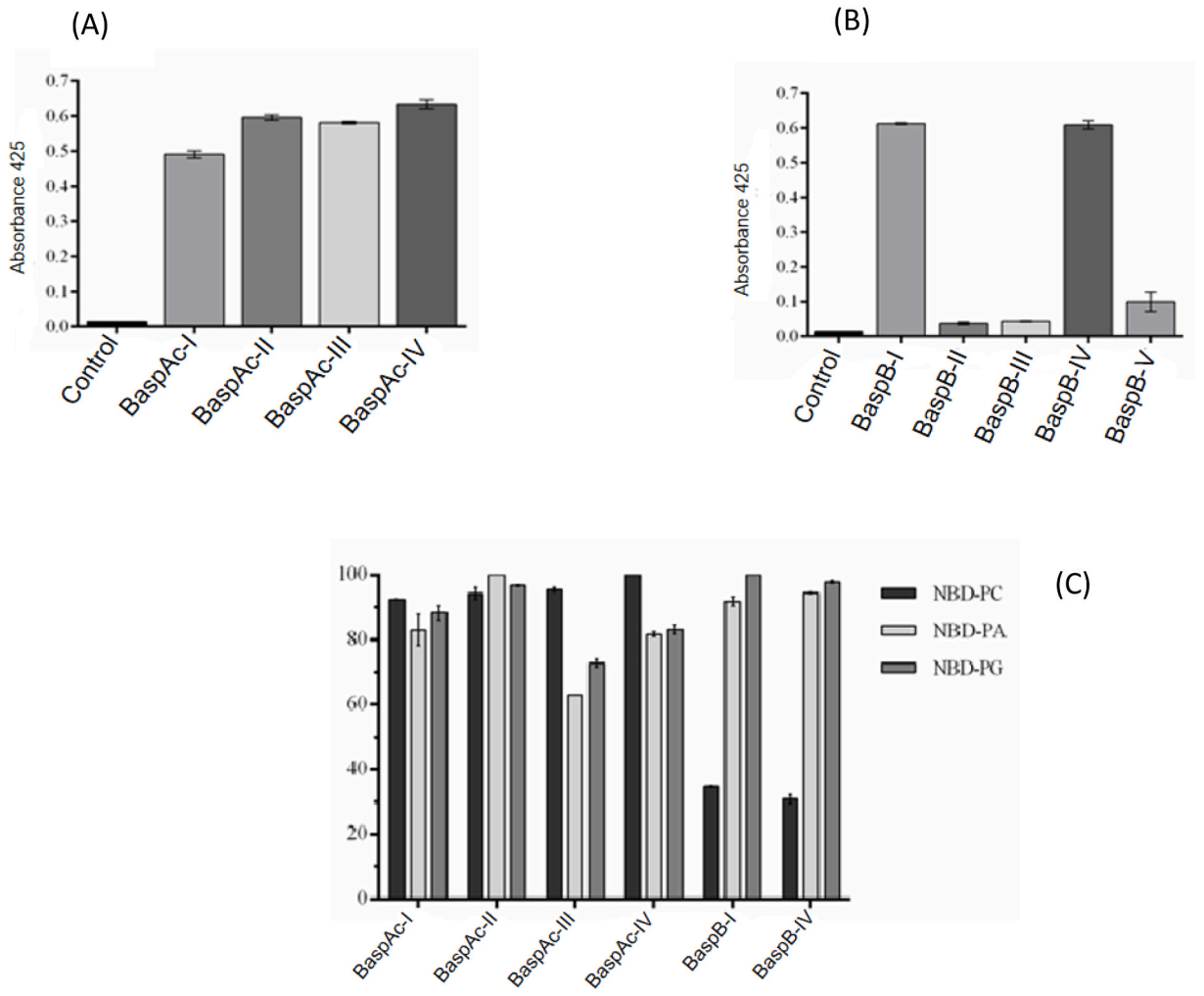

Fig. 5. Evaluation of the enzymatic activity of isolated PLA_2s: In the evaluation of phospholipase A_2 activity on the substrate 4N3OBA, considering that the acidic isoforms belong to the Asp49-PLA_2 group, the four isolated proteins showed a catalytic effect (A). Regarding the basic isoforms, only BaspB-I and Basp-IV showed enzymatic activity, while BaspB-II, III and V did not show catalytic properties (B). The results are expressed as mean and standard deviation of DO 425 nm. In both cases, deionized water was used as a negative control. When evaluated against fluorescent phospholipids, the acidic PLA_2s were able to hydrolyze the three evaluated phospholipids, while the basic isoforms showed greater specificity toward phosphatidic acid (PA) and phosphatidylglycerol (PG). The results are expressed as the mean and standard deviation of enzymatic activity (C).

activity with one compound leveraging the other. Thus, the effect of BaspB-II together with BaspB-IV was considered a synergistic one, with $\sum$FICs = 0.498.

