Inhibition of a Myotoxic Phospholipase A$_2$ isolated from *Crotalus durissus cumanensis* by Pentacyclic Triterpenes

Inhibición de una fosfolipasa A$_2$ Miotóxica aislada de *Crotalus durissus cumanensis* por Triterpenos Pentacíclicos

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ABSTRACT

BACKGROUND: Ophidian accidents have been recognized as public health events in Colombia since 2004 and have been systematically reported since 2007, constituting a significant public health problem. One of the components present in the venoms of Viperidae snakes is the phospholipase A$_2$ (PLA$_2$) enzyme, playing a fundamental role in snakebite poisoning and is responsible for many of the local effects that are not totally neutralized by antivenoms. Therefore, it is necessary to research new therapeutic strategies or adjuvants in treating snakebite poisoning. Traditional herbal treatments hold promise, with ethnopharmacological studies emphasizing compounds, particularly pentacyclic triterpenes, as potential inhibitors of PLA$_2$. **OBJECTIVE:** To identify compounds with the potential to reduce or neutralize the local effects generated by PLA$_2$, present in the venom of snakes of the Viperidae family, the major cause of ophidian accidents in Colombia. **METHODS:** Four triterpenic compounds (madecassic acid, ursolic acid, betulinic acid, and oleanolic acid) were evaluated to determine the inhibitory capacity on the enzymatic activity of myotoxic phospholipase A$_2$, extracted from *Crotalus durissus cumanensis* venom and purified by molecular exclusion chromatography followed by RP-HPLC. The synthetic monodisperse substrate 4-nitro-3- (octanoloxy) benzoic acid was used to determine the inhibitory capacity of the compounds against the enzymatic activity of PLA$_2$. Molecular docking was also performed to identify the interactions between the compounds and the active site of the enzyme by visual inspection. **RESULTS:** The highest percentage of inhibition was presented by ursolic acid (47.01%). This is supported by the results of the molecular docking, where this compound was found to interact with Leu2, Phe24, Tyr52, and Lys69, amino acids involved in the catalytic activity of the enzyme. **CONCLUSIONS**: Ursolic acid was determined as the most promising compound among the four evaluated against the local effects generated by PLA$_2$. Future studies may be performed to determine other potential benefits of these compounds versus other biological actions of the enzyme.

Keywords: Phospholipase A$_2$, triterpenic compounds, molecular docking, snake venom, *Crotalus durissus cumanensis*.

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RESUMEN

INTRODUCCIÓN: Los accidentes ofídicos han sido reconocidos como eventos de salud pública en Colombia desde el año 2004 y han sido reportados sistemáticamente desde el año 2007, constituyendo un importante problema de salud pública. Uno de los componentes presentes en los venenos de las serpientes Viperidae es la enzima fosfolipasa A_2 (PLA_2), que juega un papel fundamental en el envenenamiento y es responsable de muchos de los efectos locales que no son completamente neutralizados por el antiveneno. Por lo tanto, es necesario continuar en la búsqueda de nuevas estrategias terapéuticas o coadyuvantes para el tratamiento en casos de envenenamiento por mordedura de serpientes. Los tratamientos tradicionales a base de plantas resultan prometedores, con estudios etnofarmacológicos que destacan compuestos, en particular los triterpenos pentacíclicos, como potenciales inhibidores de la PLA_2. **OBJETIVO:** Identificar compuestos con potencial de reducir o neutralizar los efectos locales generados por la PLA_2, presente en los venenos de serpientes de la familia Viperidae, mayores causantes de accidentes ofídicos en Colombia. **MÉTODOS:** Se evaluaron cuatro compuestos triterpénicos (ácido madecásico, ácido ursólico, ácido betulínico y ácido oleanólico) para determinar la capacidad inhibitoria sobre la actividad enzimática de la fosfolipasa A_2 miotóxica, extraída del veneno de *Crotalus durissus cumanensis* y purificada mediante cromatografía de exclusión molecular, seguido por RP-HPLC. Para determinar la capacidad inhibitoria de los compuestos frente a la actividad enzimática de la PLA_2, se utilizó el sustrato monodisperso sintético 4-nitro-3-(octanoloxy) ácido benzoico. Además, se realizó un docking molecular para identificar por inspección visual las interacciones entre los compuestos y el sitio activo de la enzima. **RESULTADOS:** En la capacidad inhibitoria de la actividad enzimática, el mayor porcentaje de inhibición fue presentado por el ácido ursólico (47.01%). Esto se respalda con los resultados obtenidos en el acoplamiento molecular, donde se encontró que este compuesto tiene interacciones con Leu2, Phe24, Tyr52 y Lys69, aminoácidos implicados en la actividad de la enzima. **CONCLUSIONES:** El ácido ursólico se determinó como el compuesto más promisorio entre los cuatro evaluados frente a los efectos locales generados por la PLA_2. Estudios futuros pueden realizarse para determinar otros beneficios potenciales de estos compuestos frente a otras acciones biológicas de la enzima.

