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Article

A Novel Acidic Myotoxic Phospholipase A_2 of Colombian *B. asper* Venom. Isolation and Functional Characterization

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Abstract: Myotoxic phospholipases A_2 are responsible for many clinical signs in envenomation by Bothrops snakes. A new myotoxic acidic PLA_2 Asp 49 was isolated from Colombian *Bothrops asper* venom. It was isolated by high efficiency liquid chromatography and named BaCol PLA_2. It had a molecular weight of 14180.69 Da and an isoelectric point of 4.4. The complete sequence obtained by cDNA cloning, with access number in the gene bank MF319968; this sequence evidenced a mature product of 124 amino acids with Asp in 49 position. In vivo assays in mice demonstrated profuse oedema and myotoxicity evidenced by increase of creatina kinasa in plasma and severe and diffuse damage to the muscular fibers, further vacuolization and hyalinization necrosis of the sarcoplasm showed by histopathology with hematoxylin and eosin staining of gastrocnemius muscle. In vitro studies showed cell membrane damage without phosphatidylserine exposure, an early apoptosis hallmark. Further BaCol PLA_2 evidenced high indirect hemolytic activity and moderate anticoagulant action. The toxin showed homology with others acidic PLA_2 isolated from Bothrops venoms, including one isolated from *B. asper* of Costa Rica. Unlike this, BaCol PLA_2 was myotoxic.

Keywords: snake venom; *Bothrops asper*; acidic myotoxic phospholipase A_2; myotoxicity; edema

1. Introduction

Colombia occupies the third place in ophidian biodiversity. In 2015 there were 4,232 clinically confirmed cases of snakebites in the country, with an incidence of 9.1 cases per each 100,000 habitants [1]. Prior to the 16th epidemiologic week of 2016, 1,313 cases were registered with an average of 82.1 snakebite per week. Approximately 94.6% of the snakebites are caused by snakes of the genus Bothrops [1].

Currently, it is known that *B. asper* venom contains at least eight protein families in which phospholipases A_2 stand out, these are enzymes that hydrolyze phospholipids on the sn-2 position, liberating fatty acids and forming a lysophospholipids [2]. They have evidenced diverse biological

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activities such as effects on coagulation, platelets aggregation, miotoxicity, edema, neurotoxicity, cardiotoxicity, hemolysis, antibacterial and others [3].

Based on the amino acid sequence and the pattern of the disulfide bonds, the PLA_2 of snake venoms have been classified in two groups: PLA_2 Group I, found in venoms of Elapidae and Hydrophidae families, and PLA_2 Group II, from the Viperidae family [4]. Likewise, Viperidae PLA_2s are divided in two subgroups: the Asp49 which includes catalytically active isoforms, and the Lys49, which does not show such enzymatic activity [5]. Further, Asp 49 display a wide range of isoelectric point, from acidic to basic. The acidic isoforms usually have a higher catalytic activity than basic PLA_2s, however induce less toxic effects [6- 7].

Snakebite caused by *B. asper* sometimes can lead to sequels in approximately 6- 10% of the cases [1-8]. The most important is loss of muscular mass or limb amputation [8], produced by the myonecrosis, dermonecrosis and severe edema that induced ischemia. The main toxins implicated in these effects are snake venom metalloproteinases and PLA_2s [5- 9].

Isolation and characterization of the venom components has been being constituted as an essential tool to understand the events that unfold during envenomation. Acidic PLA_2s from snake venoms have not been completely studied and many of the ones that have been characterized, have been described as non-myotoxic and as inductors of other pharmacological effects, such as platelet aggregation inhibition and hypotension [10]. This paper describes the isolation and characterization of a myotoxic acidic PLA_2 from *B. asper* venom, it was named BaCol PLA_2.

2. Results

2.1 Isolation, determination of molecular mass, sequencing and modeling of BaCol PLA_2

Fractionation of *B. asper* venom by RP-HPLC resulted in mayor 16 peaks (Fig. 1A), these were collected and evaluated in PLA_2 activity. Peak 6 with retention time of 67.48 min showing high phospholipase activity. When submitted to a purity gradient, this peak eluted as a single symmetric peak. SDS-PAGE of the purified protein under reducing conditions showing a single band migrating at ~14.5 kDa (Fig. 1B), and its isoelectric point (pI) was 4.4 (Fig. 1C). Molecular mass obtained by ESI-Q-ToF was 14180.69 Da (Fig. 2A and 2B). This protein was named BaCol PLA_2.

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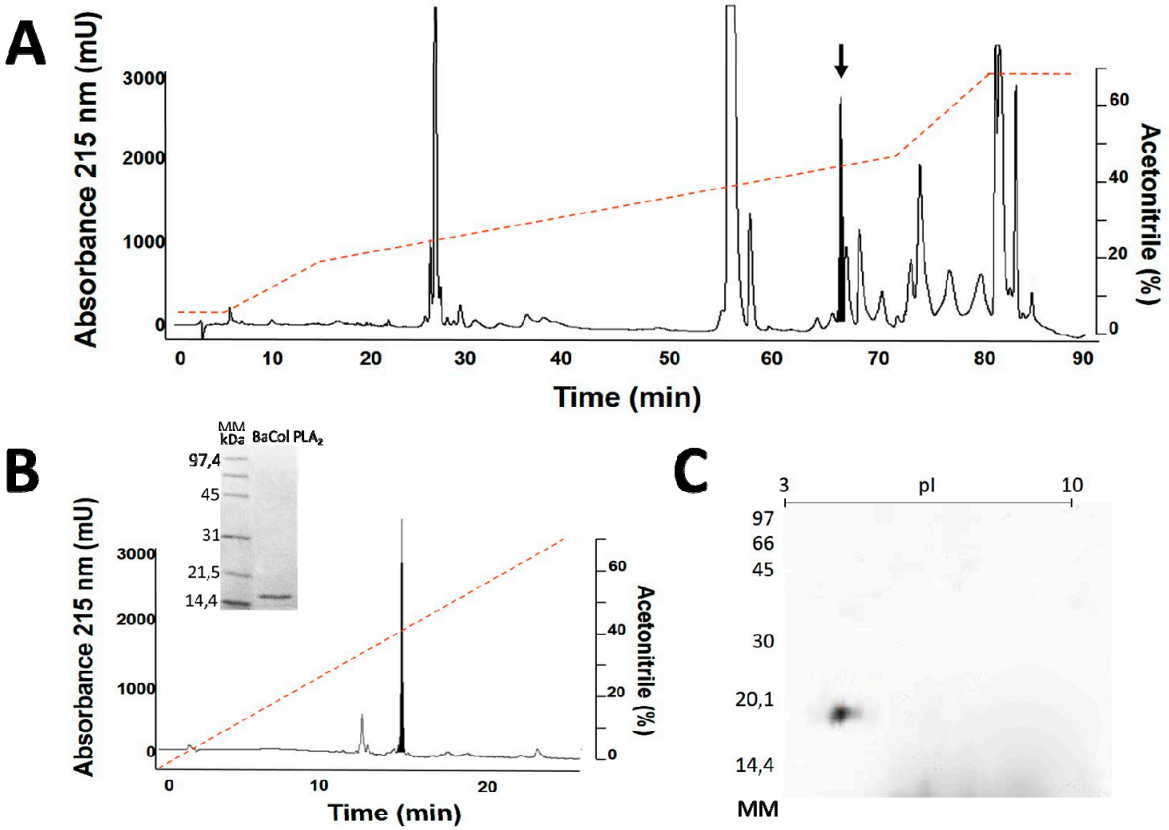

