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Original Article

Inhibitory effects of *Swietenia macrophylla* on myotoxic phospholipases A_2

Jaime A. Pereañez[a,*], *Vitelbina Núñez*[a, b], *Benjamín Rojano*[c], *Tatiana Lobo-Echeverri*[c]

[a]*Programa de Ofidismo/Escorpionismo, Universidad de Antioquia (UdeA), Medellín, Colombia*
[b]*Escuela de Microbiología, Universidad de Antioquia (UdeA), Medellín, Colombia*
[c]*Grupo de Química de los Productos Naturales y los Alimentos, Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Sede Medellín, Colombia*

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ABSTRACT

Activity-guided fractionation of an ethanol-soluble extract of the leaves of *Swietenia macrophylla* King, Meliaceae, led to several fractions. As a result, sample Sm13-16, 23 had the most promising activity against phospholipases A2 (PLA_2), Asp49 and Lys49 types. This fraction inhibited PLA_2 activity of the Asp49 PLA_2, when aggregated substrate was used. On the other hand, this activity was weakly neutralized when monodispersed substrate was used. In addition, Sm13-16, 23 inhibited, in a dose dependent manner, the cytotoxicity, myotoxicity and edema induced by PLA_{2s}, as well as the anticoagulant activity of Asp49 PLA_2. Overall, this fraction exhibited a better inhibition of the toxic activities induced by the Lys49 PLA_2 than those caused by the Asp49 PLA_2. The spectral data of Sm13-16, 23 suggested the presence of aromatic compounds (UV λ_{max} (nm) 655, 266, and 219; IR λ_{max} KBr (cm^{-1}): ~ 3600-3000 (OH), 2923.07 and 1438.90 (C-H), 1656.69 (C = O), 1618.63 and 1607.67 (C-O), 1285.47-772.60). We suggest that phenolic compounds could interact and inhibit the toxins by several mechanisms. Further analysis of the compounds present in the active fraction could be a relevant contribution in the treatment of accidents caused by snake envenomation.

Introduction

Snakebites represent a relevant public health issue in many regions of the world, particularly in tropical and subtropical countries of Africa, Asia, Latin America and Oceania (Williams et al., 2010). The pathophysiological effects observed in ophidian bites are a combination of the action of several enzymes, non-enzymatic proteins and peptides, which include phospholipases A_2, hemorrhagic metalloproteases, serine proteases neurotoxins, cytotoxins, and cardiotoxins, among others (Markland, 1997). The most important and abundant muscle-damaging components in snake venoms are phospholipases A_2 (PLA_2; EC 3.1.1.4). These enzymes hydrolyze the *sn*-2 ester bond of glycerophospholipids, releasing a fatty acid and a lysophospholipid (Kini, 2003). In addition, PLA_2 can induce several pharmacological also effects such as edema, modulation of platelet aggregation, as well as neurotoxicity, myotoxicity and anticoagulation (Kini, 2003; Six and Dennis,

* *Corresponding author.*
E-mail: andres.pereanez@siu.udea.edu.co (J.A. Pereañez).

2000). Venom PLA_2 are classified into groups I or II, based on their sequence and the pairing of the disulphide groups. Group I PLA_2 are found in the venoms of Elapidae snakes, whereas group II PLA_2 are present in the venoms of Viperidae snakes (Six and Dennis, 2000). The group II is further divided into two main subgroups: Asp49 and Lys49 (PLA_2 homologues) variants. In the latter, the aspartic acid residue at position 49, critically involved in calcium binding and essential for catalytic activity, is replaced by lysine. Due to this and other critical substitutions, the Lys49 PLA_2 can not bind calcium efficiently and are considered to be enzymatically inactive (Arni and Ward, 1996; Lomonte et al., 2003). Although catalytic activity has been shown to play a role in the toxic actions of some venom PLA_2, it is not essential in the case of Lys49 PLA_2, which uses non-enzymatic mechanisms to alter membrane homeostasis (Lomonte et al., 2003).

The therapy for snakebite envenomations has been based on the intravenous administration of antivenoms (Bon, 1996). However, it has been demonstrated that antivenoms have a limited efficacy against the local tissue damaging activities of venoms (Gutiérrez et al., 1998). Thus, there is the need to search for additional approaches that may be useful complement to conventional antivenom therapy. In this way, the search for specific PLA_2 inhibitors could complement the traditional therapy of envenomings and, in addition, may contribute to finding new anti-inflammatory agents.