Palabras clave: Fosfolipasa A_2, compuestos triterpénicos, acoplamiento molecular, veneno de serpientes, *Crotalus durissus cumanensis*.

INTRODUCTION

In Colombia, snakebites have been considered events of public health interest and established as a notifiable event since October 2004 (1,2). However, consistent reporting only began in 2007 (2). Due to the number of events registered since then, snakebites are considered a public health problem in Colombia (1). During 2023, 6,184 cases were registered in the National Public Health Surveillance System (3). In Colombia, snakebites involving snakes of the *Bothrops* genus represent the most significant number of reported cases, being considered the most epidemiologically relevant accidents in the country, with 80–85% of the cases (2). The local tissue damage observed in these envenomations can lead to several sequelae, such as limb amputation or loss of muscle mass (4–7). Currently, the only available treatment for this health problem is antivenom administration. However, it cannot neutralize local effects induced by snake venoms (1,5,6).

The venom of Viperidae family snakes can be divided into three main components: enzymes, toxins, and a non-protein component (8). Within the enzymatic group, the most representative components are phospholipases A_2 (PLA_2s), snake venom metalloproteinases (SVMPs), and snake venom serine proteinases (SVSPs) (5). PLA_2 causes hydrolysis of the bond at the sn-2 position of a cell membrane glycerophospholipids, resulting in a free fatty acid and a lysophospholipid. This reaction triggers a series of events in the organism, including local effects (such as local myotoxicity and edema) and systemic effects (such as systemic myotoxicity, hypotension, hemolysis, platelet aggregation modulation, cardiotoxicity, and direct organ damage, mainly liver, kidney, and lung) (5,7).

Traditionally, some plants have been used to treat snakebites, and these plants have been shown to have positive effects such as anti-inflammatory activity and inhibition of snake venom's PLA_2 (9,10). Ethnopharmacology has attempted to study these plants and isolate the compounds with the greatest benefits in these envenomations to evaluate them and determine their pharmacodynamics (9–12). Pharmacologically active compounds that have been described with beneficial effects in snakebites are secondary metabolites, mainly phenolic compounds (like flavonoids, phenolic acids, coumarins, and coumestanes), alkaloids, steroids, terpenoids, and polyphenols (9,11–14).

The inhibitory capacity of pentacyclic triterpenes against the main enzymes found in snake venoms has been previously assessed (12,13,15). Indeed, some of these molecules have shown inhibition on a snake venom metalloproteinase isolated from *Bothrops atrox* from Colombia (16,17). Some of these evaluations have utilized bioinformatics tools, such as molecular modeling, to identify chemical compounds in plant extracts that exhibit inhibitory effects on the PLA_2 enzyme (12,13). The results have shown that some of these compounds inhibit the activity of venom PLA_2s; in addition, other pentacyclic triterpenes have been found

to inhibit the coagulant activity induced by the serine proteinases (15); among these triterpenes are madecassic acid, oleanolic acid, ursolic acid, betulinic acid, betulin, 3-epilupeol formiate, α-amyrin acetate, 3-epilupeol acetate, lupenone, 3-epilupeol, and α-amyrin (12,13,15).