4. Discussion

4.1. Isolation and biochemical characterization of PLA_2s from B. asper venom

Molecules in snake venoms, acting individually, additively or synergistically, are responsible for the plethora of signs and symptoms observed in cases of snakebite envenoming. Studies aiming to identify and characterize the constituents of these venoms seek a better understanding of the role of these different molecules in snake envenoming and serve as prototypes of biotechnological models and tools [7,22,38].

Certainly, in order to achieve these objectives, a high degree of purity becomes imperative since there is a need to individualize these molecules for a detailed study of their activities and potential. Thus, the selection of an appropriate method for the purification of a given molecule must be based on physical-chemical or biological and functional characteristics in order to obtain satisfactory results. Information pertinent to the structure of proteins, such as homology with others that have been previously purified, must be taken into account and tend to facilitate purification processes [39,40].

PLA_2s from snake venoms are one of their most studied components, and are purified using a combination of chromatographic techniques such as molecular exclusion, ion exchange, reverse phase and affinity chromatographies using natural inhibitors, antibodies and heparin [11,41]. Thus, in the present study, nine PLA_2s were purified from *B. asper* venom (BaspAc-I, BaspAc-II, BaspAc-III, BaspAc-IV, BaspB-I, BaspB-II, BaspB-III, BaspB-IV and BaspB-V), concatenating chromatographic steps that started with cation exchange chromatography which led to the selection of fractions CM2, CM7, CM8, CM9, CM10 and CM11 as the main targets for the identification of PLA_2s from *B. asper* snake venom.

Subsequently, in order to isolate the acidic PLA_2s from the venom, fraction CM2 was fractionated in a column with a hydrophobic interaction resin (*n*-butyl Sepharose®), following the methodology proposed by Sobrinho and collaborators [17]. In this case, as well as in the first chromatographic step, the relative molecular mass profile and enzymatic activity were used as parameters for selecting the fraction of interest, identified as *n*-bS6. Finally, this fraction was submitted to RPC, which permitted the separation of four acidic PLA_2 isoforms.

The method of purifying acidic PLA_2s used in the present study (ion exchange, hydrophobic interaction and reverse phase) has been shown