Medicinal plants represent a vital source of novel bioactive compounds with several pharmacological activities that have contributed directly in the search of alternatives against ophidian envenomation or as a complement to conventional antivenom therapy (Soares et al., 2005). The species *Swietenia macrophylla* King, Meliaceae, was selected as a promising lead after the bioassay-screening of several ethanolic plant extracts, because of its significant neutralizing activity against *Bothrops* asper venom and PLA_2 isolated from the this venom (Pereañez et al., 2010). *Swietenia* species are found in neotropics regions (Brown et al., 2003; Gullison et al., 1996). The tree can grow up to a height of 40-60 m and is native to tropical America, Mexico, South America, and India (Brown et al., 2003). Organic and aqueous extracts of *S. macrophylla* seeds possess a wide array of biological properties such as anti-diabetic (Maiti et al., 2007a), anti-diarrhoeal (Maiti et al., 2007b), anti-inflammatory, anti-mutagenic, anti-tumor-promoting (Amelia et al., 1996), antimicrobial, and antimalaric (Maiti et al., 2007c; Soediro et al., 1990). Additionally, its leaves yield essential oils which contain himachalene, germacrene D, germacrene A, cadina-1, 4-diene, hexadecanoic acid, and ethyl hexadecanoate (Soares et al., 2003). Due to the promising activity of *S. macrophylla* in the preliminary screening against *Bothrops asper* venom and PLA_2 (Pereañez et al., 2010), the aim of this study was to follow up, phytochemical and biological analysis through, the ability of fractions derived from this plant to inhibit myotoxic PLA_2.

Materials and methods

Venoms, toxins and animals

Venoms of *Crotalus durissus cumanensis* (Colombian rattlesnake), *Bothrops asper* and *Bothrops atrox* were obtained from Antioquia University Serpentarium, Colombia. Cdcum6, PLA_2 (CB) from the crotoxin complex (Asp49) and *B. atrox* myotoxin I (Lys49) were purified those previously described (Núñez et al., 2004; Pereañez et al., 2009). Doses used in each experiment were chosen in order to obtain comparable effects. Swiss Webster mice of 20-22 g body weight were utilized for determination of toxic activities, in accordance with guidelines of the Universidad de Antioquia Ethics Committee (Medellín, Colombia).

Plant material

One kg of *Swietenia macrophylla* King, Meliaceae, leaves were collected in tropical and premountain forests of Medellín, Colombia, 1600 m.s.l (6°15'41" N, 75°34'35.5" W). Voucher specimen (TL-103) identified by Leon Morales, was deposited in the Herbarium of the Universidad Nacional Gabriel Gutiérrez Villegas (MEDEL).

Extraction and bioassay-guided fractionation of Swietenia macrophylla

The dried and milled plant materials was extracted three times overnight with 90% ethanol. The resultant ethanol extract was concentrated at a temperature below 40°C to a semisolid paste using a rotary evaporator (Büchi-124 Flawil, Switzerland). The ethanol-soluble extract of leaves of *S. macrophylla* was submitted to an initial fractionation using an open silica gel column eluted with a gradient of *n*-hexane: dichloromethane: methanol, which resulted in 22 fractions. The fractionation was followed by the inhibition of the PLA_2 activity bioassay, from which the most active fractions were combined into one single sample labeled as Sm13-16. A successive fractionation of Sm13-16 was carried out by silica gel and a gradient of organic solvents, which from 30 sub-fractions were obtained. The inhibition of PLA_2 enzymatic activity induced by *B. asper* whole venom (at a 1:10 w/w (venom: fraction) ratio in egg yolk assay) was performed to each of the thirty subfractions and further inhibition assays of Cdcum6 (Asp49, enzymatic activity) and *B. atrox* myotoxin I (Lys49, cytotoxic activity) were performed only on the most active fractions. Sample Sm13-16, 23 was the most promising lead, so further characterization was carried out with this fraction. For all the inhibition assays, fractions were dissolved in 3% DMSO in PBS.

Characterization of the sample

The preliminary colorimetric phytochemical analysis was carried out on the sample Sm13-16, 23, according to the methods described by Trease (2002). Additionally, an infrared (IR) spectrum of Sm 13-16, 23 was taken in KBr, using a Fourier

Transform Infrared (FT-IR) (4000 - 400 cm) (Perkin-Elmer). The UV-Vis spectrum (200 - 800 nm) of the fraction was taken in methanol, using a UV-Vis Spectrophometer (UV-1700 PharmarSpec, Shimadzu).

Inhibition of PLA_2 activity

Egg yolk as substrate

PLA_2 activity was assayed according to the method reported by Dole (1956), with titration of free fatty acids released from egg yolk phospholipids suspended in 1% Triton X-100, 0.1 M Tris-HCl, 0.01 M $CaCl_2$, pH 8.5 buffer, using 20 µg of Cdcum6 (Asp49). The time of reaction was 15 min at 37°C. The amount of protein was selected from the linear region of dose-activity curves. For inhibition experiments, 1:0.25, 1:0.5, 1:1, 1:2, 1:5 and 1:10 w/w (toxin: fraction) ratios were mixed, and incubated for 30 min at 37°C before PLA_2 activity determination. The results were indicated as inhibition percentage, where 0 % is the activity induced by PLA_2 alone.

