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Article

Correlation of the inhibitory activity of phospholipase A_2 snake venom and the antioxidant activity of Colombian plant extracts

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RESUMO: Veneno de cobra continua a ser um problema importante de saúde em muitos países da América Latina. Apesar dos avanços na terapia antiveneno, os efeitos locais causados por fosfolipases A_2 miotóxica (PLA_2) presentes no veneno, ainda persistem. Em busca de alternativas para antagonizar a atividade da PLA_2 do veneno de *Bothrops asper*, foram selecionados 36 extratos pertencentes a dezessete famílias de plantas vasculares e briófitas. Uma inibição significativa da atividade enzimática de PLA_2 presente no veneno de *B. asper* foi observada em onze extratos. Além disso, a atividade antioxidante dos extratos foi avaliada. Os resultados evidenciaram uma correlação estatisticamente significativa entre os extratos com ação inibitória contra a PLA_2 e aqueles com atividade antioxidante. Também, a quantidade de fenóis foi avaliada e foi encontrada uma relação entre a atividade biológica e a presença dessas substâncias. Nove extratos foram testados contra uma fração do veneno rico em PLA_2 básica (Fx-V *B. asper*), resultando em um efeito inibitório na atividade desta fração da PLA_2 na faixa de 30-80%. Esta atividade foi apoiada pela inibição que esses extratos apresentaram na citotoxicidade causada pelo Fx-V B. asper sobre mioblastos C2C12 de músculo esquelético de murino. Os resultados podem indicar a minimização dos esforços na busca de inibidores da PLA_2, com foco nas amostras com propriedades antioxidantes conhecidas.

Unitermos: fosfolipase A_2, veneno de cobra, *Bothrops asper*, extratos de plantas, antioxidantes, DPPH.

ABSTRACT: Snakebite continues to be a significant health problem in many countries of Latin America. Even though, there has been an improvement in the antivenom therapy, the local effects caused by myotoxic phospholipases A_2 (PLA_2) present in the venoms, still persist. In search for alternatives to antagonize the PLA_2 activity of *Bothrops asper's* venom, 36 extracts belonging to seventeen families of vascular plants and bryophytes were screened. A significant inhibition of the enzymatic activity of PLA_2 present in *B. asper's* whole venom was seen in eleven of these extracts. In addition, the antioxidant activity of all the extracts was evaluated. The results evidenced a significant statistical correlation between extracts with an inhibitory effect against PLA_2 and those with an antioxidant activity. Moreover, the amount of phenols was quantified finding a relationship between the bioactivity and the presence of these compounds. Nine extracts were screened against a fraction of the venom rich in basic PLA_2 (Fx-V *B. asper*), exhibiting an inhibitory effect on PLA_2 activity of this fraction in a range of 30-80%. This activity was supported by the inhibition that these extracts presented on the cytotoxicity caused by Fx-V *B. asper* on murine skeletal muscle C2C12 myoblasts. The results obtained, could point to minimize efforts in the search of PLA_2 inhibitors by focusing in samples with known antioxidant properties.

Keywords: phospholipase A_2, snake venom, *Bothrops asper*, plant extracts, antioxidants, DPPH.

INTRODUCTION

Bothrops asper is the main species responsible for snakebite accidents in several countries of Latin America (Fan & Cardoso, 1995; Gutiérrez, 1995). Its venom induces systemic and local effects such as swelling, haemorrhage, myonecrosis, haemostatic disorders, and nephrotoxicity. In Colombia about 3000 snakebite accidents are reported per year, 70% of which are inflicted by this species (Otero et al.,

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2002). The components of the *B. asper* venom include metalloproteinases, phospholipases A_2, and serine proteinases among others. The most important and abundant muscle-damaging components in this venom are phospholipases A_2 (PLA_2; EC 3.1.1.4). These enzymes hydrolyze the sn-2 ester bond of membrane glycerophospholipids generating fatty acids such as, arachidonic acid and lysophospholipids participating in inflammatory process (Six & Dennis, 2000).

To treat the effects caused by snakebites, intravenous administration of equine or ovine immunoglobulins constitutes the main treatment (Simpson & Norris, 2009). However, it has been demonstrated that such antivenoms generally have a limited efficacy against the damaging activities of the venoms in the local tissue (Gutiérrez et al., 1998). Thus, there is the need to search for additional approaches that may be useful to complement conventional antivenom therapy. In this way, the search for specific PLA_2s inhibitors could support the traditional therapy and contribute to find new anti-inflammatory agents.

