

Antimalarial activity of basic phospholipases A₂ isolated from Paraguayan Bothrops diporus venom against Plasmodium falciparum

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ARTICLE INFO

Keywords:

Snake venom

Bothrops diporus

Phospholipases A₂

Antiparasitic activity

Plasmodium falciparum

ABSTRACT

Malaria is a parasitic infectious disease and was responsible for 400.000 deaths in 2018. *Plasmodium falciparum* represents the species that causes most human deaths due to severe malaria. In addition, studies prove the resistance of *P. falciparum* to drugs used to treat malaria, making the search for new drugs with antiparasmodial potential necessary. In this context, the literature describes snake venoms as a rich source of molecules with microbicidal potential, including phospholipases A₂ (PLA₂s). In this sense, the present study aimed to isolate basic PLA₂s from Paraguayan *Bothrops diporus* venom and evaluate their antiparasmodial potential. Basic PLA₂s were obtained using two chromatographic steps. Initially, *B. diporus* venom was subjected to ion exchange chromatography (IEC). The electrophoretic profile of the fractions from the IEC permitted the selection of 3 basic fractions, which were subjected to reverse phase chromatography, resulting in the isolation of the PLA₂s. The toxins were tested for enzymatic activity using a chromogenic substrate and finally, the antiparasmodial, cytotoxic potential and hemolytic activity of the isolated toxins were evaluated. The electrophoretic profile of the fractions from the IEC permitted the selection of 3 basic fractions, which were subjected to reverse phase chromatography, resulting in the isolation of the two enzymatically active PLA₂s, BdTX-I and BdTX-II and the PLA₂ homologue BdTX-III. The antiparasmodial potential was evaluated and the toxins showed IC₅₀ values of: 2.44, 0.0153 and 0.59 µg/mL respectively, presenting PLA₂ selectivity according to the selectivity index results (SI) calculated against HepG2 cells. The results show that the 3 basic phospholipases isolated in this study have a potent antiparasitic effect against the W2 strain of *P. falciparum*. In view of the results obtained in this work, further research are necessary to determine the mechanism of action by which these toxins cause cell death in parasites.

1. Introduction

Malaria is an infectious parasitic disease with vector transmission involving a diversity of parasites in different ecological environments. Endemicity occurs in tropical and subtropical regions, mainly in Sub-Saharan Africa, Latin America and Southeast Asia. In 2018, the World Health Organization (WHO) reported 228 million cases of malaria, with

an estimated 405,000 deaths; children under the age of 5 represent the most vulnerable group for this endemic disease. Despite decades of efforts, this disease remains a leading cause of death worldwide (WHO, 2020).

The species capable of infection in humans are: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Cox, 2010). Among these, *P. falciparum* has a higher proportion of cases in the following

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<https://doi.org/10.1016/j.toxcx.2020.100056>

Received 15 June 2020; Received in revised form 24 July 2020; Accepted 1 August 2020

Available online 8 August 2020

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regions: Africa (99.7%), Southeast Asia (50%), Eastern Mediterranean (71%) and Western Pacific Region (65%), while *P. vivax* appears in 53% of cases in Southeast Asia and the Americas. These are species with the highest epidemiological prevalence (WHO, 2020).

Regarding clinical presentation, the species *P. falciparum* is responsible for the severest form of malaria and, consequently, causes the majority of deaths. Another factor that makes *P. falciparum* an important focus of study is the capacity of resistance development by the parasite to antimalarials, especially for the standard scheme treatment. Initially, cases of Chloroquine resistance were recorded in Southwest Asia and then in South America (Nevin and Croft, 2016; Wongsrichanalai et al., 2002). Due this fact, since 2001, combined therapy with artemisinin (ATC) has been used in the treatment of malaria (Su and Miller, 2015); however, failed treatments with ATC have been reported in strains of *P. falciparum* on the Thailand-Cambodia and Myanmar border, reaffirming the need for studies of new molecules with antiparasmodial potential (Ashley et al., 2014; Bhumiratana et al., 2013; Mathenge et al., 2020).

Given this scenario, the application of drugs with different chemical properties, the improvement of available pharmaceuticals and the development of new drugs, present new alternatives that may delay the evolution of parasitic resistance against antimalarials (Okombo and Chibale, 2016).

Regarding the identification of agents with biotechnological potential, biodiversity is very important as a source for the discovery of new molecules (Harvey et al., 2015; Newman and Cragg, 2016). In this sense, molecules of animal origin have stood out in the scientific literature for presenting microbicidal properties and within this group, snake venoms have been the target of numerous studies (Harvey et al., 1998; Koh et al., 2006). Snake venoms are characterized by the complex constitution of molecules, mainly of a protein nature, which are related to a wide spectrum of pharmacological effects (Lomonte et al., 2014; Simoes-Silva et al., 2018).

One group of molecules present in these secretions are Phospholipases A₂ (PLA₂, EC 3.1.1.4), enzymes dependent on the calcium ion that catalyze hydrolysis in the *sn*-2 position of phospholipids, triggering the release of fatty acids and lysophospholipids (Dennis et al., 2011; Gutiérrez and Lomonte, 2013). Snake venom PLA₂s (svPLA₂) are widely studied proteins and are related to several clinical manifestations of snakebite envenoming, such as myotoxicity, neurotoxicity, cardiotoxicity, inhibition of platelet aggregation, and edema, among others (Gutiérrez and Lomonte, 2013; Kini, 2003; Lomonte and Gutiérrez, 2011).

An interesting characteristic of bothropic venoms is the presence of PLA₂ isoforms. In addition to enzymatically active PLA₂s, which have an aspartic acid residue at position 49 (Asp49-PLA₂), the presence of PLA₂s with no catalytic properties has been reported, called PLA₂-homologues or Lys49-PLA₂, due to the presence of a lysine residue at position 49 (Gutiérrez and Lomonte, 2013; Kini, 2003). Within the group of homologous PLA₂s, variants have been described such as Ser49-PLA₂ (Krizaj et al., 1970), Arg49-PLA₂ (Mebs et al., 2006) and Gln49-PLA₂ (Bao et al., 2005). It should be noted that, PLA₂-homologues are devoid of enzymatic activity, and are responsible for various pharmacological effects (Lomonte et al., 2003).

In recent decades, another aspect of svPLA₂ under investigation are their potential biotechnological applications and in this context, several authors have reported the antitumor potential (Arya and Lomonte, 2007), antiviral (Cecilio et al., 2013) and antiparasitic activity of svPLA₂s (Grabner et al., 2017), which makes this group of proteins promising targets of study. In view of the above, the present study describes the isolation of basic PLA₂s from the venom of Paraguayan *Bothrops diporus* with the objective of evaluating their antiparasitic potential on intra-erythrocytic forms of *Plasmodium falciparum*.