4-Nitro-3-octanoyloxy-benzoic acid (4N3OBA) as substrate

Measurements of enzymatic activity using the monodisperse substrate 4N3OBA were performed according to the method described by Holzer and Mackessy (1996) and adapted for a 96-well ELISA plate (Ponce-Soto et al., 2002). The standard assay contained 200 µl of buffer (10 mM Tris-HCl, 10 mM CaCl2, 100 mM NaCl, pH 8.0), 20 µl of 10 mM of substrate (4NO3BA), 20 µl of sample (PLA_2) and 20 µl of water. The negative control was only buffer. The inhibition effect of the fraction on PLA_2 activity was determined through co-incubation for 30 min at 37°C of the enzyme and fraction at w/w ratios 1:1, 1:2, 1:5 and 1:10, just before assaying the residual PLA_2 activity. After the incubation period, the sample was added to the assay and the reaction was monitored at 425 nm for 40 min (at 10 min intervals) at 37°C. The quantity of chromophore released (4-nitro-3-hydroxy benzoic acid) was proportional to the enzymatic activity and the initial velocity (V_o) was calculated considering the absorbance measured in 20 min.

Inhibition of cytotoxicity induced by PLA_2

Inhibition experiments of the cytotoxic activity of PLA_2 and PLA_2 homologue by the fraction were performed on murine skeletal muscle C2C12 myoblasts or myotubes (ATCC CRL-1772), as reported in the literature (Lomonte et al., 1999). Forty micrograms of each toxin were used in the assay. Inhibition experiments on *B. atrox* myotoxin I (Lys49) were performed on myoblasts, while inhibition assays on Cdcum6 (Asp49) were carried out on myotubes. The type of cells was chosen due to the higher susceptibility of these cells to the action of the toxins (Núñez et al., 2004; Pereañez et al., 2009). Different w/w ratios (toxin: fraction, 1:2, 1:5 and 1:10) in 150 µl of assay medium (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal calf serum), were incubated at 37°C for 30 min, and added to 96-well plates. After 3 h at 37°C, a supernatant aliquot was collected for determination of lactic dehydrogenase activity (LDH; EC 1.1.1.27) released from damaged cells, using a kinetic assay (Wiener LDH-P UV). Controls of 0 to 100% toxicity consisted of assay medium and toxins, respectively.

Inhibition of myotoxic activity

Inhibition of myotoxic activity in preincubation assays

Myotoxic of the PLA_2 activity was determining estimated by the plasma levels of creatine kinase (CK; EC2.7.3.2) in groups of three mice (18-20 g body weight), after an intramuscular injection (in the gastrocnemius) of 20 µg of *B. atrox* myotoxin I (Lys49) or 3 µg of Cdcum6 (Asp49), either alone, or preincubated with the fraction at 1:1, 1:2, 1:5 and 1:10 w/w (toxin : fraction) ratios for 30 min at 37°C (Pereañez et al., 2009). Control groups received an identical injection (100 µl) of PBS, pH 7.2, alone, or fraction alone. After 3 h, blood samples were collected from the tail into heparinized capillary tubes, and the plasma CK activity of plasma was determined by a kinetic assay (Weiner Lab, CK-NAC UV-AA). Controls of 0 to 100% toxicity consisted of PBS and toxins, respectively. Experiments were carried out in duplicate.

Inhibition of myotoxic activity by independent in situ administration of fraction

The ability of fraction to inhibit myotoxicity by independent *In situ* administration was evaluated. Mice received an i.m. myotoxin injection (20 µg Myotoxin I or 3 µg of Cdcum6). Thirty seconds later several amounts of the fraction (in order to obtain 1:10, 1:5 and 1:2 w/w toxin: fraction ratios) were injected at the same site. Control animals received DMSO 3% in PBS, or fraction alone (Pereañez et al., 2009). After 3 h, plasma creatine kinase activity was determined as described above. Results are shown in percentage of inhibition, taking the toxin and PBS injections 100% and 0% of activity, respectively.

Inhibition of edema-inducing activity

Groups of three mice (18-20 g) received a subcutaneous injection of 50 µl of PLA_2 (10 µg of Cdcum6 and 32 µg of *B. atrox* myotoxin I, equivalents to two *minimum edematogenic doses*) on the right footpad. The left footpad received 50 µl of DMSO 3% in PBS as control. Inhibition studies were performed by pre-incubating fraction with PLA_2 (in order to obtain 1:10 w/w ratios) for 30 min at 37°C. After injection, the progression of edema was evaluated with a caliper at intervals of 1, 2, 3, 6, 24 h and expressed in millimeters (Pereañez et al., 2009). Control animals received fraction alone.

Inhibition of anticoagulant activity

Ten micrograms of Cdcum6, in 10 µl PBS, were mixed with 90 µl of different fraction of amounts of fraction (in order to obtain 1:10, 1:5 and 1:2 w/w ratios) and pre-incubated for 30 min at 37°C. Afterwards, 100 µl of mixtures were added to 0.3 ml of plasma, and incubated for 10 min at 37°C. Plasma aliquots incubated with PBS (fraction) or toxin used was the negative and positive controls, respectively. Then, coagulation times were recorded after adding 0.1 ml of 0.25 M $CaCl_2$ (Gutiérrez et al., 1986).