Figure 1. Isolation of BaCol PLA2. (A) Elution profile of crude *B. asper* venom by RP-HPLC on a Resteck C18 semipreparative column. (B) The highlighted in black fraction showed high phospholipase activity and was subjected purity gradient SDS-PAGE (12%) analysis of BaCol PLA2 under reduced. MW: low molecular weight markers, as indicated at the left, in kDa (C) Isoelectrofocusing in polyacrylamide 10% gel; pI. MM: isoelectric point markers, indicated at the right.

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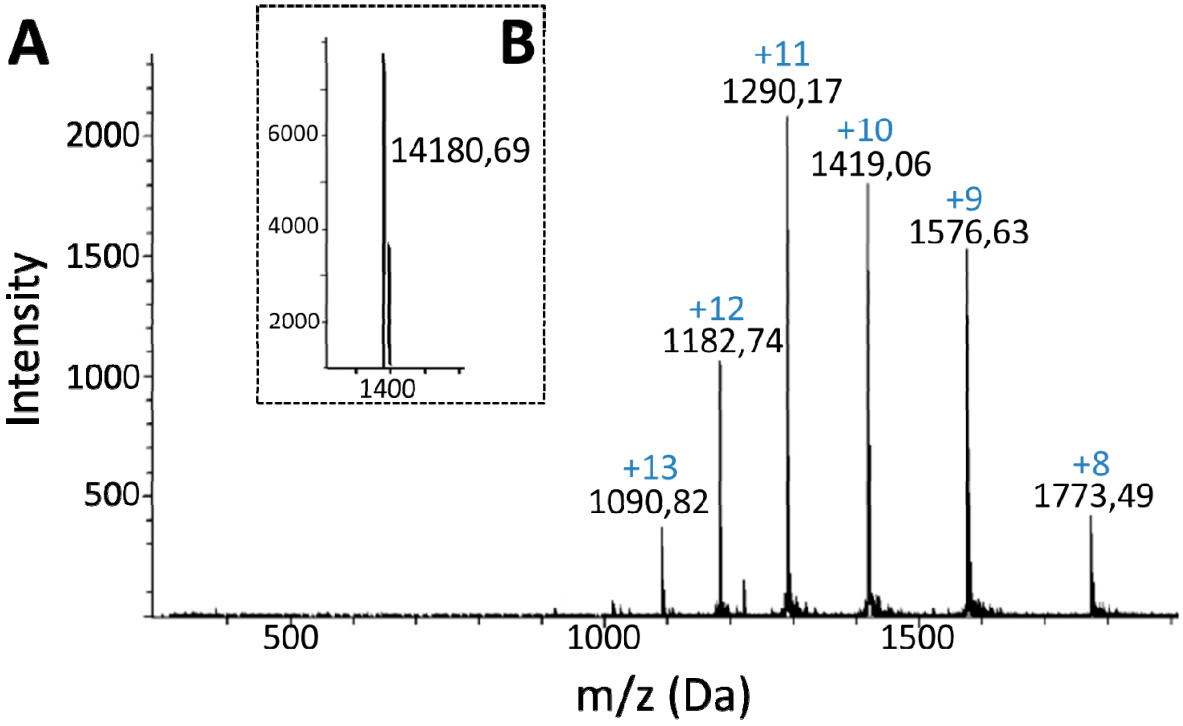

Figure 2. Molecular mass analyses of BaCol PLA2. (A) Multi-charge mode, as described in Materials and Methods. The inset (B) shows the deconvolution of the multi-charged ion series shown in (A)

N-terminal extreme obtained by Edman's degradation evidenced 25 amino acids. This sequence was used to design the primers to get the cDNA of *B. asper* venomous gland RNAm. The toxin cDNA codes for a polypeptide of 124 aminoacids (Fig. 3), with the presence of Asp at position 49 of catalytic dyad (according to numbering Renetseder, et al 1985 [11]), and theoretical pI of 4.5. The N- terminal was consistent with that obtained from cDNA (Fig. 3). The sequence of DNA has an accession number MF319968.

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Figure 3. cDNA and translated amino acid sequence of BaCol PLA2. The N-terminal sequence determined by direct protein Edman's degradation, up to the 25 residue, is underlined.

Aligning of the aminoacids sequence with other PLA2s is shown in figure 4. There was a high percentage of identity with a Costa Rican *B. asper* PLA2 named BaPLA2-II (91%) and with one of *B. jararaca* named BJPLA2 (83%), both 124 amino acids. At the same time, an 81% of identity was found with a 138 amino acids (adding the signal peptide) PLA2 obtained from *B. insularis* venom named BinTX-I and an 81% of identity with a 122 amino acids PLA2 from *B. pirajai* named BpirPLA2-I. Lower identity percentages (78%, 77% and 74%) were found with *B. jaracussu* (Bth-A-I-PLA2), *B. moojeni* (Bmoo-PLA2) and *B. alternatus*, respectively. All of them are acidic PLA2.