2. Materials and methods

2.1. Venom and authorization

Bothrops diporus snake venom was obtained from specimens kept in captivity at the Catholic University of Asuncion bioterium in Paraguay. This study was authorized by the Secretariat of the Environment, SEAM-Paraguay, Authorization N° 01/14 and pooled venom was kept refrigerated (8 °C) in the Bank of Amazon Venoms at the Center of Biomolecules Studies Applied to Health, CEBio-UNIR-FIOCRUZ-RO (Authorization: CGEN/CNPq 010627/2011-1; IBAMA 27131-2 and CEBio UNIR-FIOCRUZ-RO (register CGEN A4D12CB and IBAMA/SISBIO 64385-1).

2.2. Isolation of phospholipases A₂ from *B. diporus* venom

Fifty milligrams of *B. diporus* venom was solubilized in 1 mL of buffer A (Ammonium Bicarbonate, 50 mM, pH 8) and centrifuged at 8000×g for 10 min. The supernatant was fractionated in cation exchange chromatography, using a CM-Sepharose GE® (1 × 30 cm) column, previously equilibrated with buffer A. Fractionation was performed by applying a linear gradient of 0–100% of Buffer B (Ammonium Bicarbonate, 500 mM, pH 8), at a flow of 1 mL/min. This procedure was monitored at 280 nm, using an Akta purifier (GE®) chromatographic system. Finally, the manually collected fractions were lyophilized and stored at –20 °C.

Determination of the relative molecular masses of the proteins present in the eluted fractions was performed by 12.5% SDS-PAGE, following the methodology described by Laemmli (1970). Ten µg of the lyophilized fractions was mixed with 4% SDS (m/v), 0.2% Bromophenol Blue (m/v), 20% glycerol (v/v) 0.2 M Dithiothreitol (DTT) in 100 mM Tris pH 6.8 and heated for 5 min at 90 °C. Subsequently, the gel was incubated in a fixative solution (50% ethanol and 12% acetic acid) for 30 min, and then stained with a PhastGel™ Blue R solution (GE Healthcare) for 30 min. Finally, the gel was placed in a solution with 4% ethyl alcohol and 7% acetic acid to remove the excess stain. In order to obtain and document images of the gel, ImageScanner III (GE Lifescience Health Care) was used.

For the following step, fractions 5, 6 and 7 from the ion exchange chromatography were selected for having molecular masses compatible with phospholipases A₂. Fractions were solubilized in 0.1% aqueous trifluoroacetic acid (TFA) solution and subjected to RPC in a C18 column (Discovery-Sigma-Aldrich) using 0.1% trifluoroacetic acid (TFA) as solution A and 0.1% trifluoroacetic acid (TFA) and 99.9% acetonitrile as solution B at a 0–70% gradient with a flow of 1 mL/min. The elution was monitored at 280 nm using an Akta purifier (GE®) chromatographic system. Samples were manually collected, dried in speed vac (Lab-conco®) and stored at –20 °C. Subsequently, the eluted toxins were subjected to SDS-PAGE, 12.5% and the procedure was carried out following the steps mentioned above.

Protein quantitation was performed with DC Protein Assay kit (BIORAD®), following the manufacture's specifications.

2.3. Phospholipase activity

The phospholipase activity assay was performed according to the protocol described by Holzer and Mackessy (1996), with some modifications. Phospholipase activity of purified toxins by RPC was determined using 4-nitro-3-(octyloxy) benzoic acid (4N3OBA) as a colorimetric reagent. Initially, 200 µg of reagent was solubilized in 2 mL of a solution with 10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 10 mM CaCl₂. The toxins were diluted in Milli-Q water obtaining a concentration of 1 µg/µL. After adding the toxins, 200 µL of the mixture was incubated in 96-well plates for 30 min at 37 °C. Absorbance was determined at 425 nm using an Eon (Biotek) microplate spectrophotometer, at 3-min intervals. A Lys49-PLA₂ (BtTX-I) and an Asp49-PLA₂

(BthTX-II) from *B. jararacussu* snake venom were used as the negative and positive controls.

2.4. *In vitro* antimalarial assays against *P. falciparum*

Chloroquine-resistant and mefloquine-sensitive (W2) *P. falciparum* clones of the Indochina III/CDC strain were used for the antimalarial activity assays. Parasite cultures were grown in human red blood cells under conditions established by Trager and Jensen (1976), with modifications. Cultures were prepared in TPP bottles with 2% hematocrit, diluted in RPMI 1640 medium culture (supplemented with 25 mM HEPES, 21 mM sodium bicarbonate, 11 mM glucose, 40 µg/mL gentamicin, and 0.5% (w/v) Albumax). The cultures were maintained in desiccators at 37 °C in a standard gas mixture consisting of 5% O₂, 5% CO₂ and 90% N₂. Culture media were changed daily and parasitemia was monitored in blood smears, fixed with methanol, stained with fast panoptic segmentation and visualized under an optical microscope with an immersion objective lens (1000×).

2.4.1. Synchronization of parasites for *in vitro* tests

Cultures with a predominance of ring forms were obtained through synchronization with sorbitol as described by Lambros and Vanderberg (1979). The culture medium was removed from the bottles and 10 mL of 5% sorbitol and 0.5% glucose were added to the red blood cells containing the parasites. The volume was then transferred to a 15 mL Falcon® tube, incubated at 37 °C for 10 min and centrifuged for 5 min, 70×g at room temperature. The supernatant was removed, and the pellet resuspended in complete RPMI 1640 medium. Hematocrit and parasitemia were adjusted to 2% and 0.5%, respectively.

2.4.2. *In vitro* assay against *P. falciparum*

Parasite cultures were distributed in 96-well microplates with 180 µL/well of RPMI 1640 medium containing: 0.5% parasitemia and 2% hematocrit for the SYBR Green I test (Sigma-Aldrich®). Prior to adding the parasite suspension, 20 µL of the samples, previously diluted in PBS 1X, were added to the test plate in serial concentrations from 10 to 0.00488 µg/mL. Artemisinin (0.05 µg/mL) was used as a positive control, infected red cells were used as the negative control, and non-infected red blood cells (blank) as the experimental control. The plates were incubated at 37 °C for 48 h.

After the incubation period, a fluorescence test was performed according to Smilkstein et al. (2004). Briefly, the supernatant was removed and 100 µL of phosphate buffered saline (PBS) 1X was added to each well, and then centrifuged at 700×g for 10 min in order to wash the red blood cells. Afterwards, the supernatant was discarded and 100 µL of lysis buffer (20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% w/v saponin; w/v saponin; 0.08% Triton X-100 v/v) with SYBR Green I was added and the solution was transferred to 96-well microplates with 100 µL of 1X PBS, after which a reading was performed. After incubation for 30 min at room temperature, fluorescence was measured using a spectrophotometer (Synergy HT-BioTek) with an excitation of 485 nm and an emission of 590 nm.

2.5. *In vitro* cytotoxicity assays against HepG2 and selectivity index

Cytotoxicity was evaluated against HepG2 cells, in 96-well plates. The samples were tested in serial concentrations from 10 to 0.00488 µg/mL for BdtX-II and 100 to 0.00159 µg/mL for BdtX-I and BdtX-III, with a treatment period of 48 h. Concomitantly, the negative control consisted of untreated cells, while the positive control was cells treated with lysis buffer (20 mM Tris-HCl, 5 mM EDTA, 0.08% Triton X-100, 0.008% saponin in 1X PBS, at pH 7.5) and complete RPMI 1640 medium (control).