Inhibition of proteolytic activity

In order to determine the specific inhibition of the fraction on PLA_2 induced activities, the capacity of the compounds to inhibit the proteolytic activity induced by Batx-I, (a metalloproteinase isolated from *Bothrops atrox* venom) (Patiño et al., 2010) and trypsin, was tested. Proteolytic activity was measured on azocasein (Sigma-Aldrich, St. Louis, MO) according to Wang et al. (2004), with some modifications. Briefly, 20 µg of proteases dissolved in 5 µl of 25 mM Tris (0.15 M NaCl, 5 mM $CaCl_2$), pH 7.4, were mixed with 200 µg and 100 µg of fraction (in order to obtain 1:10 and 1:5 w/w ratios, respectively) and pre-incubated for 30 min at 37°C. Afterwards, these solutions were incubated for 90 min at 37°C with 10 mg/ml of azocasein diluted in the same buffer. The reaction was stopped by the addition of 200 µl of trichloroacetic acid. Samples were then centrifuged at 360 g for 5 min. Supernatant (100 µl) was mixed with the equal volume of 0.5 M NaOH, and the absorbances were measured at 450 nm. Results are shown in percentage of activity (absorbance at 450 nm). The assay was carried out in duplicate.

Polyacrylamide gel electrophoresis (SDSPAGE)

SDS-PAGE was performed on 15% polyacrylamide gels (Laemmli, 1970). The mixture of each toxin and several w/w ratios of toxin and the fraction (1:10, 1:5 and 1:1) was preincubated at 37°C for 30 min. Then, 20 µl of each mixture was run at 120 V for 1 h. The proteins were stained with Coomassie blue.

Statistical analysis

In order to obtain significant differences between inhibitory effects of fraction each fraction on toxin, two-way ANOVA followed by Bonferroni´s test was applied. In anticoagulation activity, the one-way ANOVA followed by Dunnet´s test was carried out. In all the cases $p < 0.05$ was considered significant, and the results shown are the mean ± SE of n indicated in each case.

Results

Bioassay-guided fractionation of Swietenia macrophylla

Altogether, 22 fractions were obtained after the initial fractionation, labeled as Sm 1 to 22. According to their similar thin layer chromatography profile, and having an inhibition of 48-60% of the enzymatic activity induced by *B. asper* whole venom (1:10 w/w ratio in egg yolk assay), fractions Sm 13 to 16 were combined as Sm13-16 and further fractionated into thirty fractions. The inhibitory ability on the PLA_2 enzymatic activity induced by *B. asper* whole venom (1:10 w/w ratio in egg yolk assay) was performed on the thirty fractions, and promising results were observed with four of them (Table 1). Further inhibition assays of Cdcum6 (Asp49, enzymatic activity) and *B. atrox* myotoxin I (Lys49, cytotoxic activity) were performed with these four fractions. Significant inhibitory activity on both myotoxins tested, was only obtained by Sm13-16, 23 (Table 1), thus this fraction was chosen for further inhibition experiments.

Table 1
Inhibition ability of the fractions obtained from Sm13-16.

Inhibition percentage of PLA_2 (%)			
Fraction	Source of PLA_2		
	B. asper[a]	Cdcum6[b]	*B. atrox* myotoxin I[c]
Sm13-16, 23	80.00 ± 5.77	93.87 ± 1.20	86.82 ± 4.93
Sm13-16, 24	66.67 ± 6.67[d]	69.93 ± 4.22[d]	8.91 ± 2.88[d]
Sm13-16, 27	50.00 ± 5.77 [d]	54.51 ± 3.58[d]	23.89 ± 5.93[d]
Sm13-16, 28	76.67 ± 8.82[d]	72.99 ± 4.35[d]	25.25 ± 7.45[d]

[a]Inhibition of *B. asper* whole venom PLA2 induced activity using egg yolk as substrate and 10 µg venom. 1:10 w/w ratio was used. Results are shown as mean ± SE, n = 8.
[b]Inhibition of Cdcum6 (Asp49) PLA2 induced activity using egg yolk as substrate and 20 µg toxin. 1:10 w/w ratio was used. Results are shown as mean ± SE, n = 8.
[c]Inhibition of *B. atrox* myotoxin I (Lys49) cytotoxic induced activity on myoblasts using 20 µg toxin. 1:10 w/w ratio was used. Results are shown as mean ± SE, n = 6.
[d]Represent statistical differences regard to Sm13-16, 23.

Characterization of Sm13-16, 23

The spectral date obtained for Sm13-16, 23 were: UV λ_{max} (nm) (MeOH): 655, 266 and 219. IR λ_{max}^{KBr} (cm): Broad and intense peak between ~ 3600-3000 (OH), 2923.07 and 1438.90 (C-H), 1656.69 (C = O), 1618.63 and 1607.67 (C-O), 1285.47-772.60 (aromatic). Furthermore, in the colorimetric preliminary assays the presence of phenolic compounds such as flavonoids, leucoanthocyanidins and tannis were detected in Sm13-16, 23.