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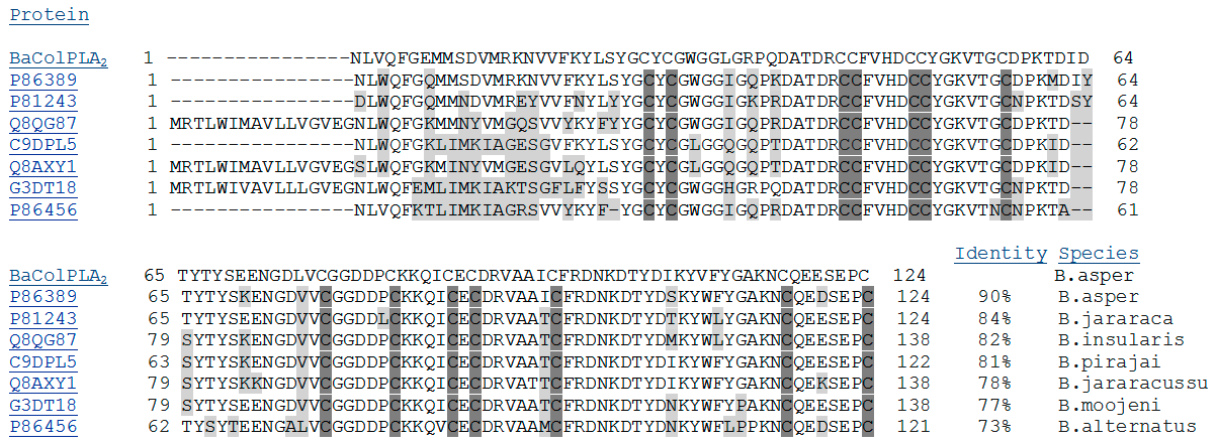

Figure 4. Multiple sequence alignment BaCol PLA2 with PLA2 isolated of Bothrops snake venoms. Protein access codes are indicated in the first column. Third column indicates the number of aminoacids. Fourth column indicates the percent identity values in comparison to BaCol PLA2 and last column indicates the species. Cysteine residues are highlighted with a dark gray background. Different positions are shaded in light gray.

The homology modeling process displayed a 3D-structure with overall characteristics of venom PLA2s, i.e., a calcium binding loop, two antiparallel helixes, a beta-wing and a C-terminal loop (Fig. 5). The reliability of the structure obtained by this approach was assayed. The quality of the homology model was evaluated by Procheck, the detailed residue-by-residue stereochemical quality of the BaCol PLA2 model was found to be good (91.5% in most favored regions, 7.5% in additional allowed regions, in generously allowed regions 1% (Arg14) and % in disallowed regions.

The Verify 3D program was used to determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D). This software considers a good score when at least 80% of the amino acids have scored equal or higher than 0.2 in the 3D/1D profile, in our case, 87.10% of the residues obtained scores that were above this value. The scores were between 0.05 and 0.53. The energetic architecture of protein folds was determined by using the program ProSA. This analysis of the model revealed a Z-score value of -5.2 and it is in the range of native conformations of the template (-4.49) (Data not shown). The energy profile of the BaCol PLA2 predicted model was found to be good.

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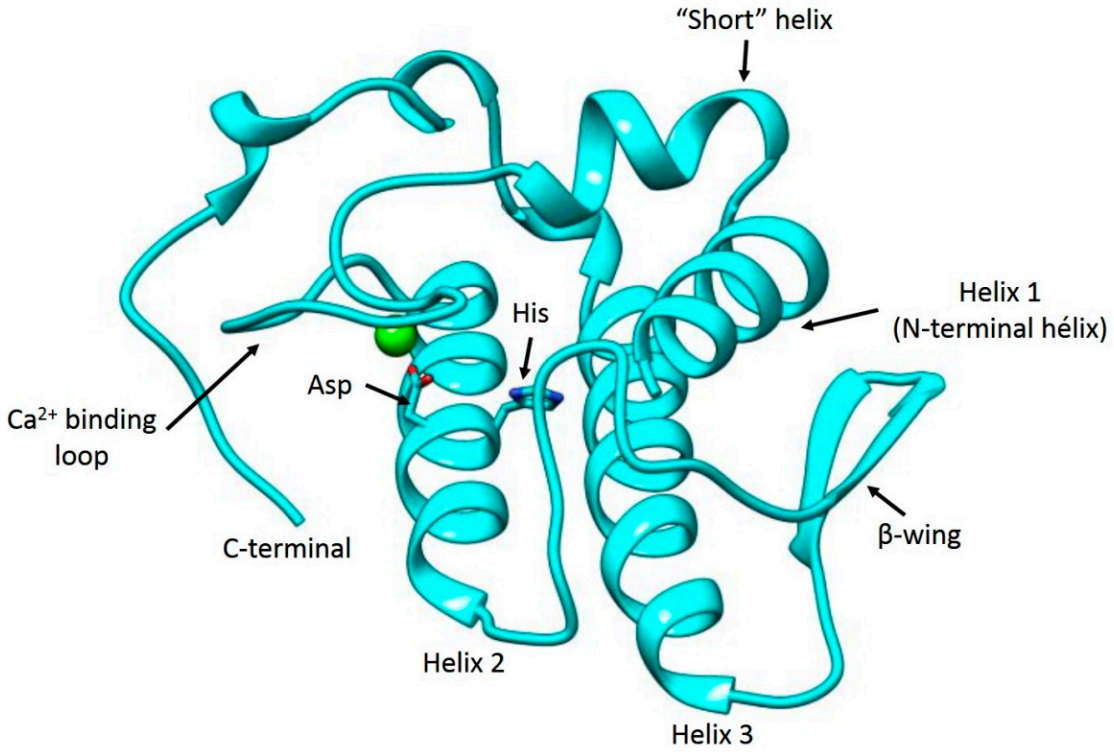

Figure 5. Three-dimensional model of BaCol PLA2. The active site residues of the enzyme are shown in sticks and Ca^{2+} is shown as a light green sphere.