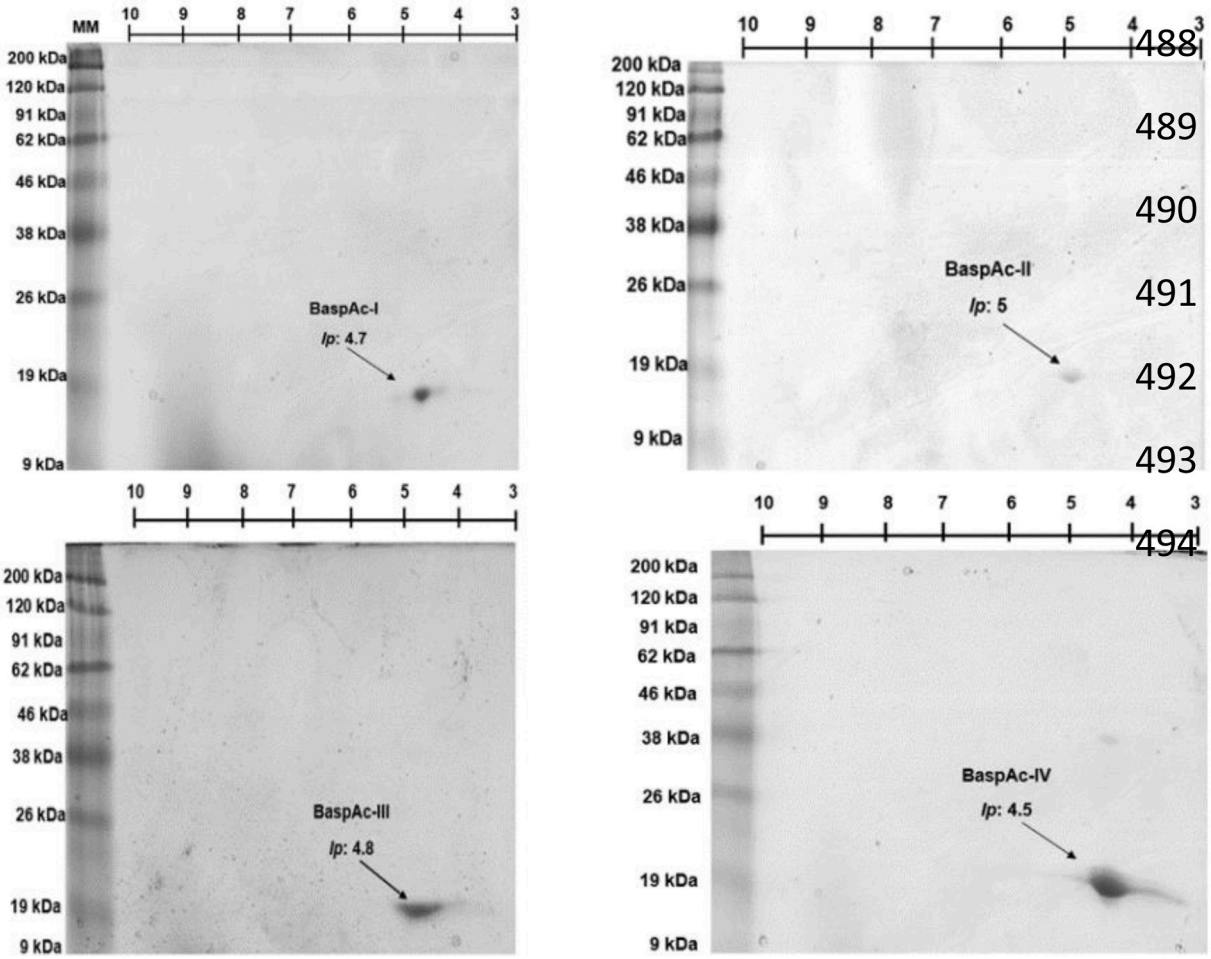

Fig. 6. Determination of the isoelectric point: As is characteristic of this group of acidic isoforms, the isoelectric point values, determined by two-dimensional electrophoresis for each protein, are between 4.5 and 5.

Table 2
Biochemical characteristics of PLA_2s isolated from *B. asper*.

Phospholipase A_2	pI	Isotope –averaged Molecular Mass (Da)
BaspAc-I	4.7	14238
BaspAc-II	5.0	14224
BaspAc-III	4.8	14204
BaspAc-IV	4.5	14214
BaspB-I	Nd	14037
BaspB-II	Nd	14000
BaspB-III	Nd	13839
BaspB-IV	Nd	14005
BaspB-V	Nd	13846

Note: Nd – Not determined.

to be efficient in isolating this type of molecule. It should be noted that the isolation of acidic isoforms of PLA_2s from the venom of *B. asper* from Panamanian specimens was previously reported by Rueda and Soares [42]. Likewise, Fernández and collaborators [15] and, more recently, Arias et al. [43] described this group of proteins isolated from specimens from Costa Rica and Colombia, respectively.

In the case of basic molecules, several PLA_2s have been isolated using processes based on cation exchange followed by reverse phase chromatographies, such as: a study carried out by Ponce-Soto and collaborators [44], who isolated two PLA_2s (BmjeTX-I and II) from *B. marajoensis* venom; a study carried out by Rueda and collaborators [45], who isolated four PLA_2s (MTX-I, II, III and IV) from the venom of the Panamanian snake *B. asper*; a study by Moura and collaborators [46], who described three PLA_2s (BmatTX-I, II and III) from the venom of *B. mattogrossensis*. Clearly, this is a widely used methodology permitting the elucidation of a large number of molecules.

SvPLA$_2$s are made up of a large group of molecules that share diverse biochemical and functional characteristics. In this context, the molecular masses, determined by mass spectrometry, confirm the relative values visualized in the monodimensional electrophoresis gel, both for acidic and basic PLA_2s. A molecular mass of approximately 14 kDa for the isolated PLA_2s is similar to those from venoms of other members of the Viperidae family such as BaspPLA$_2$-II (*B. asper*) at 14,212 Da [15], BthA-I-PLA$_2$ (*B. jararacussu*) at 13,700 Da [47], BmooPLA$_2$ at 13,601 Da [16] and BmooTX-I at 15,000 Da [48], (*B. moojeni*), BpirPLA-I at 14,500 Da (*B. pirajai*) [49] and Bp-PLA$_2$ at 15,800 Da (*B. pauloensis*) [50].

The isoelectric point is a biochemical characteristic that allows for a classic dichotomy among svPLA$_2$s, classified, as mentioned above, as acidic or basic. It is interesting to note that within these subgroups, the isoelectric point does not vary much. In this environment, the pI of several acidic PLA$_2$s were described in the venom of some bothropic snakes; it is interesting to note that the values determined for the acidic PLA$_2$s purified in this study are similar to those found for other acidic PLA$_2$s described in the scientific literature [15–17,47,48,50–56].