Cytotoxicity was determined using the MTT colorimetric method (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide-Sigma-Aldrich). After the treatment period, 20 µL of MTT was added at a

concentration of 5 mg/mL in PBS 1x (w/v) to wells and incubated for 4 h at 37 °C with CO₂ (Mosmann, 1983); after the incubation period, the supernatant was discarded and 100 µL of DMSO (Sigma-Aldrich) was added to the wells to dissolve the formazan crystals. Optical reading was performed on a plate spectrophotometer (Biochrom model: Expert plus) at wavelengths of 570 nm. Cell viability was determined according to the equation below:

$$\text{Viability (\%)} = \frac{(\text{Test absorbance} - \text{Blank absorbance}) \times 100}{(\text{Control absorbance} - \text{Blank absorbance})}$$

The selectivity index (SI) was calculated using the ratios between the CC₅₀ and IC₅₀ values of the respective samples. Samples with SI values greater than 10 were considered to be selective/non-toxic, while compounds with SI values less than 10 were considered non-selective/toxic (Nava-Zuazo et al., 2010).

2.6. *In vitro* hemolytic activity

The hemolysis assay was performed with a suspension of 1% human erythrocytes in 180 µL of incomplete RPMI 1640 medium, distributed in a 96-well microplate with “U” bottom (Kasvi). 20 µL of samples, in serial concentrations from 10 to 0.00488 µg/mL, were deposited in the microplates and then incubated for 30 min at 37 °C, with shaking every 5 min (Wang et al., 2010). The absorbances were read on a spectrophotometer (Biochrom model: Expert plus) at wavelengths of 540 nm. 0.05% saponin (Sigma Aldrich®) was used as the positive control for hemolysis and non-lysed red blood cells cultured in incomplete RPMI 1640 medium were used as the negative control.

2.7. Quality control -Z' - factor

The results of the antimalarial, cytotoxicity and hemolysis tests were subjected to a quality control parameter. The parameter used was the Z'-factor (Zhang et al., 1999), a statistical factor that indicates the reliability of the results based on the degree of difference between positive and negative controls. The Z'-factor values were calculated based on the following formula:

$$Z' - \text{factor} = 1 - 3 \left(\frac{\text{dpN} + \text{dpP}}{|\text{MN} - \text{MP}|} \right)$$

$$|\text{MN} - \text{MP}|$$

where: dpN = negative control standard deviation, dpP = positive control standard deviation, MN = negative control mean, MP = positive control mean.

2.8. Statistical analyses

The analyses were performed with the aid of GraphPad Prism version 6.0 and Origin version 9.1. The results were expressed as mean ± SD of three independent experiments carried out in triplicate. The analysis of growth inhibition of 50% of the parasites (IC₅₀) and cytotoxic concentration for 50% of the cells (CC₅₀) were calculated using dose-response curves as a function of non-linear regression, applying the formula $y = A1 + (A2 - A1) / (1 + 10^{((\text{LOG}x - x) * p)})$. ANOVA followed by the Tukey multiple comparison test were used to analyze phospholipase activity and hemolysis assays. The differences were considered significant when the p value showed a significance level <0.05.

3. Results

3.1. Isolation of PLA₂s

The result for IEC was 7 main fractions (Fig. 1A), numbered 1–7, respectively. The initial fractions 1–3, which eluted before applying the gradient, correspond to acidic proteins, while the last four fractions 4–7 correspond to basic proteins, with fraction 4 being obtained with

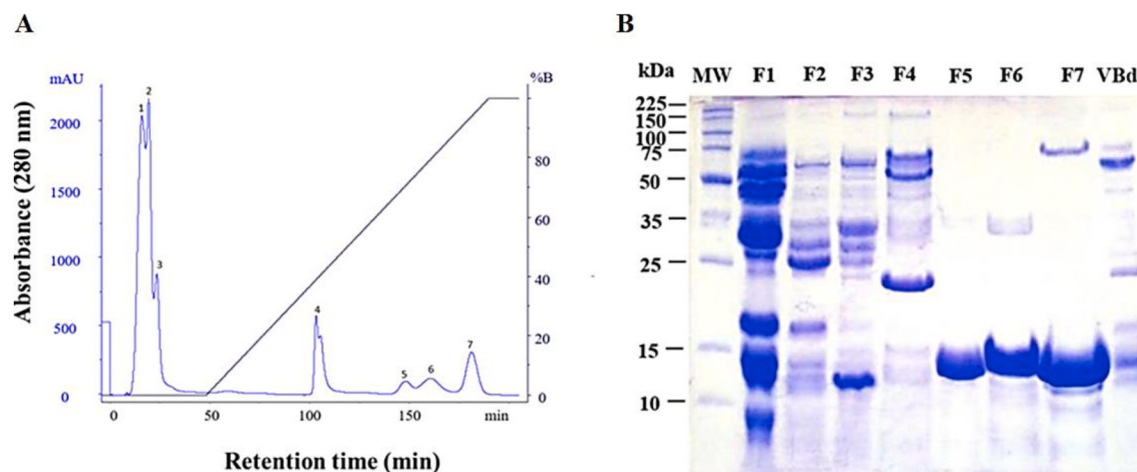


Fig. 1. Chromatographic and electrophoretic profiles of *B. diporus* venom. **(A)** This procedure allowed us to obtain seven fractions. The samples were eluted with a 0–100% gradient of buffer B, with a flow rate of 1 mL/min. Elution was monitored at 280 nm. **(B)** Electrophoretic profile of *B. diporus* venom (VBd) and seven fractions (F1–F7) eluted in IEC. It can be observed that F5, F6 and F7 fractions present protein bands with molecular mass ≈ 13 kDa, compatible with that of svPLA₂s.

approximately 30% buffer B and fractions 5, 6 and 7 eluted after approximately 65% buffer B. SDS-PAGE 12.5% was performed in order to determine the apparent molecular masses of putative PLA₂s, as well as of the molecules present in the eluted fractions (Fig. 1B). The electrophoretic profile suggests the presence of a variety of proteins with

different molecular masses, which is characteristic of bothropic venoms.