2.2 Biological activities

BaCol PLA2 did not have significant difference against the complete venom in its hemolytic activity, generating a hemolysis halo of 26 mm, compared to a 22 mm halo of whole venom (Fig. 6A) ($p>0.05$). Additionally, BaCol PLA2 hydrolyzed 4-NOBA substrate. However, non-significant difference was observed in this activity in relation to it induced by whole venom. (Fig. 6B) ($p>0.05$).

The toxin had a moderate effect on human plasma anticoagulant activity in vitro. The means for clot formation in plasma incubated with this enzyme was 446 ± 30.8 seconds, while plasma incubated with PBS coagulated to 208 ± 30.2 seconds ($p = 0.0007$).

BaCol PLA2 also induced footpad edema. Concentration of 5 and 20 µg induced percentage higher to 30% and 60% respectively, 30 min after toxin inoculation. The maximum edema was observed at two hours (Fig 6C) ($p<0.0001$), demonstrating the ability of the PLA2 to increase vascular permeability and hence the pass of liquid to the interstitial space.

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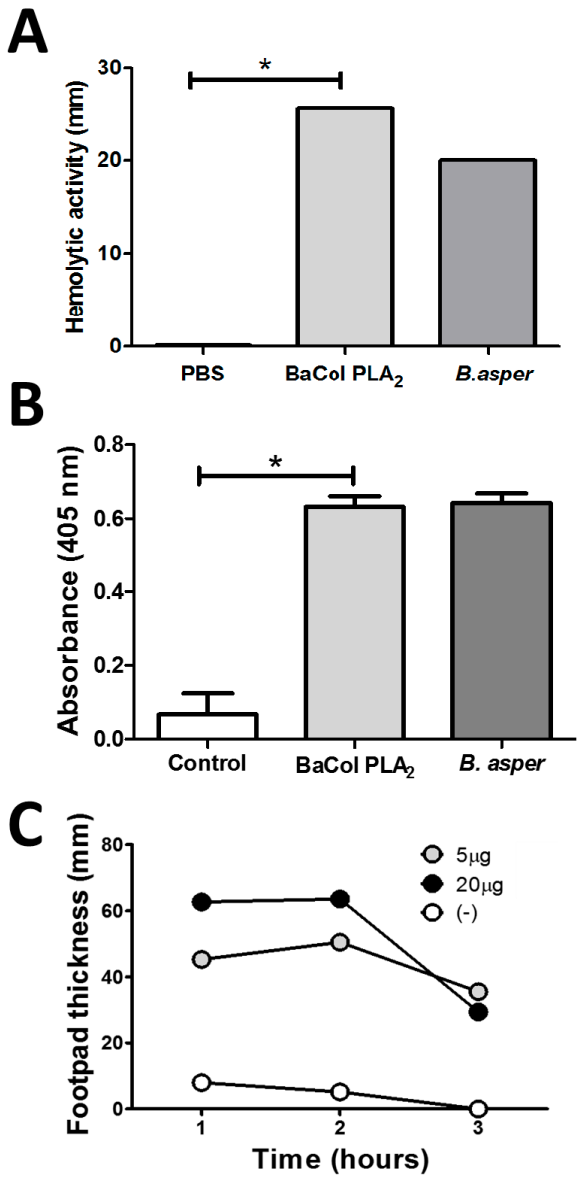

Figure 6. BaCol PLA2 phospholipase activity. (A) Indirect hemolysis test using human erythrocytes and egg yolk as substrate. (B) Hydrolysis of the substrate test using 4-nitro-3-octanoyloxy-benzoic acid as substrate. (C) Percentage of edema induced by BaCol PLA2. Negative control (white circle). BaCol PLA2 effect over the footpad at a 5 ug/100 uL concentration (gray circle). BaCol PLA2 effect over the footpad at a 20 ug/100 uL concentration (black circle).

BaCol PLA2 induced a moderate myonecrosis, evidenced by the increased of plasma CK activity after three hours of toxin injection (50 µg/mouse) in relation with control group ($p<0.05$) (Fig 7A). Additionally, the histopathological evaluation of tissue with hematoxylin and eosin staining, and analysis with Image J software to necrosis areas quantify evidenced differences significant statistically ($p<0.05$) in the damage induced by BACol PLA2 in relation with control group. (Fig 7B), where this last evidenced an incipient myositis (Fig 7C), while BACol PLA2 induced severe and diffuse damage to the muscular fibers, further vacuolization and hyalinization necrosis of the sarcoplasm (Fig 7D).

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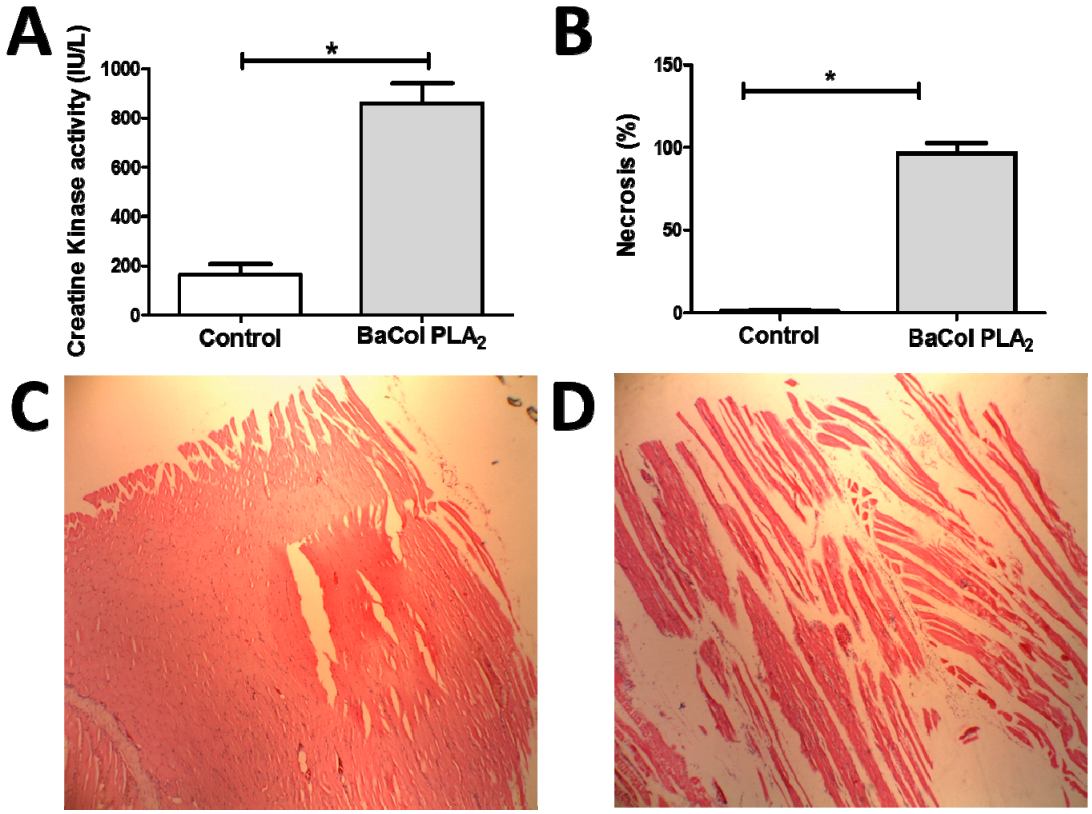