Obviously, the biochemical characteristics mentioned above are due to primary aspects, merely the composition of amino acids. Among the sequences of amino acid residues obtained in this study (Fig. 9), those that have not yet been described in the literature, that is, that can be considered new, are the four acidic isoforms and one basic PLA$_2$, BaspB-II. Multiple alignment of the N-terminal sequences of each acidic isoform

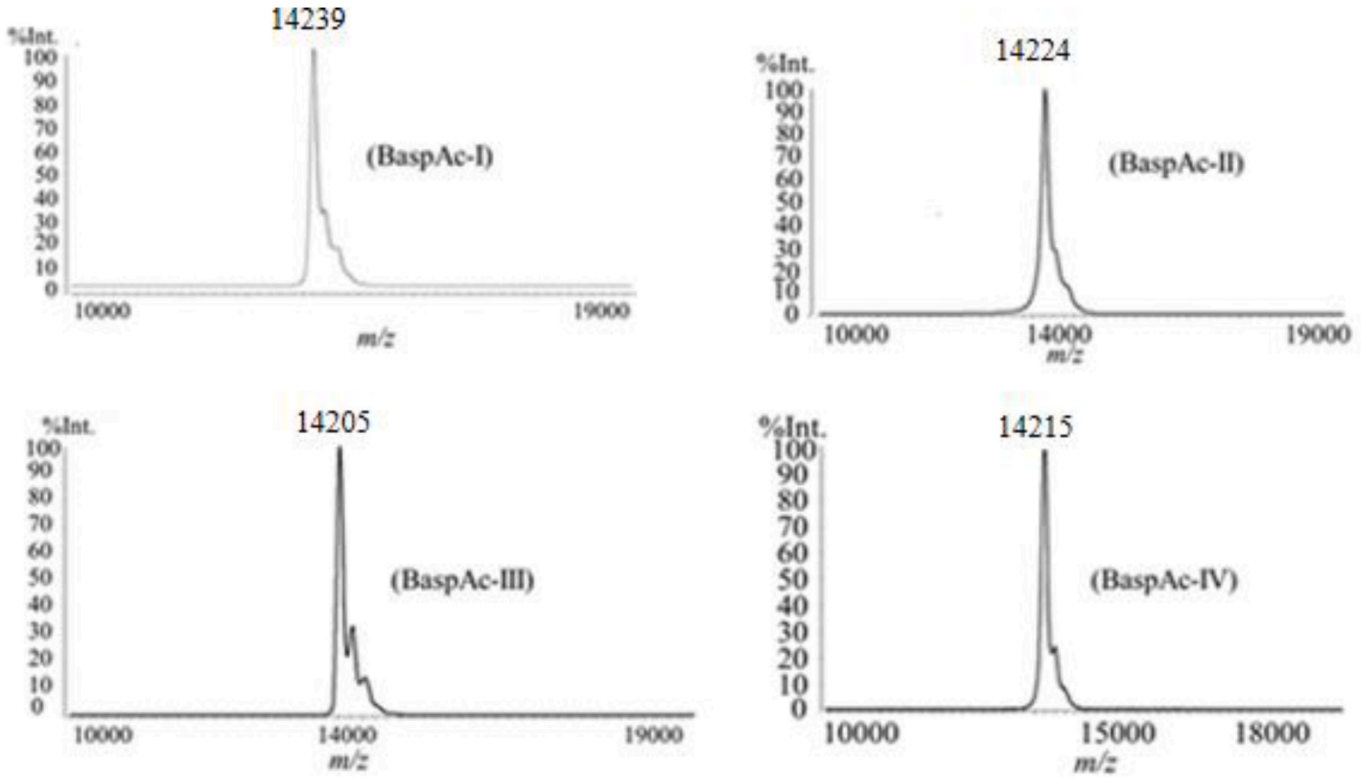

Fig. 7. Mass spectrum of acidic PLA_2s: *m/z* ratio of acidic isoforms, determined by mass spectrometry. The four proteins have a molecular mass of approximately 14.2 kDa, compatible with PLA_2s.