Based on these results and others reported in the literature of similar compounds with inhibitory activity on PLA$_2$ (15), this study aimed to evaluate the inhibitory activity of four pentacyclic triterpenes (betulinic acid, madecassic acid, ursolic acid, and oleanolic acid) on a myotoxic PLA$_2$ purified from *Crotalus durissus cumanensis* venom, and using molecular docking tool identify the possible interaction between these compounds and the active site of the enzyme.

METHODS

Chemical and reagents.

Betulinic acid, oleanolic acid, ursolic acid, and madecassic acid were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Other reagents used in this work were of the highest purity available from Sigma Aldrich (St. Louis, MO, USA) and Merck (Kenilworth, NJ, USA).

Purification of PLA$_2$

PLA$_2$ was purified from the lyophilized venom of *C. durissus cumanensis*, obtained from four specimens from the Department of Meta (southeast region of Colombia), and kept in captivity at the serpentarium of the University of Antioquia (Medellín, Colombia). PLA$_2$ was purified following the method described by Pereañez *et al.* (18) with modifications. Briefly, a bioguide fractionation was performed by quantifying the PLA$_2$ activity of each fraction. The enzyme was purified by molecular exclusion chromatography on Sephadex G-75, followed by reverse-phase HPLC on a C$_{18}$ column eluted at 1.0 mL/min with a gradient from 0% to 100% acetonitrile in 0.1% trifluoroacetic acid (v/v). A micro-UV/VIS NanoDrop Spectrophotometer was used to quantify the toxin. Absorbance in effluent solution was recorded at 215 nm.

Purity Evaluation of the obtained PLA$_2$

RP-HPLC was used to determine protein purity, on a C$_{18}$ column eluted at 1.0 mL/min with a gradient from 0% to 70% acetonitrile in 0.1% trifluoroacetic acid (v/v) for 25 minutes. A micro-UV/VIS NanoDrop Spectrophotometer was used to quantify the toxin. Absorbance in effluent solution was recorded at 215 nm.

Inhibition of PLA$_2$ activity using 4-nitro-3-octanoyloxybenzoic acid (4N3OBA) as a synthetic monodisperse substrate.

Measurements were performed according to the method described by Holzer *et al.* (19), using a 96-well ELISA plate method with 25 µL of 4N3OBA (Santa Cruz Biotechnology, Dallas, TX, USA) (1 mg/mL) each. Four pentacyclic triterpenes were used to evaluate their inhibitory capacity of PLA$_2$ activity: betulinic, oleanolic, ursolic, and madecassic acids. For the wells carrying the compounds, 80 µg of PLA$_2$ and a 500 µM solution of each compound dissolved in buffer (10 mM Tris-HCl, 10 mM CaCl$_2$, 100 mM NaCl, pH 8.0) were used. Positive (PLA$_2$ + buffer), negative for each compound (compound + buffer), and negative (only buffer) controls were performed. The mixture was incubated for 60 min at 37 °C, and absorbance was measured at 425 nm, n=4. Results are described in percentage of inhibition, taking toxin and PBS as 100% and 0% of activity, respectively.