Based on the chromatographic and electrophoretic profiles (Fig. 1), the basic fractions F5, F6 and F7 were submitted to RPC. Fig. 2A–C shows the elution profile for the three isolated molecules. The electrophoretic profile allows for the visualization of main bands at

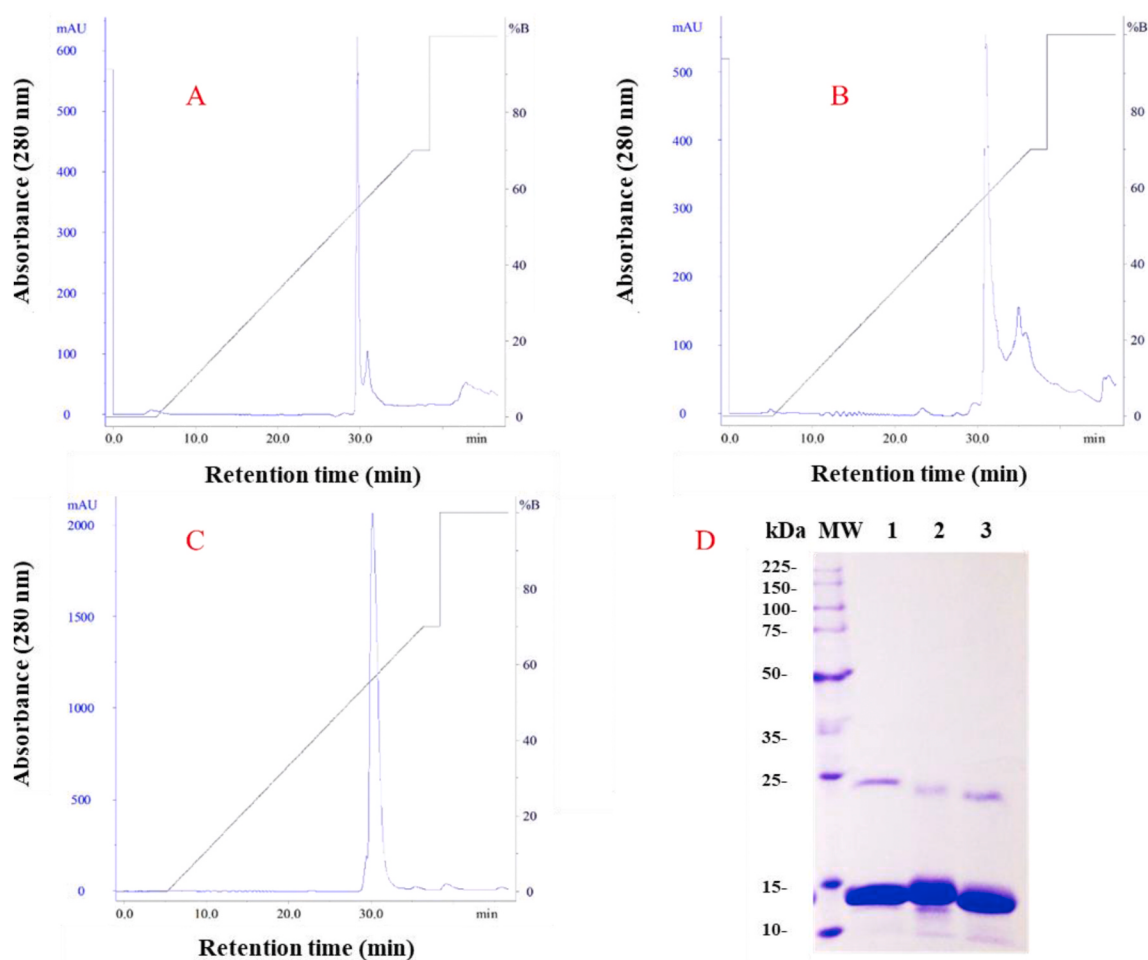


Fig. 2. Chromatographic profile in RPC of F4–6 IEC fractions from *B. diporus* venom. Fig. 2A–C shows the RPC elution profile of the three isolated basic PLA₂s: BdTX-I (A), BdTX-II (B) and BdTX-III (C), respectively. The electrophoretic profile (D) demonstrates the presence of main bands with proteins ≈ 13 kDa, compatible with the molecular weight of svPLA₂s. Lanes: 1- BdTX-I, 2- BdTX-II and 3- BdTX-III.

approximately 13 kDa for each isolated toxins, putative PLA₂s, which were named BdTX-I, BdTX-II and BdTX-III, respectively. It is possible to observe bands with proteins at approximately 25 kDa, corresponding to aggregates of PLA₂s in dimeric forms.

Phospholipase activity was evaluated using the chromogenic substrate 4N3OBA. In this step, it was demonstrated that BdTX-I showed enzymatic activity; however, the activity of BdTX-II was even more intense (Fig. 3). The enzymatic activity of these toxins was compared with the positive control (CP) BthTX-II (Asp49-PLA₂). BdTX-III showed no activity on the substrate, comparable to the negative control (CN) (BthTX-I, a Lys49-PLA₂-homologue). Both controls correspond to toxins isolated from *B. jararacussu* venom.

The PLA₂s isolated from fractions 5, 6 and 7 tested *in vitro* against strains of *P. falciparum*, showed IC₅₀ values of 2.44 µg/mL, 0.0153 µg/mL and 0.59 µg/mL, respectively. Cytotoxicity assays on HepG2 cells indicated that the PLA₂s were not toxic at the concentrations tested, with CC₅₀ ≥ 10 and 100 µg/mL for both. The exact Selectivity Index (SI) cannot be calculated; however, with these results, the fractions were selective and non-toxic, showing SI ≥ 41, ≥653.6 and ≥169.5, respectively (Table 1).

Regarding the hemolytic capacity of these PLA₂s, as a result, proteins did not show hemolytic action at the tested concentrations (10–0.0048 µg/mL). This result demonstrates that the PLA₂s are selective against the parasite and do not cause damage to erythrocytes.

4. Discussion

The diversity of proteins found in snake venoms explains the motivation of research groups who seek to develop studies aimed at elucidating the physical, chemical, structural and biological characteristics of the molecules that make up these venom and the relationship that these molecules have with the pathophysiology of snake envenoming and biotechnological applications (Kini and Fox, 2013; Simoes-Silva et al., 2018). Thus, the aim of this study was to evaluate the antiparasitic potential of basic isoforms of PLA₂s isolated from *B. diporus* venom.

Several authors have reported the purification of svPLA₂s through the application of different chromatographic techniques (Stábili et al., 2012). In the present study, we chose to use cation exchange chromatography as the first fractionation step, a procedure from which 7 fractions were eluted (Fig. 1A), which were subjected to 12.5% SDS-PAGE. The electrophoretic profile presents the relative molecular masses of the proteins present in the seven eluted fractions (Fig. 1B).

Both the characteristic elution profile of fractions 5, 6 and 7 corresponding to basic proteins, as well as the relative molecular masses of

Table 1

Bioactivity of PLA₂s from *B. diporus* venom against *P. falciparum*.

PLA ₂	IC ₅₀ (µg/mL) <i>P. falciparum</i>	Standard deviation (±)	CC ₅₀ (µg/ mL) HepG2	SI
BdTX-I	2.44	0.15	≥100	≥41
BdTX-II	0.0153	0.005	≥10	≥653.6
BdTX-III	0.59	0.03	≥100	≥169.5
Artemisinin	0.0125	0.3	≥1000	≥80.000

IC₅₀ = half maximal inhibitory concentration.

CC₅₀ = half maximal cytotoxic concentration.

SI= Selectivity Index.

the proteins observed in the aforementioned fractions, suggest that the molecules eluted in the last three fractions correspond to basic PLA₂s present in *B. diporus* venom. Cation exchange chromatography has already been reported by several authors as an efficient step for the fractionation and isolation of the proteins that make up these venoms (Andrião-Escarso et al., 2000; Soares et al., 1998, 2000a, 2000b).