Inhibition of PLA_2 activity by Sm13-16, 23

In the bioassays using egg yolk and 4N3OBA as substrates, PLA_2 activity induced by Cdcum6 was inhibited in a dose dependent manner by Sm13-16, 23. However, the higher inhibitory activity was observed when aggregated substrate was used (egg yolk) (Table 2). In this assay, inhibition values between 73 to 95% were shown at 1:1, 1:2, 1:5 and 1:10 w/w ratios. Nevertheless, minor neutralization of PLA_2 activity was observed when the monodisperse substrate (4N3OBA) was used, showing inhibition values between 0-21% when using the w/w ratios mentioned above.

Inhibition of cytotoxic activity by Sm13-16, 23

The sample Sm13-16, 23 showed a dose dependent inhibition of the cytotoxic activity induced by Asp49 and Lys49 PLA_2 (Fig. 1). Significant difference between neutralization values obtained at 1:10 and 1:5 w/w ratios (toxin: sample) was observed inter and intra-toxin. However, the best inhibition percentages were always shown toward *B. atrox* myotoxin I. The fraction was not cytotoxic on C2C12 muscle cells (data not shown).

Table 2
Inhibition of PLA_2 activity by Sm13-16, 23.

	PLA_2 activity inhibition percentage (%)					
	W/W ratio[a]					
Substrate	1:10	1:5	1:2	1:1	1:0.5	1:0.25
Egg yolk[b]	93.87 ± 1.20	87.38 ± 1.48	76.96 ± 1.37[d]	75.78 ± 3.68[d]	30.66 ± 2.11[d]	5.76 ± 1.06[d]
4N3OBA[c]	21.51 ± 1.42	12.31 ± 1.72[e]	2.59 ± 1.59[e]	0.0 ± 0.0[e]	0.0 ± 0.0[e]	0.0 ± 0.0[e]

[a]Twenty micrograms of Cdcum6 were used in all experiments. Different w/w ratios Cdcum6:fraction were assayed. Results are shown as mean ± SE as indicated in each case.
[b]Assay with egg yolk as substrate, n = 8.
[c]Assay with 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA) as substrate, n = 12.
[d]Represents statistical differences regard to 1:10 w/w ratio when egg yolk was used as substrate.
[e]Represents statistical differences regard to 1:10 w/w ratio when 4N3OBA was used as substrate.

Inhibition of myotoxic activity by Sm13-16, 23

The inhibition of myotoxic activity induced by toxins was dose dependent (Fig. 2A and 2B). In both assays (preincubation and *in situ* administration) significant difference between neutralization values obtained at 1:10 and 1:5 w/w ratios (toxin: sample) was observed inter and intra-toxin. In contrast, only in preincubation assay significant difference at 1:2 and 1:1 ratios was observed (Fig. 2A). In both experiments, Cdcum6 was not inhibited by Sm13-16, 23 at a lowest ratios used (Fig. 2A and 2B).

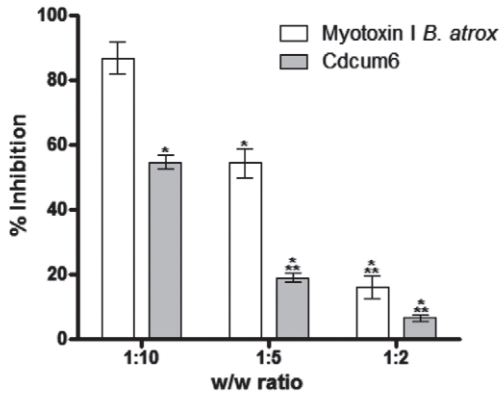

Figure 1 - Inhibition of cytotoxicity of PLA_{2s} Sm13-16, 23. Forty micrograms of each toxin and different w/w ratios were used, n = 6. Results are shown as mean ± SE. a Represents statistical differences regard to inhibition values 1:10 w/w ratio on cytotoxic activity induced by myotoxin I of B. atrox. b Represents statistical differences regard to inhibition values 1:10 w/w ratio on cytotoxic activity induced by Cdcum6.