Molecular docking

Molecular docking was performed using AutoDock Vina (20). The structure of phospholipase A$_2$ from the venom of *Crotalus durissus terrificus* (PDB code 2QOG) was used as a model structure for the computational studies, which was prepared using the programs UCSF Chimera 1.14 (21) and AutoDockTools. In UCSF Chimera 1.14, foreign elements such as water molecules and residual solvent present from crystallization were removed from the protein, whereas in AutoDockTools, polar hydrogen atoms were added, atoms were assigned the AD4 type, and atomic charges were assigned. A charge of +2 was set to the calcium cofactor of phospholipase A$_2$. Likewise, the structures of the four compounds (betulinic acid, madecassic acid, ursolic acid, and oleanolic acid) were downloaded from the MolView database (22) and prepared using the Avogadro program (23), and hydrogen atoms were automatically added according to the ionized form found under physiological conditions. The coupling was performed in AutoDock Vina, using the calcium ion as the center of the grid (X= 61.179, Y= 42.712, and Z= 47.465), an exhaustivity of 20 and a mesh size of 16 Å^3. Finally, the interactions between the compounds and the enzyme active site were visually inspected using the UCSF Chimera

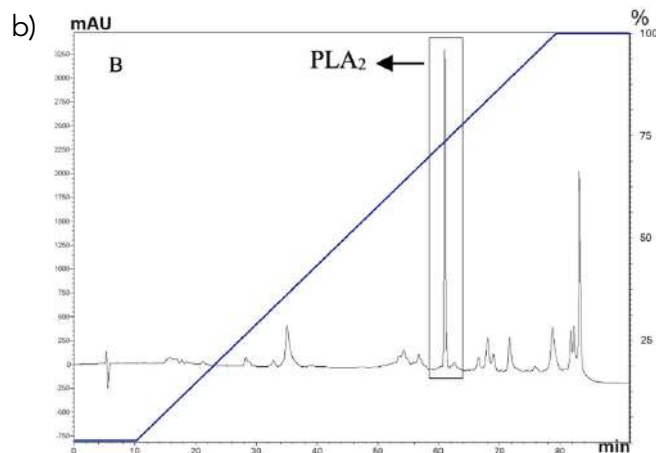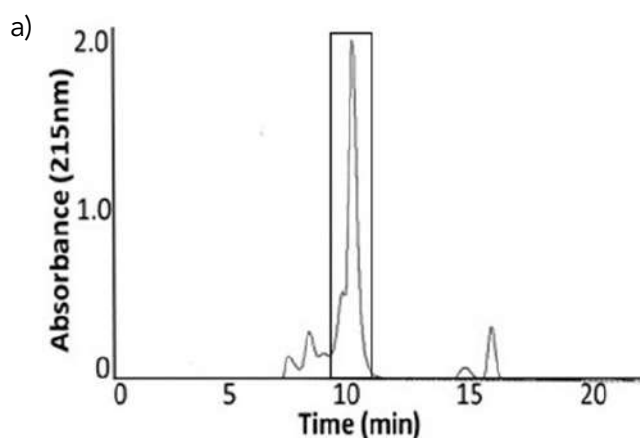1.14 (21), and PLIP (24) was used to generate the docking images.

Statistical analysis.

A Shapiro–Wilk test was used to determine whether the data sets adjusted to a normal distribution. The data obtained were subjected to a one-way ANOVA, followed by a Bonferroni post hoc test. The significance level was 0,05. Data are shown as mean ± standard deviation, n=4. Analysis was performed with Python (Version 3.10).
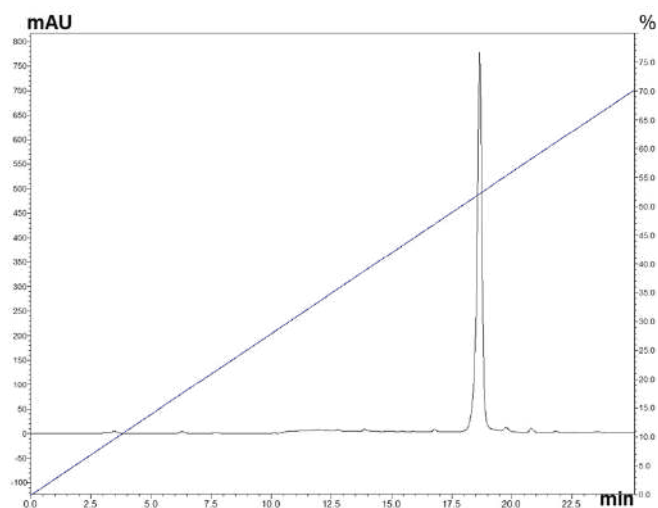
RESULTS