Plant extracts constitute rich sources of pharmacologically active compounds, and some of them have been reported to antagonize the activity of various crude venoms and purified toxins (Coe & Anderson, 2005; Mors et al., 2000; Otero et al., 2000). This activity has been attributed to different compounds such as flavonoids, coumarins, and other polyphenolic metabolites widely distributed in different plant families (Lindahl & Tagesson, 1993, 1997; Pithayanukul et al., 2005; Toyama et al., 2009). These classes of compounds are known to be powerful antioxidant agents both in hydrophilic and lypophilic environments. Antioxidants are substances that can prevent, stop or reduce oxidation damage. They have diverse pharmacological properties, such as anti-inflammatory activity (Biesalski, 2007; Rahman, 2008). In this regard, some authors have shown that flavonoids present in different plant families induce a dose-dependent inhibition of *in vitro* phospholipid hydrolysis by secretory PLA_2s (Lindahl & Tagesson, 1997). Recently, studies concluded that α-tocopherol (Vitamin E), a well known antioxidant, is an effective inhibitor of PLA_2 (Chandra et al., 2002; Takeda et al., 2004).

In this investigation, we examined the effect of several extracts among vascular plants and bryophytes, on some phospholipase A_2 activities induced by *Bothrops asper's* whole venom. The resulting active leads were also assayed against a refined fraction rich in PLA_2 (Fx-V *B. asper*), and its cytotoxicity was tested. Due to the evidence pointing to a relationship between antioxidant activity and PLA_2 inhibitory activity, plant extracts were also tested for its antioxidant potential using the DPPH method. Additionally, in all of the samples, phenols were quantified to have an idea of the type of compounds that could be responsible for the results obtained.

MATERIAL AND METHODS

Venom, chemicals and reagents

The snake venom was obtained by manual extraction of several species of *B. asper* from Antioquia and Chocó, which are maintained in captivity at the Universidad de Antioquia Serpentarium (Medellín, Colombia). Venoms were centrifuged at 800 g for 15 min, and supernatants were lyophilized and stored at -20 °C until used. For antioxidant experiments, DPPH free radical (2,2-diphenyl-1-picrylhydrazyl), methanol (Aldrich Chem.), Folin reagent and sodium carbonate (Merck), were used. All chemicals and reagents employed in this work were analytical. For plant extraction, distilled ethanol was used.

Plant material

Plant material for this investigation was selected according to preliminary studies in its antioxidant properties (Rojano, Personal communication). For vascular plants, samples of leaves and twigs were collected in tropical and premountain forests of Antioquia (Colombia) between 900-2000 m.s.l. For bryophytes, the whole sporophyte was collected in mountain forests at the locality of Santa Elena (2200-2600 m.s.l.), Antioquia (Colombia). From each species, 1000-2000 g of fresh plant material was collected and air-dried in mesh bags. Collections were supported by a voucher sample and were deposited at the Herbarium of the Universidad Nacional, Gabriel Gutiérrez Villegas (MEDEL), which were identified by Leon Morales; Herbarium of the Botanical Garden, Joaquin Antonio Uribe (JAUM), identified by Alvaro Cogollo; or the Universidad de Antioquia Herbarium (HUA), identified by Adriana Corrales (Table 1).

Extraction

For each of the selected plants 500-700 g of dried and milled plant material was extracted overnight with 90% ethanol, three times. The resultant ethanol extract was concentrated to a semisolid paste using a BÜCHI-124 rotavapor (Flawil, Switzerland) and an amount of 1-3 mg was used to perform bioassays, the remaining amount was saved for reference purposes.

Inhibition of phospholipase A_2 activity

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 10 µg of *B. asper's* venom or 50 µg of a fraction of the venom that contained

Table 1. Inhibitory ability against the phospholipase A_2 activity of *B. asper* snake whole venom, antioxidant activity and concentration of total phenols. *Positive control (quercetin) showed a IC50 0.815±0.04 µg/mL.