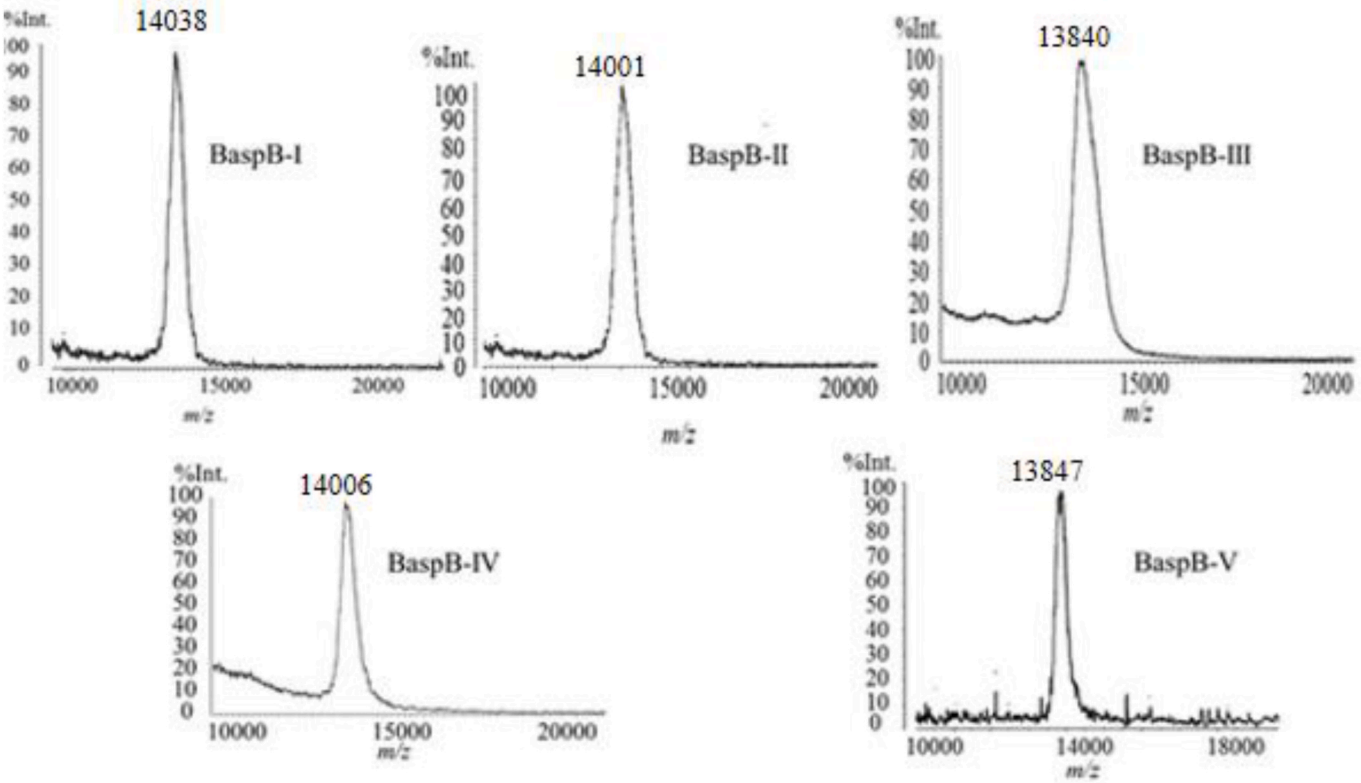

Fig. 8. Mass spectrum of basic PLA_2s: The spectrum demonstrates the mass/charge (*m/z*) ratio of the basic isoforms. These five proteins have molecular mass values ranging from 13.8 to 14.2 kDa.

```
                    10        20        30        40        50
BaspAc-I      NLWQFGQMMNDVMRQNVVFQYLSYGCYCGAGGIGQPQDATDRCCQVHDCC
BaspAc-II     NLWQFGQMMNDVMRQNVVFQYLSYGCYCGDGGIGQPRDATDRCCFVHDCC
BaspAc-III    QLWQFGQMTSDVMRQDVVFQYLSYGCYCGDGGIGQPQDATDRCCQVHDCC
BaspAc-IV     NLWQFGEMMSDVMRDNVVFQYLSYGCYCGDGGIGQPRDATDRCCFVHDCC
BaspB-I       QLIEFAYMILEATQRLPFPYYTQYGCYCGAGGQGQPKDATDRCCFVHDCC
BaspB-II      SLVELGKMILQETGKNPLTSYGVYGCNCGVGSRHKPKDATDRCCYVHKCC
BaspB-III     SLVELGKMILQETGNNPVTSYGAYGCNCGVLGRGQPQDATDRCCYVHKCC
BaspB-IV      SLIEFAKMILEGTFRLPFPYYTTYGCYCGQGGQGQPKDATDRCCFVHDCC
BaspB-V       SLFELGKMILQETGKNPAKSYGAYGCNCGVYGRGKPKDATDRCCYVHKCC
              .* ::. *  :          *  *** **  .  :* ******* **.**
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Fig. 9. Multiple alignment of 50 amino acid residues obtained from the N-terminal region of the isolated svPLA$_2$s: In red are the acidic isoforms, which demonstrate the presence of an aspartic acid residue at position 49 (blue box). Below are the basic isoforms (in blue). In the group of basic isoforms, BaspB-I and IV present an Asp49 residue, while in BaspB-II, III and V, a Lys49 residue can be observed (yellow box). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
In vitro antiparasitic activity of *B. asper* venom and the isolated acidic PLA$_2$s.

Sample	*IC$_{50}$ against* T. cruzi *epimastigotes (μg/mL)*	*IC$_{50}$ against* L. infantum *promastigotes (μg/mL)*
B. asper	*34.7*	*8.6*
Acidic PLA$_2$s	*>100*	*>100*
Benzanidazole	*0.5*	—
Pentamidine	—	*2.6*

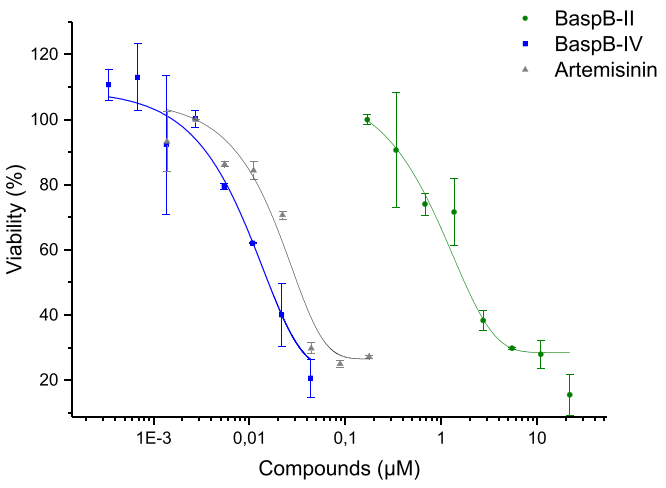

Fig. 10. Dose-response curves. The antiplasmodial activity of phospholipases BaspB-II, BaspB-IV and artemisinin against the *P. falciparum* strain W2.

showed high levels of identity with previously described snake PLA$_2$s (Supplementary material S1). Interestingly, even when comparing the sequences of the acidic PLA$_2$s isolated in the present study with those of the PLA$_2$s isolated from *B. asper* venom previously described in the literature, no sequence with 100 % identity was found. One fact that may explain the presence of various isoforms in the venom is the geographic distribution of the snakes, considering that several studies indicate that the variation in the location where certain species are found can cause the characteristics of their venoms to vary considerably [4,57].

To date, the literature reports that all isolated acidic svPLA$_2$s have an Asp residue at position 49. In this context, all the acidic phospholipases showed catalytic activity (Fig. 5A), which is in line with what has been described by several authors [15,47,54]. However, as expected, the same was not found for all the basic phospholipases A$_2$, since only BaspB-I and BaspB-IV showed enzymatic activity (Fig. 5B). The proteins BaspB-II, BaspB-III and BaspB-V did not show catalytic properties and in this sense, in addition to the presence of a lysine at position 49, shown in the sequencing (Fig. 9), the absence of enzymatic activity confirms that these toxins belong to the group of Lys49-PLA$_2$s