Subsequently, basic fractions compatible with svPLA₂ molecular weights were subjected to reverse phase chromatography (Fig. 2A–C). The combination of IEC followed by RPC has already been reported by several authors, who mention that these steps contribute considerably to the isolation of basic PLA₂s from bothropic venoms, guaranteeing the purification of the molecules of interest with a high degree of purity (Alfonso et al., 2019; Grabner et al., 2017; Nunes et al., 2013). The electrophoretic profile of the isolated toxins can be seen in Fig. 2D. The eluted molecules were named BdTX-I, BdTX-II and BdTX-III, and for each of them, a main band of approximately 13 kDa can be observed, compatible with svPLA₂ molecular masses.

The test performed to evaluate the enzymatic activity of these toxins (Fig. 3) revealed that both BdTX-I and BdTX-II exert enzymatic activity, which indicates that these enzymes belong to the group of Asp49-PLA₂s. On the other hand, considering that BdTX-III did not show catalytic properties, it is suggested that this protein is a homologous PLA₂, with a lysine residue at position 49 (Maraganore et al., 1984; Ownby et al., 1999).

In this study, protein quantification suggests that the isolated proteins correspond to approximately 15% of the *B. diporus* venom. The literature describes PLA₂s as representing approximately 24% of the *B. diporus* venom proteome, with approximately 65% of PLA₂s corresponding to the Asp49-PLA₂ group (Gay et al., 2015). Recently, Teixeira and coworkers (2018) reported the isolation of a Lys49-PLA₂ homologue from *B. diporus* named BdpTX-I. The authors describe this toxin as having pharmacological effects such as myotoxicity and edema induction.

Subsequently, from the venom of the same species, Bustillo and coworkers (2019) describe the isolation of PLA₂-I (Asp49) and PLA₂-II (Lys49), reporting myonecrotic, cytotoxic action and suggesting that proteins have a synergistic effect that can potentiate their pharmacological effects.

As previously described, svPLA₂s present in *B. diporus* venom are responsible for different pharmacological effects. While the literature reports the antiparasitic activity of this group of proteins (Alfonso et al., 2019; Grabner et al., 2017; Nunes et al., 2013; Stábili et al., 2006), this study aimed to evaluate the cytotoxic capacity of PLA₂s against the intra-erythrocytic forms of *P. falciparum*, becoming the first report in scientific literature to perform antiplasmodial assays of toxins isolated from Paraguayan *B. diporus*.

Some authors have demonstrated that antiplasmodial action is more pronounced in enzymatically active PLA₂s than inactive ones. However, BmajPLA₂-II (a Lys49-PLA₂-homologue), isolated by Grabner and coworkers (2017) presents an IC₅₀ value comparable to that of the enzymatically active PLA₂s obtained in this study.

Previously, Zieler and coworkers investigated the antiplasmodial activity of a phospholipase isolated from *Crotalus adamanteus*; the authors describe that a concentration of 1 µmol.L⁻¹ of PLA₂ is effective to

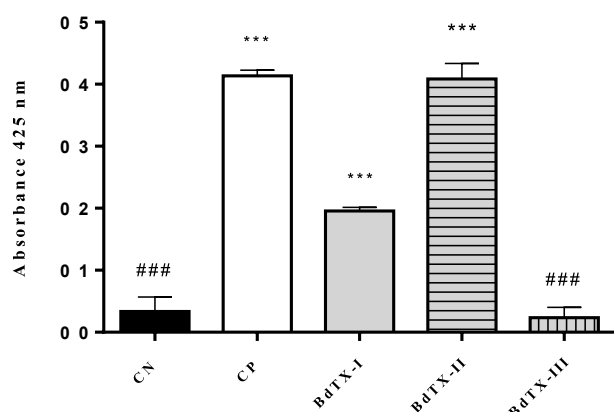


Fig. 3. Phospholipase activity of toxins isolated from *B. diporus* venom using the substrate 4N3OBA. CN: Negative control BthTX-I, CP: Positive control BthTX-II. Columns marked with * differ statistically from CN and columns marked with # differ statistically from CP based on ANOVA (***/#p < 0.0001), followed by the Tukey post-test.

block the *in vitro* development of ookinetes, intestinal forms of *P. falciparum* found in the mosquito (Zieler et al., 2001). In this context, Guillaume et al. also report antimalarial effects against intra-erythrocytic forms of *P. falciparum* of seven PLA₂s, with IC₅₀ values between 512.4 and 0.1 nM, including the snakes *Agkistrodon halys*, *Naja mossambica mossambica*, *Notechis scutatus scutatus* and *Vipera ammodytes* (Guillaume et al., 2004).

Upon analysis of the activities presented by different bothropic venoms, it is noted that the PLA₂s of these venoms are relevant in terms of their antiprotozoal action and antiplasmodial potential as shown in the present study.

The cytotoxicity assessment of the PLA₂s BdTX-I, BdTX-II and BdTX-III was performed against HepG2 cells. This cell line was chosen because hepatocytes are cells that naturally metabolize drugs and where *Plasmodium* replicates during a phase of its cycle in the vertebrate host (Thieleke-Matos et al., 2016). The CC₅₀ values observed for the three PLA₂s was ≥ 10 and 100 $\mu\text{g/mL}$; with this result the exact SI cannot be calculated; however, the fractions can be considered potentially safe regarding cytotoxicity parameters, since the SI results were greater than 10 (Bézivin et al., 2003; Katsuno et al., 2015).

The cytotoxic profile obtained in this study agrees with results previously obtained (Castillo et al., 2012). These authors tested an Asp49-PLA₂ purified from the venom of *B. asper*, which presented a CC₅₀ of 26.98 $\mu\text{g/mL}$ against PBMC and an SI of 19 against *P. falciparum*. In this context, Grabner et al. reported that BmajPLA₂-II has a CC₅₀ of 53.07 $\mu\text{g/mL}$ and an SI of 8.28. It is noted that homologous PLA₂s tend to have lower SI than enzymatically active PLA₂s; however, in the present study the three protein isoforms presented CC₅₀ and SI values characterizing them as selective and non-toxic (Grabner et al., 2017).

In order to observe that the antiplasmodial activity of PLA₂s is not due to hemolytic action, an *in vitro* hemolysis assay was performed, using the same concentrations tested in the antiplasmodial assay. BdTX-I, BdTX-II and BdTX-III did not present hemolysis. The correlation of these results shows that the antiplasmodial activity of these PLA₂s may occur directly on the intra-erythrocytic forms of *P. falciparum* and not cause destabilization or lysis of the red blood cell membrane.

5. Conclusion

Natural animal and plant products can provide compounds that have therapeutic biotechnological potential in the face of biological tests; among these include snake venoms, which have been successful in obtaining new drugs. Bothropic venoms are biochemically complex, consisting mostly of protein components, such as metalloproteases, serinoproteases, type C lectins, L-amino acid oxidases (LAAO), myotoxins, disintegrins and phospholipases A₂ (PLA₂).

The results presented herein show that PLA₂s isolated from *B. diporus* have applicable biotechnological potential regarding their antiplasmodial activity. New studies should be carried out in order to understand the mechanism of death of the parasite in the presence of these proteins.