Figure 7. In vivo effects of BaCol PLA2. (A) CK meassurements at 3 hours post BaCol PLA2 vs Physiological Saline Solution inoculation. (B) Percentage of necrosis. Meassurements made to the histological plates with Image J software (C). Necrosis evaluated by histopathology. Gastrocnemiuos muscle. Hematoxylin and Eosin staining. 10X. Control group animals, inoculated with saline solution. (D) Necrosis evaluated by histopathology. Gastrocnemiuos muscle. Hematoxylin and Eosin staining. 10X. Animals from the group inoculated with BaCol PLA2.

In vitro the effect of BaCol PLA2 on cellular line U937 included a high (82%) cellular membrane damage, evidenced by high intensity fluorescence for PI in the cells treated with BaCol PLA2 in a concentration of 1,6 ug/uL, indicating cell dead by damage to the cytoplasmic membrane (figure 8A, 8B and 8C), in comparison to the negative control group (15%), which had high intensity fluorescence for DIOC6 and negative for PI, proper of alive cells. At a concentration of 0.16 ug/uL, plasma membrane damage was of approximately 22% (Figures 8D and 8F) was observed; additionally, in this concentration, for the fikoeritrin annexin V conjugate marking, evidenced the low annexin marking indicated that there was no phosphatidylserine externalization, nor early apoptosis. This way, the death mechanism induced by BaCol PLA2 was more compatible with necrosis (figure 8D), annexin V control without BaCol PLA2 (Fig 8E).

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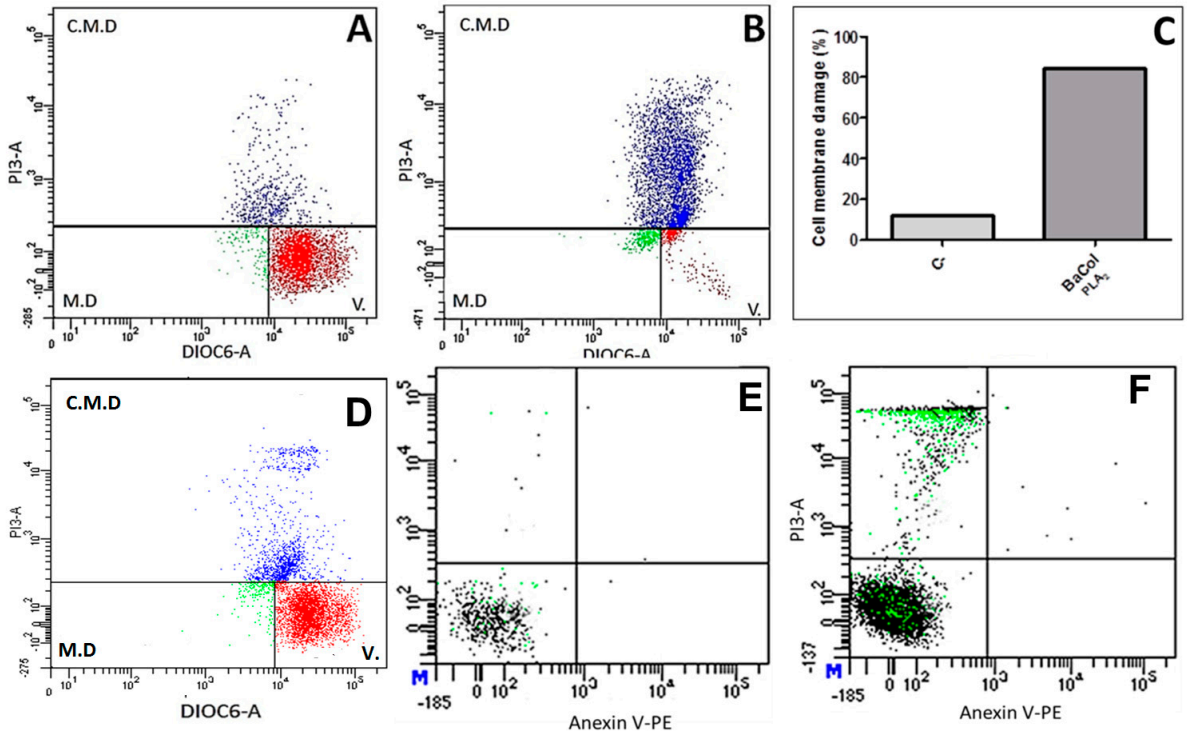

Figure 8. BaCol PLA2 effect over the lymphocytes U937. (A) Negative control. Cells without BaCol PLA2 treatment 85% viable cells. (B) Cells treated with BaCol PLA2. 82% of cell membrane damage. (C) Percentage of cell membrane damage induced by BaCol PLA2 versus the negative control. D) Percentage of cell membrane damage by BaCol PLA2 at a concentration of 0.16ug/uL. (E) Negative control for Annexin V conjugated with fikoeritrin marking. (F) Cells treated with BaCol PLA2 at a concentration of 0.16 ug/uL. There was not phosphatidylserine exposure that would indicate an early apoptosis process.