In addition to the method using an artificial chromogenic substrate 4N3OBA to determine phospholipase activity, other substrates can be tested for this purpose, adding relevant information related to the specificity of the enzyme for a certain class of phospholipids [50]. Thus, the enzymatic activity of the acidic and basic Asp49 phospholipases A$_2$ from *B. asper* snake venom was tested with a variety of phospholipids [phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidic acids (PA)] and all the acidic phospholipases hydrolyzed the tested phospholipids in a non-selective manner. For the basic phospholipases, it was observed that there was greater specificity for phospholipids with a negative residual charge (phosphatidylglycerol and phosphatidic acid).

4.2. Biotechnological potential and synergism of PLA$_2$s isolated from B. asper venom

Over the past few years, several studies have shown that svPLA$_2$s have a wide range of biological activities that can be used to benefit human health. Research with this group of toxins from the venom of different snakes indicates that these molecules have microbicidal potential [21–23,58]. Based on this, we decided to analyze the activity of the PLA$_2$s found against the parasites that cause Chagas disease, Leishmaniasis and Malaria.

Regarding the antiparasitic activity using *T. cruzi* epimastigotes as a model, it was observed that *B. asper* venom presented IC$_{50}$ values of 34.7 μg/mL. The trypanocidal potential of bothropic venoms has been demonstrated, suggesting that they can cause several changes in the parasites, causing the death of these microorganisms [59,60]. Likewise, an evaluation of the trypanocidal effect of the isolated acidic PLA$_2$s showed that the highest concentration (100 μg/mL) reached approximately 30 % of antiparasitic activity against epimastigotes (BaspAc-II, BaspAc-III and BaspAc-IV), results similar to those obtained by Sobrinho and collaborators [17], who determined the trypanocidal effect of acidic isoforms of PLA$_2$s isolated from *B. brazili* venom.

In addition, it is notable that the leishmanicidal potential of venom and its components is frequently discussed in the scientific literature. The antiparasitic effect of snake venom against Leishmania spp. *has been reported by several authors* [61–63], *and in this study, using* L. infantum *promastigotes as a model, the IC$_{50}$ value reached by B. asper venom was 8.*6 μg/mL.

The leishmanicidal activity of svPLA$_2$s, mainly of the basic isoforms, has also been evaluated and proven, and different species of Leishmania *have shown susceptibility when treated with this group of proteins* [27,64]. *Regarding the leishmanicidal activity of acidic isoforms, at a concentration of* 100 μg/mL, *BaspAc-I and BaspAc-IV showed approximately 30 % antipromastigote activity, which is similar to the effect caused by Braziliase-I,*

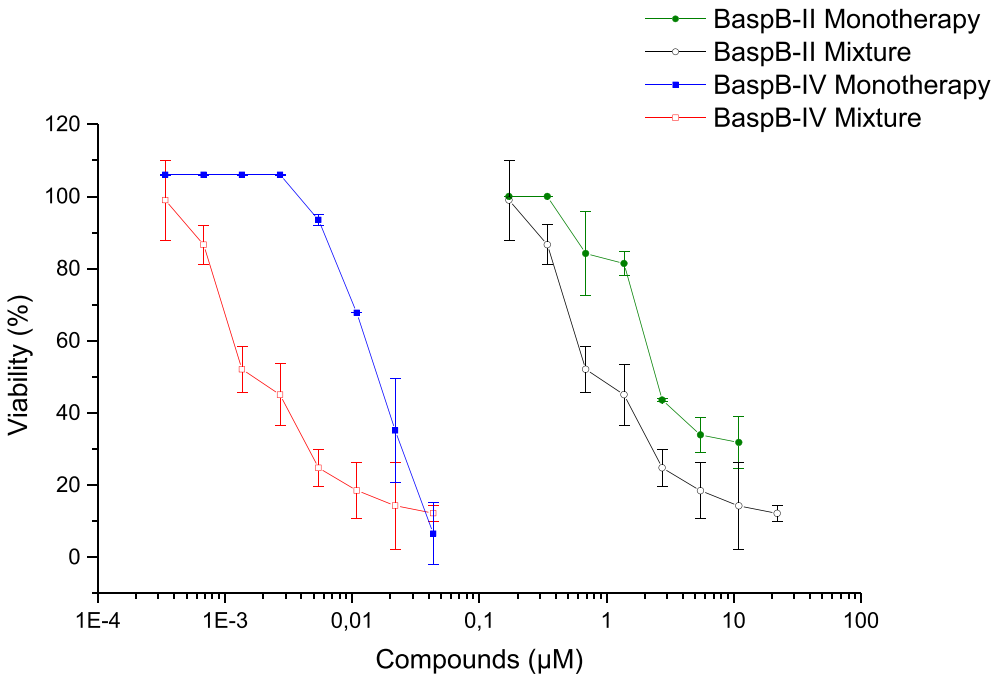