CRediT authorship contribution statement

Keila A. Vitorino: Conceptualization, Investigation, Methodology. **Jorge J. Alfonso:** Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing, Visualization. **Ana F. Gómez:** Conceptualization, Methodology, Validation, Writing - original draft, Visualization. **Ana Paula A. Santos:** Conceptualization, Methodology, Validation, Writing - original draft, Writing - review & editing, Visualization. **Ygor R. Antunes:** Methodology, Validation. **Cleópatra A. da S. Caldeira:** Conceptualization, Investigation, Methodology. **Celeste V. Gómez:** Visualization, Supervision. **Carolina B.G. Teles:** Conceptualization, Validation, Writing - original draft, Funding acquisition. **Andreimar M. Soares:** Writing - original draft, Project administration, Funding acquisition. **Leonardo A. Calderon:** Project

administration, Funding acquisition.

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.

Acknowledgments

The authors are grateful to the Catholic University of Asuncion, Paraguay for collecting the venom and making it available to the Plataforma de Bioensaios de Malária e Leishmaniose (PBML) of the Oswaldo Cruz-Rondonia Foundation (FIOCRUZ-RO) for carrying out the *in vitro* tests. The authors express their gratitude to the Conselho de Gestão do Patrimônio Genético (CGEN/MMA) and the Programa de Desenvolvimento Tecnológico em Ferramentas para a Saúde, PDTIS-FIOCRUZ.

Abbreviations

PLA ₂	phospholipase
A ₂ ; svPLA ₂	snake venom phospholipase A ₂
IEC	Ionic Exchange Chromatography
SDS-PAGE	Poly-Acrylamide Gel Electrophoresis in the presence of Sodium Dodecyl Sulfate
RPC	Reverse Phase Chromatography
TFA	trifluoroacetic acid
ACN	Acetonitrile
4N3OBA	4-nitro- 3-(octyloxy) benzoic acid
RPMI-1640	Roswell Park Memorial Institute
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
IC ₅₀	half maximal inhibitory concentration
CC ₅₀	half maximal cytotoxic concentration
SI	Selectivity Index

Availability of data and materials

Not applicable.

Funding

The authors express their gratitude to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/MCTIC), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/MEC), Fundação Rondonia de Amparo ao Desenvolvimento das Ações Científicas e Tecnológicas de Pesquisa do Estado de Rondonia (FAPERON), Instituto Nacional de Epidemiologia da Amazônia Ocidental (EpiAmO), and the National Research Incentive Program (PRONII-229/2011) of the National Council for Science and Technology (CONACYT) for financial support.

Authors' contributions

KAV, JJA, AFG, YRA, AMS, LAC performed the isolation and biochemical characterization. KAV, APAS, CBGT carried out the plasmonic assays. KAV, JJA, AFG, YRA, AMS, LAC, CVG, APAS, CBGT analyzed the results. KAV, JJA, AFG, YRA, CASC, CVG, APAS, CBGT, AMS and LAC participated in the discussion of the results, carried out a critical review of the work and assisted in drafting and structuring the manuscript. JJA, AFG, LAC, CBGT, CVG and AMS were responsible for the conception of the study and supervised the experimental work. All the authors read and approved the final manuscript.

Ethical statement

All experimental venom collection procedures were approved by the Secretariat of the Environment, SEAM-Paraguay, Authorization No. 01/14.

Consent for publication

Not applicable.