Plant families	Species	Herbarium & Voucher number	PLA_2 Inhibition (%)	Antioxidant activity DPPH (IC50 µg/mL)*	Total phenols mg gallic acid/100 g
Anacardiaceae	*Anacardium excelsum* (Kunth) Skeels	MEDEL TL-100	52.00±2.00	8.00±0.40	320±20.0
Annonaceae	*Ephedranthus colombianus* Maas & Setten	MEDEL LECA-3365	36.21±3.43	6.00±0.25	170±15.0
	Oxandra xylopioides Diels	JAUM 037852	5.26±0.58	>100	60±2.0
	Sapranthus isae J.G. Vélez & Cogollo	JAUM 037855	26.00±0.00	>100	130±8.5
Araceae	*Xanthosoma undipes* (K. Koch & C.D. Bouché) K. Koch	HUA LA-052	0.00±0.00	>100	30±2.0
Arecaceae	*Oenocarpus mapora* H. Karst.	HUA LA-025	9.33±3.51	36.00±2.00	40±1.3
Euphorbiaceae	*Euphorbia cotinifolia* L.	HUA LA-046	11.83±6.81	>100	50±3.0
	Hevea nitida Mart. ex Müll. Arg.	HUA LA-051	60.67±14.29	20.00±2.00	220±6.0
Fabaceae	*Hymenaea courbaril* L.	MEDEL TL-101	9.33±3.51	13.00±0.50	740±25.0
Hippocastanaceae	*Billia hippocastanum* Peyr.	MEDEL LECA-4518	23.00±7.94	3.00±0.10	320±18.0
Magnoliaceae	*Dugandiodendron guatapense* Lozano	JAUM 4168	52.00±3.46	22.00±3.00	602±32.0
	Dugandiodendron yarumalense Lozano	JAUM 4015	44.00±0.00	16.90±0.30	405±21.0
	Dugandiodendron sp.	JAUM 4111	49.00±1.73	26.00±1.30	550±28.0
	Talauma espinalii Lozano	JAUM 4172	37.67±7.50	5.30±0.03	527±12.0
	Talauma hernandezii Lozano	JAUM 1634	37.04±0.00	3.00±0.02	420±14.0
	Talauma polyhypsophylla Lozano	JAUM 3396	15.00±14.53	17.00±1.05	220±15.0
	Talauma silvioi Lozano	JAUM 3388	10.00±8.71	32.50±2.00	50±2.0
Meliaceae	*Carapa guianensis* Aubl.	MEDEL TL-107	36.33±1.53	7.80±0.002	600±17.0
	Cedrela odorata L.	MEDEL TL-106	21.50±13.44	45.70±3.00	170±8.0
	Guarea guidonia (L.) Sleumer	MEDEL TL-105	7.33±0.58	>100	10±0.1
	Swietenia humilis Zucc.	MEDEL TL-102	22.33±1.50	8.30±0.30	340±11.0
	Swietenia macrophylla King	MEDEL TL-103	59.33±3.51	4.30±0.10	310±13.0
	Swietenia mahagoni (L.) Jacq.	MEDEL TL-104	42.00±5.00	15.60±0.09	910±42.0
Myristicaceae	*Virola flexuosa* A.C. Sm.	MEDEL LECA-5067	16.00±3.0	21.00±1.05	30±2.00
	*Virola sebifer*a Aubl.	MEDEL LECA-2514	0.00±0.00	22.70±1.30	760±33.0
Rutaceae	*Murraya paniculata* (L.) Jack	MEDEL TL-108	56. 00±1.73	>100	20±1.8
Sapindaceae	*Cupania americana* L.	MEDEL TL-112	25.00±1.00	25.80±2.00	10±0.3
	Dilodendron costaricense (Radlk.) A.H. Gentry & Steyerm.	MEDEL TL-110	4.00±4.00	5.50±0.10	40±1.4
	Melicoccus bijugatus Jacq.	MEDEL TL-109	0.00±0.00	28.70±2.00	40±2.5
	Sapindus saponaria L.	MEDEL TL-111	20.67±9.50	>100	124±6.5
Bartramiaceae (Bryophyte)	*Breutelia chrysea* (Müll. Hal.) A. Jaeger	HUA VL-160111	6.67±6.50	>100	20±1.3
Dicranaceae (Bryophyte)	*Dicranum frigidum* Müll. Hal.	HUA VL- 160138	28.33±19.55	>100	20±1.1
Pottiaceae (Bryophyte)	*Leptodontium luteum* (Taylor) Mitt.	HUA VL- 160159	0.00±0.00	>100	20±2.3
Sphagnaceae (Bryophyte)	*Sphagnum recurvum* P. Beauv.	HUA VL-160143	4.67±4.51	>100	60±2.4
	Sphagnum sp	HUA VL-160146	13.64±4.54	>100	60±3.3
Thuidiaceae (Bryophyte)	*Thuidium peruvianu*m Mitt.	HUA VL- 160170	10.67±2.52	>100	10±2.00

active enzymatic basic PLA_2. The time of reaction was 15 min at 37 °C. The amount of protein was selected from the linear region of activity curves. For inhibition experiments, 100 or 500 µg of crude extracts were mixed with the venom or fraction, respectively, and incubated for 30 min at 37 °C before PLA_2 activity determination. The results are indicated as inhibition percentage, where 100% is the activity induced by venom or fraction alone.