Fig. 11. *In vitro* interaction of phospholipases. Dose-response curves for phospholipases BaspB-II and BaspB-IV alone or in combination against *P. falciparum* strain W2.

isolated from B. brazili *venom* [17].

Another potential biotechnological application for phospholipases A_2 is related to their effect on the parasites that cause malaria. Several studies have shown that these toxins may become an important tool in the fight against this disease [65–67].

In this sense, Zieler et al. [67], upon observing that a PLA_2 from *Crotalus adamanteus* venom was able to inhibit the formation of oocysts, found that these molecules drastically reduced the level of association between the ookinete and the intestinal wall cells of the mosquito *in vitro*, and therefore, the authors consider PLA_2s to be excellent candidates in the search for tools with the potential to inhibit malaria transmission.

On the other hand, Guillaume and collaborators [65] demonstrated that PLA_2s from venoms of different species, even in low concentrations, are potent inhibitors of the intra-erythrocytic development of *P. falciparum*. Likewise, *P. falciparum* was reported to be susceptible when treated with BmajPLA$_2$, a Lys49-PLA$_2$-like isolated from *B. marajoensis* venom [68].

In this study, it was observed that the basic PLA_2s BaspB-II and BaspB-IV from *B. asper* venom presented IC_{50} values of 2.46 and 0.019 μM, respectively (Fig. 10). It is worth mentioning that, when compared to the IC_{50} of the drug Artemisinin, BaspB-IV (a Asp49-PLA$_2$), showed greater activity against *P. falciparum*. Similar results were obtained by Castillo et al. (2012), who reported that both the fraction of *B. asper* venom formed by enzymatically active PLA_2s and that formed by Lys49-PLA_2s showed antiplasmodial activity; however, the fraction with Asp49-PLA$_2$ showed greater antiparasitic activity.

It is important to highlight that at the same concentrations evaluated in the parasites, the PLA_2s were not able to cause hemolysis in uninfected erythrocytes, which makes it more interesting to explore these molecules as potential tools in the search for antimalarial agents.

Another aspect addressed in this study was the potential synergism between the toxins from *B. asper*. Although it is very interesting and has already been demonstrated in venoms of various species, including snake venoms, this synergism is poorly studied [69,70]. With regard specifically to the synergism between svPLA$_2$s, a potent *in vitro* synergistic effect has been demonstrated between Asp49-PLA$_2$s and PLA_2 homologues, which causes an increase in plasma membrane permeability causing cell death [71]. Later, Mora-Obando and collaborators [72] confirmed the synergistic effect in *vivo* experiments. According to the authors, an increase in myonecrosis was observed due to the joint action of the two toxins, thus demonstrating a clear synergistic action between svPLA$_2$s. Recently, it was also reported by Bustillo and collaborators [73] that PLA$_2$s isolated from *B. diporus* venom act synergistically and cause damage to C_2C_{12} cells.