Purification of PLA$_2$

Lyophilized venom of *C. durissus cumanensis* was first subjected to molecular exclusion chromatography on Sephadex G-75. The fraction with PLA$_2$ activity on 4N3OBA eluted in about 10 minutes and is consistent with the previous isolation (18). This fraction was concentrated in a SpeedVac. Then, 6 mg of this fraction was submitted to a RP-HPLC, under the conditions described in methodology, and the average yield of PLA$_2$ obtained was around 20%. Figure 1 shows the molecular exclusion chromatography and RP-HPLC profiles corresponding to the purification of the toxin, in which the peak obtained around 60 min with a concentration of acetonitrile at 75% belongs to PLA$_2$, which is also the major compound in the fraction.

Purity Evaluation of the obtained PLA$_2$

An RP-HPLC run was performed to verify the purity of PLA$_2$ obtained, in which a single peak was observed around 18 min with a concentration of acetonitrile at 52%, indicating that the toxin had been successfully isolated from the venom of *C. durissus cumanensis* (Figure 2).

Figure 2. Verification of the purity of the isolated phospholipase A$_2$. The RP-HPLC chromatogram shows a single response (peak) at approximately 18 min, indicating that the enzyme was successfully purified.

Figure 1. Isolation of PLA$_2$ from C. d. cumanensis venom. **A.** Molecular exclusion chromatography. The fraction containing the PLA$_2$ is marked. **B.** RP-HPLC of the fraction containing PLA$_2$. The enzyme is highlighted.

Inhibition of PLA2 activity using 4-nitro-3-octanoyloxybenzoic acid (4N3OBA) as a synthetic monodisperse substrate.
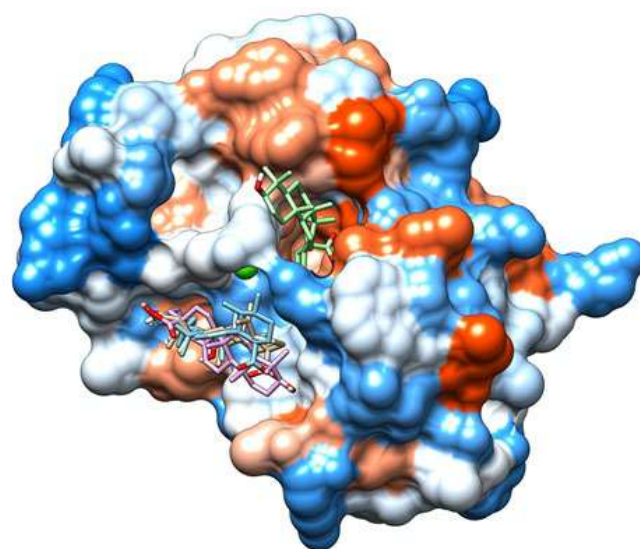
In this method, p-nitroanilide is released from the substrate after the hydrolytic action of the PLA$_2$. This chromophore has its maximum absorbance at 425 nm. Therefore, it is expected that when inhibitors are tested, the absorbance decreases. Absorbance obtained in the test with the synthetic substrate for each compound, including the positive control (PLA$_2$), are recorded Figure 3. The best inhibitory activity was those for ursolic and madecassic acids (p<0.001, regarding positive control). In contrast, oleanolic acid (p<0.01) and betulinic acid (p<0.05) showed lower inhibitory ability on PLA$_2$ of the toxin.