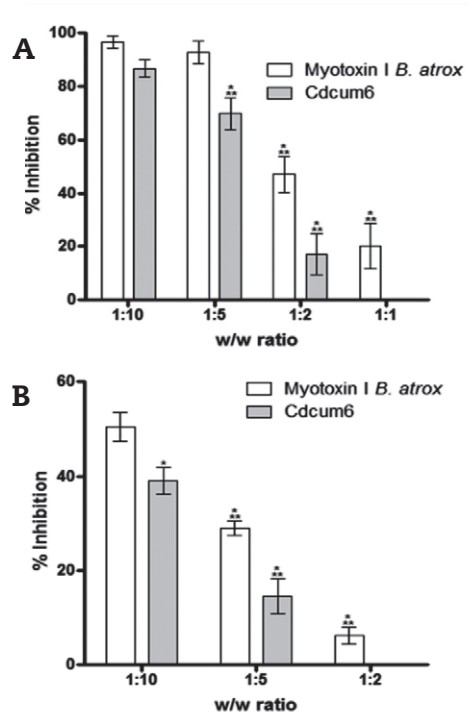

Figure 2. - Inhibition of PLA_2-induced myotoxicity by Sm13-16, 23. A, Inhibition of myotoxicity in preincubation assay; B, Inhibition of myotoxic activity by locally administered fraction. Twenty µg of *B. atrox* myotoxin I and 3 µg of Cdcum6 and different w/w ratios were used, n = 6. Results are shown as mean ± SE. a Represents statistical differences regard to inhibition values 1:10 w/w ratio on cytotoxic activity induced by myotoxin I of *B. atrox*. b Represents statistical differences regard to inhibition values 1:10 w/w ratio on cytotoxic activity induced by Cdcum6.

Inhibition of anticoagulant activity by Sm13-16, 23

This sample exhibited a dose dependent inhibition on anticoagulant activity induced by Cdcum6 (Fig. 3). At all w/w ratios used, a significant inhibition was observed ($p < 0.05$). Sample Sm13-16, 23 did not display coagulant effect, showing comparable coagulation times with negative control.

Inhibition of edema-inducing activity by Sm13-16, 23

The highest edema-inducing activity of the two toxins was shown two hours after injection (Fig. 4). At this time, at 1:10 w/w ratio, edema caused by Cdcum6 was inhibited ~ 26% by Sm13-16, 23 ($p < 0.001$). Likewise, the edema induced by *B. atrox* myotoxin I was inhibited ~ 53% ($p < 0.001$). Significant edema-inducing activity was not shown by 332 micrograms of the fraction (the highest amount of fraction used in this assay).

Inhibition of proteolytic activity by Sm13-16, 23

At all w/w ratios assayed, the proteolytic activity caused by Batx-I and Trypsin was not inhibited by Sm 13-16, 23 (Fig. 5).

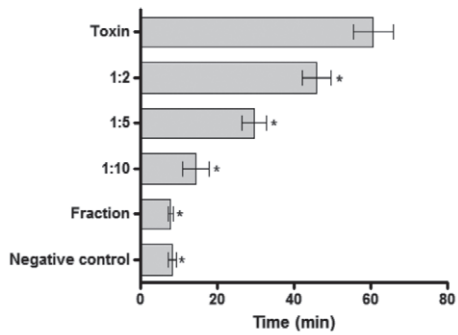

Figure 3 - Inhibition of anticoagulant activity of aan Asp49 PLA_2 by Sm13-16, 23. Ten micrograms of Cdcum6 and different w/w ratios were used, n = 6. a Represents statistical significant difference respect to toxin. Results are shown as mean ± SE.

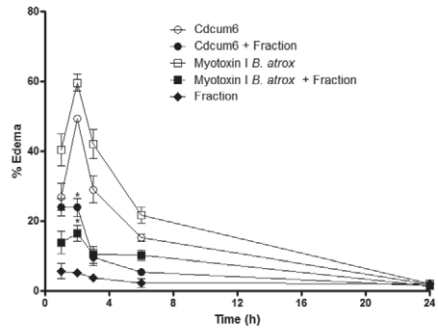

Figure 4 - Inhibition of edema-inducing activity of PLA_2 by Sm13-16, 23. Ten micrograms of Cdcum6 and 32 of B. atrox myotoxin I (equivalents to two minimum edematogenic doses) were used. 1/10 w/w ratio was tested, n = 6. a Represents statistical significant difference respect to each toxin at 2 h. Results are shown as mean ± SEM.

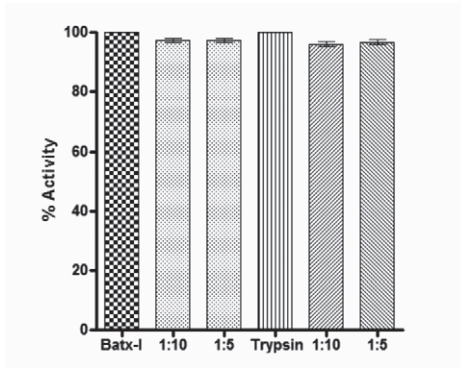

Figure 5 - Inhibition of proteolytic activity by Sm13-16, 23. Twenty micrograms of each protease were used. 1:10 and 1:5 w/w ratios was tested, n = 6. a Represents statistical significant difference respect to each protease. Results are shown as mean ± SE.

Polyacrylamide gel electrophoresis (SDSPAGE)

Sm13-16, 23 did not change the migration pattern of the toxins (Fig. 6A and 6B). In addition, the fraction apparently did not modify the molecular mass of the toxins, and was not stained with Coomassie Blue, evidencing the lack of proteins in this fraction (Fig. 6A and 6B).