References

- Alfonso, J.J., Kayano, A.M., Garay, A.F.G., Simões-Silva, R., Sobrinho, J.C., Vourliotis, S., Soares, A.M., Calderon, L.A., Gómez, M.C.V., 2019. Isolation, biochemical characterization and antiparasitic activity of BmatTX-IV, A basic Lys49-phospholipase A2 from the venom of *Bothrops mattogrossensis* from Paraguay. *Curr. Top. Med. Chem.* 19, 2041–2048. <https://doi.org/10.2174/1568026619666190723154756>.
- Andrião-Escarso, S.H., Soares, A.M., Rodrigues, V.M., Angulo, Y., Díaz, C., Lomonte, B., Gutiérrez, J.M., Giglio, J.R., 2000. Myotoxic phospholipases A₂ in Bothrops snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*. *Biochimie* 82, 755–763. [https://doi.org/10.1016/S0300-9084\(00\)01150-0](https://doi.org/10.1016/S0300-9084(00)01150-0).
- Araya, C., Lomonte, B., 2007. Antitumor effects of cationic synthetic peptides derived from Lys49 phospholipase A₂ homologues of snake venoms. *Cell Biol. Int.* 31, 263–268. <https://doi.org/10.1016/j.cellbi.2006.11.007>.
- Ashley, E.A., Dhorda, M., Fairhurst, R.M., Amaratunga, C., Lim, P., Suon, S., Sreng, S., Anderson, J.M., Mao, S., Sam, B., Sopha, C., Chuor, C.M., Nguon, C., Sovannaroth, S., Pukrittayakamee, S., Jittamala, P., Chotivanich, K., Chutasmit, K., Suchatsoonthorn, C., et al., 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N. Engl. J. Med.* 371, 411–423. <https://doi.org/10.1056/NEJMoa1314981>.
- Bao, Y., Bu, P., Jin, L., Hongxia, W., Yang, Q., An, L., 2005. Purification, characterization and gene cloning of a novel phospholipase A₂ from the venom of *Akistrodon blomhoffii ussuriensis*. *Int. J. Biochem. Cell Biol.* 37, 558–565. <https://doi.org/10.1016/j.biocel.2004.05.022>.
- Bézivin, C., Tomasi, S., Lohezic-Le Devehat, F., Boustie, J., 2003. Cytotoxic activity of some lichen extracts on murine and human cancer cell lines. *Phytomedicine* 10, 499–503. <https://doi.org/10.1078/094471103322331458>.
- Bhumiratana, A., Intarapuk, A., Sorosjinda-Nunthawarasilp, P., Maneekan, P., Koyadun, S., 2013. Border malaria associated with multidrug resistance on Thailand-Myanmar and Thailand-Cambodia borders: transmission dynamic, vulnerability, and surveillance. *BioMed Res. Int.* (13) <https://doi.org/10.1155/2013/363417>, 2013.
- Bustillo, S., Fernández, J., Chaves-Araya, S., Angulo, Y., Leiva, L.C., Lomonte, B., 2019. Isolation of two basic phospholipases A₂ from *Bothrops diporus* snake venom: comparative characterization and synergism between Asp49 and Lys49 variants. *Toxicon* 168, 113–121. <https://doi.org/10.1016/j.toxicon.2019.07.004>.
- Castillo, J.C.Q., Vargas, L.J., Segura, C., Gutiérrez, J.M., Pérez, J.C.A., 2012. In vitro antiplasmodial activity of phospholipases A₂ and a phospholipase homologue isolated from the venom of the snake *Bothrops asper*. *Toxins* 4, 1500–1516. <https://doi.org/10.3390/toxins4121500>.
- Cecilio, A.B., Caldas, S., De Oliveira, R.A., Santos, A.S.B., Richardson, M., Naumann, G. B., Schneider, F.S., Alvarenga, V.G., Estevão-Costa, M.I., Fuly, A.L., Eble, J.A., Sanchez, E.F., 2013. Molecular characterization of Lys49 and Asp49 phospholipases A₂ from snake venom and their antiviral activities against Dengue virus. *Toxins* 5, 1780–1798. <https://doi.org/10.3390/toxins5101780>.
- Cox, F., 2010. History of the discovery of the malaria parasites and their vectors. *Parasites Vectors* 3, 1–9. <https://doi.org/10.1186/1756-3305-3-5>.
- Dennis, E.A., Cao, J., Hsu, Y.-H., Magriotti, V., Kokotos, G., 2011. Phospholipase A₂ enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* 111, 6130–6185. <https://doi.org/10.1021/cr200085w>.
- Gay, C., Sanz, L., Calvete, J.J., Pla, D., 2015. Snake venomomics and antivenomics of *Bothrops diporus*, a medically important pitviper in northeastern Argentina. *Toxins* 8, 1–13.
- Grabner, A.N., Alfonso, J., Kayano, A.M., Moreira-Dill, L.S., dos Santos, A.P.D.A., Caldeira, C.A.S., Sobrinho, J.C., Gómez, A., Grabner, F.P., Cardoso, F.F., Zuliani, J.P., Fontes, M.R.M., Pimenta, D.C., Gómez, C.V., Teles, C.B.G., Soares, A.M., Calderon, L. A., 2017. BmajPLA2-II, a basic Lys49-phospholipase A₂ homologue from *Bothrops marajoensis* snake venom with parasitocidal potential. *Int. J. Biol. Macromol.* 102 <https://doi.org/10.1016/j.ijbiomac.2017.04.013>.
- Guillaume, C., Deregnaucourt, C., Clavey, V., Schrével, J., 2004. Anti-Plasmodium properties of group IA, IB, IIA and III secreted phospholipases A₂ are serum-dependent. *Toxicon* 43, 311–318. <https://doi.org/10.1016/j.toxicon.2004.01.006>.
- Gutiérrez, J.M., Lomonte, B., 2013. Phospholipases A₂: unveiling the secrets of a functionally versatile group of snake venom toxins. *Toxicon: Off. J. Int. Soc. Toxinol.* 62, 27–39. <https://doi.org/10.1016/j.toxicon.2012.09.006>.
- Harvey, A.L., Bradley, K.N., Cochran, S.A., Rowan, E.G., Pratt, J.A., Quillfeldt, J.A., Jerusalinsky, D.A., 1998. What can toxins tell us for drug discovery? *Toxicon* 36, 1635–1640. [https://doi.org/10.1016/S0041-0101\(98\)00156-1](https://doi.org/10.1016/S0041-0101(98)00156-1).
- Harvey, A.L., Edrada-Ebel, R., Quinn, R.J., 2015. The re-emergence of natural products for drug discovery in the genomics era. *Nat. Rev. Drug Discov.* 14, 111–129. <https://doi.org/10.1038/nrd4510>.
- Holzer, M., Mackessy, S.P., 1996. An aqueous endpoint assay of snake venom phospholipase A₂. *Toxicon* 34, 1149–1155. [https://doi.org/10.1016/0041-0101\(96\)00057-8](https://doi.org/10.1016/0041-0101(96)00057-8).
- Katsuno, K., Burrows, J.N., Duncan, K., Van Huijsduijn, R.H., Kaneko, T., Kita, K., Mowbray, C.E., Schmatz, D., Warner, P., Slingsby, B.T., 2015. Hit and lead criteria in drug discovery for infectious diseases of the developing world. *Nat. Rev. Drug Discov.* <https://doi.org/10.1038/nrd4683>.
- Kini, R.M., 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A₂ enzymes. *Toxicon: Off. J. Int. Soc. Toxinol.* 42, 827–840. <https://doi.org/10.1016/j.toxicon.2003.11.002>.
- Kini, R.M., Fox, J.W., 2013. Milestones and future prospects in snake venom research. *Toxicon* 62, 1–2. <https://doi.org/10.1016/j.toxicon.2012.09.002>.
- Koh, D., Armugam, A., Jeyaseelan, K., 2006. Snake venom components and their applications in biomedicine. *Cell. Mol. Life Sci.* 63, 3030–3041. <https://doi.org/10.1007/s00018-006-6315-0>.
- Krizaj, L., Bieber, A.L., Anka, R., Franc, G., 1970. The primary structure of ammodytin L, a myotoxic phospholipase A₂ homologue from *Vipera ammodytes* venom. *Biochem. Soc. Symp.* 30, 29–41.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lambros, C., Vanderberg, J.P., 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J. Parasitol.* 65, 418–420.
- Lomonte, B., Angulo, Y., Calderón, L., 2003. An overview of lysine-49 phospholipase A₂ myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. *Toxicon*. <https://doi.org/10.1016/j.toxicon.2003.11.008>.
- Lomonte, B., Fernández, J., Sanz, L., Angulo, Y., Sasa, M., Gutiérrez, J.M., Calvete, J.J., 2014. Venomous snakes of Costa Rica: biological and medical implications of their venom proteomic profiles analyzed through the strategy of snake venomomics. *Journal of Proteomics*. <https://doi.org/10.1016/j.jprot.2014.02.020>.
- Lomonte, B., Gutiérrez, J.M., 2011. Phospholipases A₂ from viperidae snake venoms: how do they induce skeletal muscle damage? *Acta Chim. Slov.* 58, 647–659.
- Maraganore, J.M., Merutka, G., Cho, W., Welches, W., Kézdy, F.J., Heinrikson, R.L., 1984. A new class of phospholipases A₂ with lysine in place of aspartate 49. Functional consequences for calcium and substrate binding. *J. Biol. Chem.* 259, 13839–13843.
- Mathenge, P.G., Low, S.K., Vuong, N.L., Mohamed, M.Y.F., Faraj, H.A., Alieldin, G.I., Al khudari, R., Yahia, N.A., Khan, A., Diab, O.M., Mohamed, Y.M., Zayan, A.H., Tawfik, G.M., Huy, N.T., Hirayama, K., 2020. Efficacy and resistance of different artemisinin-based combination therapies: a systematic review and network meta-analysis. *Parasitol. Int.* 74 <https://doi.org/10.1016/j.parint.2019.04.016>.
- Mebs, D., Kuch, U., Coronas, F.I.V., Batista, C.V.F., Gumprecht, A., Possani, L.D., 2006. Biochemical and biological activities of the venom of the Chinese pitviper *Zhaeria mangshanensis*, with the complete amino acid sequence and phylogenetic analysis of a novel Arg49 phospholipase A₂ myotoxin. *Toxicon: Off. J. Int. Soc. Toxinol.* 47, 797–811. <https://doi.org/10.1016/j.toxicon.2006.01.031>.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63.
- Nava-Zuazo, C., Estrada-Soto, S., Guerrero-Álvarez, J., León-Rivera, I., Molina-Salinas, G. M., Said-Fernández, S., Chan-Bacab, M.J., Cedillo-Rivera, R., Moo-Puc, R., Mirón-López, G., Navarrete-Vázquez, G., 2010. Design, synthesis, and in vitro antiparasitic, antimicrobial activities of N-{2-[(7-chloroquinolin-4-yl)amino]ethyl}ureas. *Bioorg. Med. Chem.* 18, 6398–6403. <https://doi.org/10.1016/j.bmc.2010.07.008>.
- Nevin, R.L., Croft, A.M., 2016. Psychiatric effects of malaria and anti-malarial drugs: historical and modern perspectives. *Malar. J.* <https://doi.org/10.1186/s12936-016-1391-6>.
- Newman, D.J., Cragg, G.M., 2016. Natural products as sources of new drugs from 1981 to 2014. *J. Nat. Prod.* <https://doi.org/10.1021/acs.jnatprod.5b01055>.
- Nunes, D.C., Figueira, M.M., Lopes, D., De Souza, D., Al, E., 2013. BnSP-7 toxin, a basic phospholipase A₂ from *Bothrops pauloensis* snake venom, interferes with proliferation, ultrastructure and infectivity of *Leishmania (Leishmania) amazonensis*. *Parasitology* 140, 44–54. Nunes DC, Figueira MM, Lopes DS, DE Souza DL, et al. *Parasitology* 2013.
- Okombo, J., Chibale, K., 2016. Antiplasmodial drug targets: a patent review (2000–2013). *Expert Opin. Ther. Pat.* 26, 107–130. <https://doi.org/10.1517/13543776.2016.1113258>.
- Organization, W.H., 2020. World malaria report [WWW Document]. URL. <https://www.who.int/news-room/fact-sheets/detail/malaria>.
- Owby, C.L., Selistre de Araujo, H.S., White, S.P., Fletcher, J.E., 1999. Lysine 49 phospholipase A₂ proteins. *Toxicon: Off. J. Int. Soc. Toxinol.* 37, 411–445.
- Simoes-Silva, R., Alfonso, J., Gomez, A., Holanda, R.J., Sobrinho, J.C., Zaqueo, K.D., Moreira-Dill, L.S., Kayano, A.M., Grabner, F.P., da Silva, S.L., Almeida, J.R., Stabeli, R.G., Zuliani, J.P., Soares, A.M., 2018. Snake venom, A natural library of new potential therapeutic molecules: challenges and current perspectives. *Curr. Pharmaceut. Biotechnol.* 19, 308–335. <https://doi.org/10.2174/1389201019666180620111025>.
- Smilkstein, M., Sriwilaijaroen, N., Kelly, J.X., Wilairat, P., Riscoe, M., 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob. Agents Chemother.* 48, 1803–1806. <https://doi.org/10.1128/aac.48.5.1803-1806.2004>.
- Soares, A.M., Andrião-Escarso, S.H., Angulo, Y., Lomonte, B., Gutiérrez, J.M., Marangoni, S., Toyama, M.H., Arni, R.K., Giglio, J.R., 2000a. Structural and functional characterization of myotoxin I, a Lys49 phospholipase A₂ homologue