3. Discussion

Many of acidic PLA2 isolated from snake venoms seem to be devoid of pharmacological activities or toxicity including myotoxicity [7-12-13]. This study presents structural and functional characteristics of a new acidic myotoxic Asp 49 phospholipase A2 isolated from Colombia *B. asper* venom. The toxin was obtained by one chromatographic step by HPLC and was named BaCol PLA2. BaCol PLA2 presented a molecular weight of 14,180.69 Da and with a PI of 4.4. The complete sequence obtained from *B. asper* gland cDNA, indicated that it is different from other acidic PLA2 previously reported of this same species [7]. BaCol PLA2 contained Asp at position 48 (position 49 is the catalytic diad, according to Renetseder et al. , 1985 [11]) numeration, being catalogued as an Asp49 PLA2. We used the homology modeling to suggest a three dimensional structure of BaCol PLA2. The methodology used suggested that BaCol PLA2 three-dimensional model is a reliable model, since the stereochemical quality, the compatibility of the 3D structure with its amino acid sequence and the energy profile of the generated model were similar of those described for other acidic PLA2s [12]. In

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addition, our model evidenced the presence of the residues involved in calcium binding (Tyr28, Gly30, Gly32, His48 and Asp49) and in the catalytic net (His48, Asp99, Tyr52, and Tyr73) [11]. At the same time, it was concluded that BaCol PLA2 has highly conserved amino acids such as fourteen cysteines, involved in the formation involved in seven disulfide bonds. Similar findings were obtained for other acidic PLA2 using the homology modeling method [7, 12].

To sum up, the stereochemical quality, the compatibility of the 3D structure with its amino acid sequence and the energy profile of the BaCol PLA2 three-dimensional model confirm that this is a reliable model. The modeled structure of BaCol PLA2 was similar to those previously described.

When toxin was test in their myotoxic effect, the PLA2 evidenced an increased creatine kinase activity correlated with a severe and diffuse damage to the muscular fibers evidenced by vacuolization and hyalinization necrosis in the hispotaphological analysis.

The myotoxic effect has not been a constant characteristic in most acidic PLA2 isolated from Bothrops snakes. Cogo and collaborators, 2006 [10] reported the BinTX-I, isolated from *B. insularis* venom; Rodrígues and collaborators, 2007 [14] reported a PLA2 isolated from *B. pauloensis* and Santos Filho and collaborators, 2008 [15] reported another PLA2 isolated from *B. moojeni*, all three with myotoxic inductive characteristics. This was not the same for the PLA2s isolated from *B. jararaca* [16], Costa Rican *B. asper* [7], *B. pirajai* [12] and *B. moojeni* [17], which did not caused myotoxicity.

It is interesting to point out that the higher percentage of identity of BaCol PLA2 was with Costa Rican BaspPLA2-II (91%); indicating that variations in some residues amino acids could play an important role in the induction of myonecrosis.

BaCol PLA2 was characterized as an edema inductive source, in a dosage depending way and reaching its highest peak after two hours (50.5% and 63.6% with 5 and 20 ug respectively), agree with other acidic PLA2 with whom BaCol PLA2 had greater percentage of identity, [6-7-10-12-17]. Additionally, with *B. pauloensis* [14] and from *B. moojeni* [15] PLA2s, which it was only had been reported the N-Terminal, and were not included in the lining.

BaCol PLA2 caused an increase in CK values at three hours post-inoculation and this was consistent with the histopathological findings which showed severe and diffuse damage in the muscular fibers, evidenced by vacuolization and hyalinization necrosis of the sarcoplasm. Similar results were found with BmooTX-I obtained from *B. moojeni*, in which extensive cellular destruction and abundant leukocitary infiltrate were described and, further displaying contracted and clumped fibers in different stages of degeneration [15]. Bp-PLA2 from *B. pauloensis* [14], whose myotoxicity was confirmed by the increase in the CK activity was similar to induced by whole venom. In the same way, BinTX-I from *B. insularis*, also increased CK activity. Nevertheless, morphological analysis, showed BinTX-I produced less damaged proportion of fibers than the venom [10].

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BaCol PLA2 further, evidenced prominent damage to the cell membrane of U937 line lymphocytes, in different concentrations, from 0.016 (data not shown) to 1.6 ug/uL of toxin in 24 hours. Santos Filho et.al., 2008 [15], demonstrated that BmooTX-I was able to damage plasmatic membrane formed by some types of phospholipids, mainly phosphatidylcholine, which were hydrolyzed by BmooTX-I producing free fatty acids and lysophospholipids, causing cellular damage. By the contrary, Bl-PLA2 not evidenced cell viability damage on human peripheral blood mononuclear cells [18]. In the same way, BaspPLA2-II did not caused damage on cell membranes of skeletal C2C12 myoblasts in culture [7].

Experiments with annexin V and propidium iodide on U937 not showed early apoptosis, evidenced by no exposition of phosphatidylserine, indicating that death cellular was by necrosis. Mora and collaborators, 2014 [19] reported that a basic Lys 49 PLA2 from Costa Rican *B. asper* induced apoptosis with toxin levels of 5-25 ug/mL, while necrosis was observed with doses of 50 ug/mL. However, our results did not evidenced differences between different doses used (from 0.16 µg/µL to 1.6 µg/µl).

The assays showed moderated anticoagulant activity of BaCol PLA2 (446 ± 30.79 seconds, p = 0.0007). This effect had not been evaluated to other acidic PLA2, except to BaPLA2-II described by Fernández et. al. 2010 [7] who obtained negative results for anticoagulant activity. However, when compared to the basic anticoagulant activity of basic PLA2s, the weak capacity of BaCol PLA2 was evidenced. This could be explained by was proposed by Kini et. al. , 2005 [20] regarding a possible anticoagulant site which is probably between residues 54 and 77, this region is positively charged in in the PLA2s with high anticoagulant activity contrary to PLA2 with moderate or low anticoagulant activity, which have a predominance of negative or neutral charges in this region, which is in accordance with the presence of three aspartic acid and two glutamic acid in this segment for BaCol PLA2.

In Colombia there are few studies for *B. asper* acidic myotoxic PLA2 and therefore it is necessary to deepen in order to understand better its action mechanism and its relationship with the effects induced by venom.