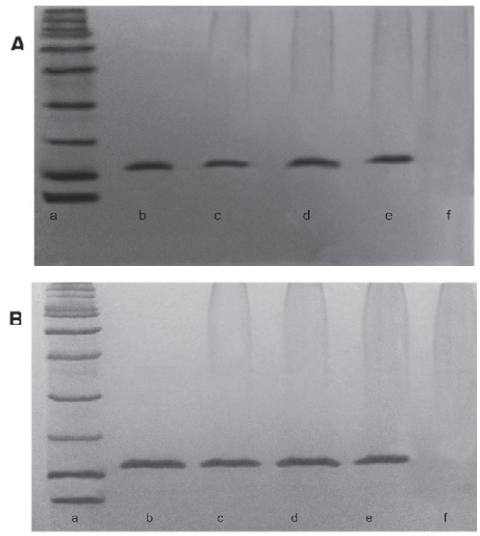

Figure 6 - SDS-PAGE of the Sm13-16, 23 preincubated with myotoxic PLA2. Twenty micrograms of each toxin were preincubated (30 min at 37°C) with several amounts of Sm13-16, 23, in order to obtain 1:10, 1:5 and 1:1 w/w ratios. A, Sm13-16, 23 mixed with Cdcum6, a. Molecular mass markers (200, 116.2, 97.4, 66.2, 45, 31, 21.5, 14.4, 6.5), b. 20 µg Cdcum6; c.1:10 w/w ratio cdcum6: Sm13-16, 23; d. 1:5 w/w ratio cdcum6: Sm13-16, 23; e.1:1 w/w ratio cdcum6: Sm13-16, 23; f. 200 µg Sm13-16, 23, the highest quantity used in this assay. B, Sm13-16, 23 mixed with *B. atrox* Myotoxin I, a. Molecular mass markers (200, 116.2, 97.4, 66.2, 45, 31, 21.5, 14.4, 6.5), b. 20 µg *B. atrox* Myotoxin I; c.1:10 w/w ratio *B. atrox* Myotoxin I: Sm13-16, 23; d. 1:5 w/w ratio *B. atrox* Myotoxin I: Sm13-16, 23; e.1:1 w/w ratio *B. atrox* Myotoxin I: Sm13-16, 23; f. 200 µg Sm13-16, 23, the highest quantity used in this assay.

Discussion

PLA_2-induced myotoxicity occurs in two clinical patterns: local and systemic (Warrell, 1996). The action of these enzymes may result in irreversible lesions which might lead to dysfunction and even amputation of the affected limb (Otero et al., 2002). Moreover, it has been demonstrated that antivenoms, the current therapy for snakebite, have a limited efficacy against the local tissue damaging activities of venoms (Gutiérrez et al., 1998). Thus, there is a need to search for additional inhibitors and approaches that may be useful counterparts to conventional antivenom therapy. In this direction, the plant *Swietenia macrophylla* King, Meliaceae, was chosen from a pool of plants as a promising lead because of its neutralizing activity against *Bothrops asper* venom and a PLA_2 isolated from it (Pereañez et al., 2010). After several bioassay-guided fractionation steps, the fraction Sm13-16, 23 was obtained as the most active for its inhibitory ability of the myotoxic effect induced by PLA_{2s}. In preincubation assays, the myotoxicity was successfully inhibited by 1:10 w/w ratios (~ 90%). However, when Sm13-16, 23 was injected immediately after poisoning, the myotoxicity was inhibited only to a minor extent. Nevertheless, the reduction in muscle damage is still significant since the major limitation for the development of any clinically effective inhibitor of myotoxins is the dramatic speed at which PLA_2 affects skeletal muscle (Gutiérrez and Ownby, 2003). In addition, inhibition experiments using cell cultures, such as rodent lines of skeletal muscle myoblasts/myotubes, appear to correlate with their *in vivo* myotoxicity (Lomonte et al., 1999). This relationship was used to confirm the results described above, since when PLA_2 were pre-incubated with Sm13-16, 23 at different w/w ratios to the previous cell cytotoxic assay, the damage was significantly reduced.