- from *Bothrops moojeni* (Caissaca) snake venom. Arch. Biochem. Biophys. 373, 7–15. <https://doi.org/10.1006/abbi.1999.1492>.
- Soares, A.M., Guerra-Sá, R., Borja-Oliveira, C.R., Rodrigues, V.M., Rodrigues-Simioni, L., Rodrigues, V.M., Fontes, M.R., Lomonte, B., Gutiérrez, J.M., Giglio, J.R., 2000b. Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A₂ homologue from *Bothrops neuwiedi pauloensis* venom. Arch. Biochem. Biophys. 378, 201–209.
- Soares, A.M., Rodrigues, V.M., Homsí-Brandeburgo, M.I., Toyama, M.H., Lombardi, F.R., Arni, R.K., Giglio, J.R., 1998. A rapid procedure for the isolation of the LYS-49 myotoxin II from *Bothrops moojeni* (caissaca) venom: biochemical characterization, crystallization, myotoxic and edematogenic activity. Toxicon 36, 503–514.
- Stábeli, R.G., Amui, S.F., Sant'Ana, C.D., Pires, M.G., Nomizo, A., Monteiro, M.C., Romão, P.R.T., Guerra-Sá, R., Vieira, C.a., Giglio, J.R., Fontes, M.R.M., Soares, A.M., 2006. *Bothrops moojeni* myotoxin-II, a Lys49-phospholipase A₂ homologue: an example of function versatility of snake venom proteins. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 142, 371–381. <https://doi.org/10.1016/j.cbpc.2005.11.020>.
- Stábeli, R.G., Simões-Silva, R., Kayano, A.M., Gimenez, G.S., Moura, A.A., Caldeira, C.A. da S., Coutinho-Neto, A., Zaqueo, K.D., Zuliani, J.P., Soares, A.M., Calderon, L.A., 2012. Purification of phospholipases A₂ from American snake venoms. In: Calderon, Leonardo de Azevedo (Ed.), Chromatography - the Most Versatile Method of Chemical Analysis.
- Su, X.Z., Miller, L.H., 2015. The discovery of artemisinin and the nobel prize in physiology or medicine. Science China. Life Sci. 58, 1175–1179. <https://doi.org/10.1007/s11427-015-4948-7>.
- Teixera, L.F., de Carvalho, L.H., de Castro, O.B., Bastos, J.S.F., Nery, N.M., Oliveira, G.A., Kayano, A.M., Soares, A.M., Zuliani, J.P., 2018. Local and systemic effects of BdipTX-I, a Lys-49 phospholipase A₂ isolated from *Bothrops diporus* snake venom. Toxicon 141, 55–64. <https://doi.org/10.1016/j.toxicon.2017.11.007>.
- Thieleke-Matos, C., Lopes da Silva, M., Cabrita-Santos, L., Portal, M.D., Rodrigues, I.P., Zuzarte-Luis, V., Ramalho, J.S., Futter, C.E., Mota, M.M., Barral, D.C., Seabra, M.C., 2016. Host cell autophagy contributes to *Plasmodium* liver development. Cell Microbiol. 18, 437–450. <https://doi.org/10.1111/cmi.12524>.
- Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675. <https://doi.org/10.1126/science.781840>.
- Wang, C., Qin, X., Huang, B., He, F., Zeng, C., 2010. Hemolysis of human erythrocytes induced by melamine-cyanurate complex. Biochem. Biophys. Res. Commun. 402, 773–777. <https://doi.org/10.1016/j.bbrc.2010.10.108>.
- Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug-resistant malaria. Lancet Infect. Dis. [https://doi.org/10.1016/S1473-3099\(02\)00239-6](https://doi.org/10.1016/S1473-3099(02)00239-6).
- Zhang, J.-H., Chung, T.D.Y., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen 4, 67–73.
- Zieler, H., Keister, D.B., Dvorak, J.A., Ribeiro, J.M.C., 2001. A snake venom phospholipase A₂ blocks malaria parasite development in the mosquito midgut by inhibiting ookinete association with the midgut surface. J. Exp. Biol. 204, 4157–4167.