4. Materials and Methods

4.1. Venoms and isolation of toxins

Whole *B. asper* venom from middle Magdalena in Antioquia was donated by Antioquia University Serpentarium. The venom pool was obtained by manual milking of 30 specimen, keeping in captivity. The venom was centrifuged, lyophilized and frozen at -70 ˚C upon use.

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4.2 Animals

Swiss-Webster mice were used for all in vivo experiments were supplied by Animal House - SIU, (Sede de Investigacion Universitaria, Universidad de Antioquia) and maintained under standard conditions with diet and water *ad libitum*. The experimentation protocol was approved by the committees for use and care of research animals from Universidad de Antioquia, License number 70, 2011, and modified in the license number 102, 2016.

4.3 Isolation of BaCol PLA2

Venom (10 mg) were dissolved in 200 µL of water/trifluoroacetic acid (TFA) at 0.1% (solvent A), and evaluated by reverse-phase high performance liquid chromatography (RP-HPLC) on a C18 semipreparative column (250 × 10.0 mm, 5 µm particle; Restek), using a Shimadzu Prominence-20A Chromatograph. Elution was performed at 2 mL/min by applying solution B (acetonitrile, containing TFA 0.1%) as follows: 5% B for 5 min, 15% B over 15 min, 45% over 75 min, 70% B over 85 min, 90 min 70%. The elution profile was monitored at 215 nm in a UV/VIS photodiode array detector (Shimadzu). The peaks were collected and evaluated by SDS PAGE 12% and peak with bands around 15 kDa were posteriorly proved in their hemolytic activity. Later the fraction was analyzed through a purity gradient 0-100% on an analytical C18 column.

Electrophoretic homogeneity of BaCol PLA2 was evaluated by SDS-PAGE, 20 µg of protein was loaded onto a 12% gel, and run in a Mini-Protean Tetra® electrophoresis system (Bio-Rad) at 150 v. Proteins were visualized by Coomassie blue R-250 staining [21].

4.4 Molecular mass and N- terminal determination

To evaluate isolated protein's purity and molecular mass, an ESI-Q-ToF type mass spectrometry equipment, MicrQ-TOF III model, was used (Bruker Daltonics), coupled to a LC-20AT liquid chromatographer (Shimadzu). Two mobile phases were used: Water (A) and acetonitrile (B), both in presence of 0.1% formic acid (v/v). Chromatographic separation was performed by a C18 reverse phase column (4.5 mm x 100 mm, 1.8 um). The elution conditions were optimized on a 0 to 85% linear gradient B solvent for 60 min, on a 0.2 mL/min flow. Sample's column and automatic applicator were held at 25 °C and 10 °C, respectively. Mass spectrometer acted with 4.5 kV with 180 °C solvation temperature, positive mode on an ionization interval between 100 m/z and 3000 m/z, nitrogen flow of 6 L/min and 0.8 bar pressure. Data were processed using Bruker Data Analysis software (version 3.3).

Determination of N-terminal was done by a Shimadzu protein automatic sequencer (PPSQ-23A model). A solution with approximately 1 mg/mL of the sample was applied into the sequencer, being the sequence determined by the Edman degradation method [22]. After, N-terminal determination was analyzed by lining it with other snake venom PLA2s using BLAST [23] and MultAlin programs [24].

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4.5 cDNA and nucleotide sequencing

A venomous gland of a dead specimen of an adult *B. asper* of the serpentarium of the Antioquia University was used to obtain the complete sequence of the toxin. The gland total RNA was extracted with QIAzol® following manufacturer´s instructions. The obtained RNA was submitted to a retrotranscription reaction using the Superscript III enzyme, Invitrogen®, and following the indications of the manufacturer. The mRNA was transformed into cDNA using a dNTP mix and specifically designed N-terminal based primers: External primer 5'GTTTGGCCAGATGATGAGCG3' and the internal primer 5'GGCGATGATCCGTGCAAAAA3'. The cDNA was cloned on the cloning vector pGEM-T Easy (Promega) and DH5-α *E. coli* were transformed. PCR was performed to detect the presence of the vector with the toxin's sequence in the colonies. From these transformed colonies the plasmid was obtained, using the construct and employing the QIA prep® Spin Miniprep kit (Qiagen). The product of this extraction was sequenced (Macrogen), specifying that it corresponded to whole plasmids with the gen inserted in the multiple cloning site, to be sequenced from the vector T7 and SP6 promoters. DNA Sequence data were analyzed and translated to amino acid sequence with the Mega 6 software [25]. Protein sequence homology in the Swiss-Prot database was searched using FASTA3 [26], and sequence alignments were generated with CLUSTAL W 2.1. The theoretical isoelectric point was calculated with ExPASy [27].

4.6 Bidimensional electrophoresis to evaluate isoelectric point

To make the first dimension, 30 µg of the BaCol PLA_2 were submitted to a 7 cm long Immobilized pH Gradients tape with pH of 3 to 10. Rehydration of the stripe happened overnight at room temperature, in the presence of 125 µL of the rehydration solution that contains urea at 8M, CHAPS 2% (m/v), 1% of Inmobilized pH gradient (IPG) tampon solution, DithioThreitol (DDT) at 19 nM and bromophenol blue. The isoelectric focusing happened using three steps: 1st step: 500 V for 30 min, 2nd step: 1000 V for 30 min, and 3rd step: 5000 V for 2 hours. After that, the stripe was balanced for 20 min in presence of DDT at 19 mM, Trisat 50 mM, urea at 6 M, 30% glycerol (v/v) and 2% SDS (m/v) and later for 20 min more in the same solution, for the exception of the substitution of DDT for iodioacetamide at 0.2 M. For the second dimension, the isoelectric focusing stripe was set at the top of a 10% (m/v) polyacrylamide gel of the next dimensions 10 x 10 cm, 1.5 mm thick. Electrophoresis was performed at room temperature, using three steps: 10 mA per gel for 15 min and then 20 mA per gel for 1 hour. The protein was seen by Coomassie Brilliant Blue (CBB) coloration. The biodimensional gel was digitalized using an ImageScanner III (GE Healthcare Life Sciences) scanner on transmission mode. On the other hand, the image was analyzed through Image Master 2D Platinum v 7.05 (GE Healthcare) software to obtain the isoelectric point.