Figure 4. Molecular docking results. Betulinic acid is shown in beige, madecassic acid in blue, oleanolic acid in purple and ursolic acid in green. The red areas of the surface represent the acid regions, the white areas represent the neutral, and the blue areas are basic regions. The green sphere represents Ca^{2+}. Images were obtained using UCSF Chimera (21).

Figure 3. Absorbance results for the test with the synthetic substrate, the triterpenic compounds, and the positive control (80 µg PLA$_2$). Negative for each compound (compound + buffer), and negative (only buffer) controls were also performed. (*) Statistically significant differences compared with the absorbance induced by the positive control with a p-value < 0.05. (**) P-value < 0.01 (***) P-value < 0.001.; n=4.

Molecular docking

A molecular docking study was performed to suggest a mode of action for the pentacyclic triterpenes. The results indicated that all compounds could bind to the PLA$_2$ (Figure 4). Three of the four compounds had a similar conformation and binding site; however, ursolic acid had a different mode of action and binding site.

When evaluating the type of interactions between the inhibitors and the enzyme, it is observed that most of these interactions are Van der Waals; however, hydrogen bonds are observed between madecassic acid and Tyr28 and Asp49; betulinic acid and Tyr28; and oleanolic acid and Gly33 and Thr68 (Figure 5 and Table 1).
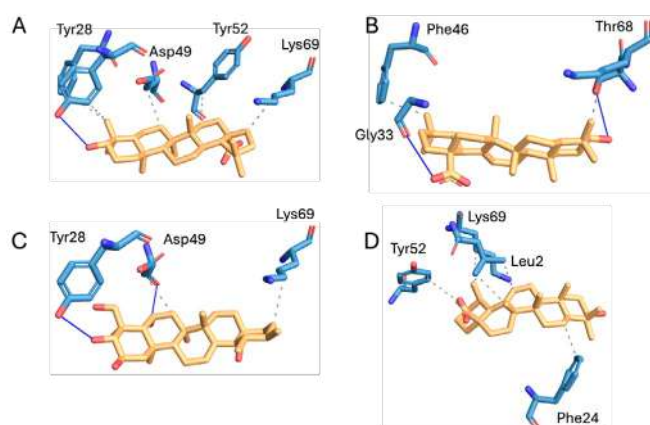
Figure 5. Binding modes of triterpenic acids into PLA$_2$ binding pocket. A*:* Betulinic acid. B*:* Oleanolic acid. C*:* Madecassic acid. D*:* Ursolic acid. Gray dashed lines represent Van der Waals interactions*; b*lue lines represent hydrogen bonds. Images were obtained with PLIP (24).

Table 1. Suggested interaction for each evaluated compound and PLA$_2$.

Interaction	Van der Waals	Hydrogen bond	Affinity (kcal/mol)
Madecassic acid	Asp49 and Lys69	Tyr28 and Asp49	- 5,8
Betulinic acid	Tyr28, Phe46, Asp49, Tyr52 and Lys69	Tyr28	- 6,6
Oleanolic acid	Phe46 and Lys69	Gly33 and Thr68	- 6,2
Ursolic acid	Leu2, Phe24, Tyr52, Lys69		- 6,8

DISCUSSION

The PLA$_2$s are the most abundant enzymes in Viperidae snake venoms (7,25). In addition, they contribute to the local tissue damage that can derive in sequelae, such as amputations and tissue loss (4,7). Therefore, an important goal in snakebites is to inhibit or neutralize the catalytic activity of the PLA$_2$s (25,26). An enzyme from *C. durissus cumanensis* was isolated to test the inhibitory potential of triterpenic acids on snake venom PLA$_2$. According to the results, the purification of the PLA$_2$ enzyme was consistent with the previously reported procedure in terms of enzyme activity and purification parameters (retention times and acetonitrile concentration) (18).