It is known that the action of Asp49 PLA_2 over membrane phospholipids requires interfacial activation of the enzyme (Berg et al, 2001). Sm13-16, 23 inhibited PLA_2 activity of Cdcum6 on egg yolk (aggregated substrate). However, this activity was weakly neutralized when 4N3OBA (monodispersed substrate) was used. These results could be explained taking into account that in egg yolk assay, PLA_2 performs interfacial activation and further phospholipid hydrolysis (Berg et al, 2001), whereas in 4N3OBA assays only the hydrolytic activity of the enzyme is measured (in this assay PLA_2 does not require interfacial activation to perform its activity). Therefore, these results indicate the possible interaction of Sm13-16, 23 compounds either on the interfacial binding surface (i-face) or the active site of the enzyme. The first interactions could also explain the inhibition of PLA_2-induced anticoagulation. The mechanism of this type of enzymes is related to their capacity to interact by means of their "anticoagulation site" (residues 57-74) with factor X a during the coagulation cascade (Kini, 2005). However, it is known that some of these residues are implicated also in interfacial activation of the enzyme (Jain and Berg, 2006). In the same way, the inhibition of other activities induced by Lys49 PLA_{2s}, such as myotoxicity, could also be explained by a change in the overall charge of the interfacial binding surface of the PLA_2 homologues. This was described by means of structural and *in vivo* studies for suramin. This compound caused 60% of the inhibition of myotoxicity induced by bothropstoxin I (Lys49 PLA_2) (Murakami et al., 2007). On the other hand, edema caused by Asp49 and Lys49 PLA_2 may be due to their combined effect to hydrolyze phospholipid membrane - resulting in the loss of membrane integrity - as well as their metabolic activity generating pro-inflammatory products such as eicosanoids, whose function is to amplify the inflammatory event (Teixeira et al., 2003). In addition, Lys49 PLA_2-induced edema is related to their ability to interact, and destabilize biological membranes (Lomonte et al., 2003). Thus, the inhibitory effect showed by

Sm13-16, 23 could be related to the inhibition of enzymatic activity and the change of the overall charge of the interfacial binding surface, as described above.

In the effort to characterize Sm13-16, 23, the preliminary phytochemical qualitative analysis of Sm13-16, 23, was consistent with the detection of phenolic compounds. Additionally, the detection of the aromatic system by the UV and IR data, were comparable to those previously reported in the literature for phenolic compounds (Silverstein and Webster, 1998). Therefore, the signals for the hydroxyl groups and the aromatic rings observed in IR spectrum support the detection of phenolic compounds in sample Sm13-16, 23. This is relevant, since these molecules can interact with the enzymes in several ways. This has been concluded, in several studies, in which the mode of interaction of polyphenolic compounds on PLA_2 is due to the interactions between the enzyme and the hydroxyl groups present in this type of metabolites, through hydrogen bonds, that result in the formation of a stable complex (Chandra et al., 2002; Da Silva et al., 2009; Lindahl et al., 1997). However, the activity of polyphenolic compounds may involve varying degrees of interactions such as hydrophobic connections mediated by aromatic rings, which should also be considered.

In the specific case of polyphenols such as tannins, the inhibition on PLA_2 could be due to unspecific binding and further precipitation of proteins (Haslam, 1996; Wall et al., 1996). However, this is not likely to be the case in this study, because Sm13-16, 23 did not inhibit the enzymatic activity of other enzymes (snake venom metalloproteinase and trypsin). Therefore, these results indicated some specificity of this type of tannins toward PLA_{2s}. Nevertheless, the presence of smaller molecules derived from gallotannins, such as ellagic acid and its O-methyl derivatives could be considered, since it has been reported to high inhibition ability of these molecules on snake venom PLA_2 (da Silva et al., 2008). In the same way, results of SDS-PAGE suggests that Sm13-16, 23 does not form large complexes with the toxins, which also indicates that tannins of Sm13-16, 23 not unspecifically bind and precipitate proteins.

Considering the presence of other type of polyphenols in Sm13-16, 23, such as flavonoids, it these metabolites have been shown to exert inhibition of enzymatic and toxic effects of PLA_{2s} (Iglesias et al, 2005; Lindahl et al., 1997). The inactivation of the enzyme by competitive inhibition has been proposed for these compounds (Lättig et al., 2007). Hence, this is in accordance with one of the possible mode of action for Sm13-16, 23 described above (interaction of Sm13-16, 23 compounds with active site of the enzyme). Other phenols like leucoanthocyanidins with a very similar structure as flavonoids, known systematically as flavan-3, 4-diols, could have a similar mode of action as well. Moreover, these molecules are structurally and biosynthetically related to anthocyanidins (Springob et al., 2003), which have a considerable inhibitory activity over secreted PLA_{2s} (Dreiseitel et al., 2009).

In conclusion, we obtained a fraction from *S. macrophylla* leaves extract with promising inhibitory ability on myotoxic PLA_2. This fraction, named as Sm13-16, 23, with a high content of phenolic compounds, inhibited enzymatic and anticoagulant activities of Asp49 PLA_2 and also inhibited myotoxic and edema-forming activities of Asp49 and Lys49 PLA2s. However, Sm13-16, 23 was much more efficient to inhibit the toxic activities induced by the Lys49 PLA_2 than the Asp49 PLA_2. Our next goal is the isolation and elucidation of the chemical structure of compounds that occur in Sm13-16, 23 in order to contribute to the development of a novel product that could reduce the local effects in the snakebites, such as myotoxicity and edema.

Authors' contributions

JAP contributed in running the laboratory work, analysis of the date and drafted the paper. TLE contributed in collecting plant sample and identification, confection of herbarium, and chromatographic processes to critical reading of the manuscript. BR prepared plant material and critical reading of the manuscript. VN designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

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