4.7 Molecular modeling

The NCBI Basic Local Alignment Search Tool (BLAST), for the sequence similarities was used for searching the crystal structures of the closest homologues available in the Brookhaven Protein

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Data Bank (PDB). The results yielded by NCBI BLAST revealed chain X of PLA_2 from *Bothrops jararacussu* (PDB ID: 1WMV_X) with a resolution of 1.79 Å as a suitable template and with an identity score of 78%, an E value 7 x10-67 and coverage of 100%. The three-dimensional model of BaCol PLA_2s was performed using the program Modeller (9.17) [28, 29]. This program is completely automated and is capable of generating energy minimized protein models by satisfying spatial restraints on bond distances and dihedral angles extracted from the template PDB file. Modeller performs an automatic loop modeling and model optimization. Many runs of Modeller were carried out in order to obtain the most plausible model. The stereochemical excellence of the protein structure and overall structural geometry were confirmed by Procheck program [30]. The energy of residues was checked by ProSA, using the web service ProSA-web [31, 32]. The Verify 3D program was used to determine the compatibility of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class on the basis of its location and environment (alpha, beta, loop, polar, non-polar, etc.) as well as by comparing the results with good database structures [33].

4.8 PLA_2 Activity

Phospholipase activity was evaluated in vitro using two methods: by indirect hemolysis on agar gel containing human erythrocytes and egg yolk, as previously described Gutiérrez et al., 1986 [34]; and the method described by Cho and Kézdy, 1991 [35] and Holzer and Mackessy, 1996 [36], modified for 96 well plates. In the last, the standard assay mix contained 200 µL of buffer (10 mM Tris–HCl, 10 mM CaCl2, 100 mM NaCl, pH 8.0), 20 µL of substrate 4-NOBA (4-nitro-3-octanoyloxy-benzoic acid), at different concentrations 20 µL of water and 20 µL of PLA_2 (1 µg/ µL) in a final volume of 260 µL. After the addition of the BaCol PLA_2 (20 µg), the mix was incubated at 37 °C for 60 min, and the absorbance was determined at 405 nm (Awareness, Stat Fax 3200). All the tests were conducted in triplicate. Crude venom from *B. asper* and substrate 4-NOBA, were used as positive and negative controls, respectively.

4.9 Edematogenic activity

Doses of 5 and 20 µg of BaCol PLA_2 diluted in 50 µL of phosphate buffered saline (PBS) was injected subcutaneously, in the foodpad of the right paw, to two groups of four mice. 50 µL of PBS were injected on the right hind limb to one control group of four mice. Footpad measurement in millimeter was conducted after 1, 2 and 3 h with a caliper. Edema was expressed as a thickness percentage increase on the right hind limb footpad compared to the control group [37]. The experiments were conducted in duplicate.

4.10 Myotoxic activity

A group of four mice was inoculated on the right gastrocnemius muscle with 50 µg of BaCol PLA_2 diluted in 100 µL of physiologic saline solution. After 3 hours, a 70 µL blood sample, was collected from the tail vein in heparinized capillaries. The plasma were separated and used to quantify the activity of the creatine kinase (CK) (CK-NAC UV, Wiener Lab®) following

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manufacturer's instructions and absorbance was measured at 280 nm [38]. Gastrocnemius muscle dissection was performed, and a fragment was taken for histopathological examination with hematoxylin and eosin staining to evaluate myonecrosis and signs of inflammation such as edema, congestion, and leukocyte infiltrate. For negative control, a group of four mice received 100 µL of physiological saline solution [39].

4.11 Anticoagulant activity

The anticoagulant activity of BaCol PLA_2 venoms was tested by preincubating 20 µg (in 50 µL PBS) with 500 µL of citrated human plasma for 10 min at 37 °C, in duplicates. Clotting times were recorded after adding 100 µL of 0.25 M CaCl2. Plasma aliquots preincubated with PBS were used as controls [34].

4.12 Cell viability and type of cellular death

U937 cellular line was used to evaluate alterations on the mitochondrial permeability transition (MPT) and damage to the cell membrane. The cells were suspended on a RPMI 1640 medium with 10% fetal bovine serum in a concentration of 300.000 cells/well (total volume per well was 300 uL). The cells were incubated at 37°C during 24 hours. $DIOC_6$ and propidium iodide (PI) dyes were used to determine MPT and cell membrane damage, respectively, by flow cytometry (FACSCanto II). The cells that presented high intensity fluorescence for $DIOC_6$ and negative for PI were considered as alive, whereas, cells with high intensity fluorescence for PI were considered as dead due to damage to the cytoplasmic membrane. Additionally, to determine the type of cellular death induced by PLA_2, cells were suspended in a medium containing propidium iodide (PI) and annexin V (Invitrogen, Carlsbad, CA, USA). Cells were considered as apoptotic when shown positive marking for annexin V and negative for PI; non apoptotic cells were the ones with negative marking for both; cells in early stages of apoptosis were positive for annexin V and negative for PI and necrotic cells were double positive.

4.13 Statistical analysis

Descriptive statistic of each group is expressed as mean. The significance differences between experimental groups where assessed with t- test and one way ANOVA, where a P value <0.05 was considered significant.

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Author Contributions: Silvia Posada Arias performed the isolation and characterization of PLA_2 by HPLC; SDS-PAGE; phospholipase activity; edematizing activity; myotoxic activity; anticoagulant activity; cell viability; sequencing; analysis and discussion of the information; writing and organization of the article; Paola Rey Suárez performed HPLC, SDS-PAGE; phospholipase activity, myotoxic activity, anticoagulant activity, figures in the article; Andrés Pereáñez Jiménez participated in the experiments of phospholipase activity and performed the molecular modeling of the protein; Cristian Acosta performed experiments of cDNA, sequencing (primer design, PCR, cloning in E. coli; alignment); Mauricio Rojas designed and performed the experiments of flow cytometry; cell viability testing; evidence of cell death mechanism; Luzilene Delazari Dos Santos performed 2D electrophoresis and mass spectrometry. Rui Seabra Ferrerira Junior determinated the N-terminal by Edman degradation. Vitelbina Núñez Rangel participated in the experiments of edematizing activity; phospholipase activity, myotoxic activity; anticoagulant activity. All authors contributed with the discussion of results.

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