Based on the results observed by the aforementioned authors, the present study evaluated whether a potential synergistic effect between the two toxins isolated in this study (BaspB-II and BaspB-IV) could cause an increase in their antiparasitic effect. In this sense, it was observed that the IC_{50} of the Lys49-PLA$_2$ BaspB-II showed a reduction from 2.46 μM to 0.98 μM, that is, an increase of about 2.5 times in antiplasmodial activity, whereas BaspB-IV's IC_{50} value of 0.019 μM was reduced to 0.0019 μM, with a 10-fold increase in antiparasitic activity. Considering the value of the sum of the FICs = 0.498, it is suggested that the increase in cell death observed in the *P. falciparum* culture is the product of a synergistic effect among the toxins evaluated, making this the first study to report an increased plasmodicidal effect due to synergism between PLA_2 isoforms.

Considering these results, it is important to highlight that further studies are needed to achieve a better understanding of the mechanisms by which the toxins have an antiparasitic effect, especially against *P. falciparum*. Clarification of the mechanisms of potential synergistic effects and their relationship with an increase in the intensity of pharmacological effects, in addition to contributing to better understanding the behavior of the components of the venom in snakebites, can generate important knowledge in the search for agents with antiparasitic effects.

5. Conclusions

Therefore, based on the above, the purification methodology adopted in this study made it possible to purify nine PLA_2s from Panamanian *B. asper* venom, five of which are new isoforms of svPLA$_2$s. With that, it was possible to carry out the biochemical characterization of the nine isolated isoforms, and tests on *Trypanosoma cruzi*, *Leishmania infantum* and *Plasmodium falciparum* demonstrated that the tested PLA_2s are important targets in the search for new molecules with antiparasitic

potential. In addition, for the first time, synergistic antiplasmodial activity between an Asp49 PLA_2 and a PLA_2-like was demonstrated. Despite this, new studies need to be carried out in order to elucidate the mechanisms by which these molecules act on these microorganisms.

6. Credit authorship contribution statement

RSS and JJA: Formal analysis, Data curation, Writing - original draft, designed the study, performed the experiments, collected analyzed the data and performed the figures, wrote the manuscript. AFG, JCS and AMK: Writing - original draft, performed the biochemical experiments. DSSM and CBGT: Formal analysis, Data curation, Writing - original draft, performed the plasmonicidal experiments, collected analyzed the data and performed the figures. ALF and CVG: Writing - original draft, performed the experiments, collected analyzed the data and performed the figures, wrote the manuscript. AQ: Writing - original draft, provided reagents. CVG, SSP, SLS, RGS, and AMS: Supervision, performed and supervised the biochemical procedures, provided reagents, Writing - original draft and review & editing, designed the study, wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2021.109581.

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