The tested triterpenic acids inhibited the PLA$_2$ activity of the enzyme in different percentages, 47,01% (ursolic acid), 43,77% (madecassic acid), 33,57% (oleanolic acid), and 29,60% (betulinic acid). These results may be explained by the binding modes suggested by molecular docking results. All compounds exhibited a Van der Waals interaction with Lys69, which is a conserved residue involved in the interfacial binding surface of the enzyme that contributes to the activation in the lipid-water interface (26,27). Other important interactions are Van der Waals and hydrogen bonds of betulinic and madecassic acids with Asp49, respectively. This amino acid is responsible for Ca^{2+} coordination, which is required for the oxyanion stabilization during catalytic cycle (26,27).

The most active compound was the ursolic acid, which showed several Van der Waals interactions with Leu2, Phe24, Tyr52, and Lys69. Phe24 is involved in the interfacial binding surface, therefore, affecting substrate recognition by the enzyme (26,27). In contrast, Leu2 is located in the hydrophobic channel of the PLA$_2$, which is involved in the stabilization of the glycerophospholipid in the active site of the enzyme (26,27). The other interaction of ursolic acid was with Tyr52, which is responsible for maintaining the hydrogen bond network of the catalytic site of the enzyme (26,27). Finally, the interaction with Lys69 was described previously.

Results obtained in this work agree with those reported by Dharmappa *et al.*, who reported the ability of oleanolic acid to inhibit the PLA$_2$ activity of *Naja naja* and *Vipera russelli* venoms. The authors determined that the inhibition process is independent of the amount of substrate or the presence of calcium ions, also demonstrated that oleanolic acid interacts directly with the enzyme, forming a complex, that irreversibly inhibits PLA$_2$ activity (28). On the other hand, Nataraju *et al.*, reported similar findings for ursolic acid by identifying that this compound has inhibitory action against phospholipase A$_2$ isolated from the venoms of *Vipera russelli* and *Naja naja* snakes (29).

Triterpenic acids also have been reported by their inhibitory effects on SVMPs, by inhibiting the catalytic, hemorrhagic, myotoxic, and edema-forming activities of a PI-SVMP from *B. atrox* venom (16). In addition, it was demonstrated that the carboxylate ion interacts with the zinc ion, an essential cofactor for SVMP activity (17). Therefore, these compounds are promising molecules that can find active leads against local tissue damage induced by snake venoms.

CONCLUSION

The method used for the extraction, purification, and evaluation of the toxin was effective, allowing us to determine the inhibitory activity of different triterpenic compounds against the toxin. Through the method using the synthetic substrate to evaluate the inhibition of PLA$_2$ enzymatic activity, it was determined that ursolic acid presented greater inhibitory capacity compared to the other compounds evaluated, with a percentage of 47,01 % (p < 0.001), being the most promising compound to reduce or inhibit the local effects that PLA$_2$ can cause. With the results obtained in the molecular

docking, a hypothesis can be established as to why ursolic acid presents a greater inhibitory capacity against the enzymatic activity of PLA$_2$, since it presents the highest affinity with the enzyme and shows interactions with the amino acids Leu2, Phe24, Tyr52, Lys69, all involved in important functions on the enzyme structure and catalysis (hydrophobic channel, active site, and interfacial binding surface).

Further studies should be conducted to determine other potential benefits of these compounds against other biological actions of PLA$_2$ that could not be addressed in the present project, as well as formulations studies to develop therapeutic strategies as coadjutants of antivenoms.

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CONFLICTS OF INTEREST

The authors of the manuscript declare that there are no conflicts of interest associated with this work.

AUTHORS' CONTRIBUTIONS

The authors confirm contribution to the paper as follows: Study conception and design: LMPR, and JAP; data collection: LCA, LMPR, IBS, and VCC; analysis and interpretation of results: LCA, LMPR, IBS, VCC, and JAP; draft manuscript preparation: LCA, LMPR, IBS, VCC, and JAP. All authors reviewed the results and approved the final version of the manuscript and have agreed to the publication.

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