Antioxidant activity by the DPPH method

The radical scavenging activity was measured according to the method reported by Brand-Williams et al. (1995) with some modifications (Rojano et al., 2008). Each sample (10 µL) was added to 990 µL of DPPH solution and after 30 min, the absorbance was determined at 517 nm (Jemway 6405, UV/VIS spectrophotometer). Samples were evaluated at different concentrations and its 50% inhibition index (IC50) was determined. Quercetin was used as positive control.

Determination of phenols

This was determined by the colorimetric method of Folin-Ciocalteu (Dewanto & Adom, 2002). A base curve was done using gallic acid as a standard, and the plant extracts were diluted to a concentration within the curve. The results were expressed as mg of gallic acid/100 g of plant extract.

Cytotoxic activity of plant extracts in mice muscle cells

Cytotoxicity of the extracts was measured on murine skeletal muscle C2C12 myoblasts (ATCC CRL-1772) (Lomonte et al., 1999) by adding 100 µg of each active extract diluted in 150 µL assay medium (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal calf serum) to 96-well plates. Controls of 0 to 100% toxicity consisted of assay medium and 0.1% Triton X-100, respectively. 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). Experiments were carried out in triplicate.

Isolation and purification of fraction with PLA_2 activity Fx-V *B. asper*

A fraction rich in basic PLA_2s (Fx-V *B. asper*) and with enzymatic activity was purified using cationic interchange chromatography. For this, 250 mg of *B. asper's* venom were diluted in 0.05 M Tris, 0.1 M KCl (pH: 7.0) and applied to a Carboxymethyl-Sephadex C 25 column, which had been pre-equilibrated with the same buffer. Proteins were eluted at a flow rate of 1.0 mL/min with a KCl gradient from 0.1 to 0.75 M, and elution profile was monitored at 280 nm. The fractions corresponding to main peaks were pooled, lyophilized, evaluated in phospholipase A_2 activity and evaluated in SDS PAGE electrophoresis.

Inhibition of the cytotoxicity induced by Fx-V *B. asper*

Inhibition experiments of the cytotoxic activity of Fx-V *B. asper* by the extracts were performed on murine skeletal muscle C2C12 myoblasts (ATCC CRL-1772) as reported in the literature (Lomonte et al., 1999), by mixing 100 µg of each extract with 10 µg of such fraction in 150 µL of medium. Solutions were incubated at 37 °C for 30 min, and 150 µL were added to 96-well plates. The cytotoxic activity was calculated as described above, and all experiments were carried out in triplicate. Controls of 0 to 100% toxicity consisted of assay medium and Fx-V *B. asper*, respectively. The results are indicated as LDH activity (U/L).

Statistical analysis

To analyze the relationship between antioxidant activity of each extract and phospholipase A_2 inhibitory activity, a nonparametric correlation analysis using the Spearman method was used. The same analysis was carried out to compare total phenols and phospholipase A_2 inhibitory activity. To determine significant differences in the cytotoxicity assays an ANOVA followed by a Dunnett's test was applied. In all cases significance was tested using a probability level of 95% ($p \leq 0.05$).

RESULTS

Preliminary experiments searching for the phospholipase A_2 inhibitory activity of *B. asper`s* whole venom, established that 32 of the 36 whole extracts evaluated, demonstrated some percentage of activity (Table 1). For the selection of the plant extracts for further evaluation, two criteria were considered; (a) to have an inhibition percentage of PLA_2 activity higher than 40%, or (b) to present a combination of an inhibition percentage of PLA_2 activity higher than 20% and an antioxidant activity with an IC50 less than 5 µg/mL. Considering the selection criteria, eleven extracts were chosen for further experimental work. This included the evaluation of its *in vitro* cytotoxicity, in which only two of them, *Dugandiodendron guatapense* and *Dugandiodendron* sp. induced this effect (Figure 1). Subsequently, the nine remaining, non-cytotoxic extracts, were evaluated against a fraction of *B. asper`s* venom with basic PLA_2 (Fx-V *B. asper*) resulting in a reduction of the enzymatic and cytotoxic activity induced by this fraction (Figure 2 and 3). In the same way 25% of the all extracts demonstrated antioxidant activity, measured by the DPPH method, with an IC50

under 9.00 µg/mL (Table 1). When a nonparametric correlation between the inhibitory percentage of the venom's PLA_2 enzymatic activity and the antioxidant activity was performed by the Spearman method, a negative and significant correlation between both variables was evidenced [p=0.0200, r=-0.3863 (-0.6404 to -0.05599)].

The concentration of polyphenoles in the extracts was variable (Table 1), nevertheless a positive and significant relationship between inhibitory activity against PLA_2 and a high concentration of this type of compounds was found when the same nonparametric correlation analysis (Spearman method), was applied in this case [p=0.0053, r=0.4546 (0.1382 to 0.6868)].

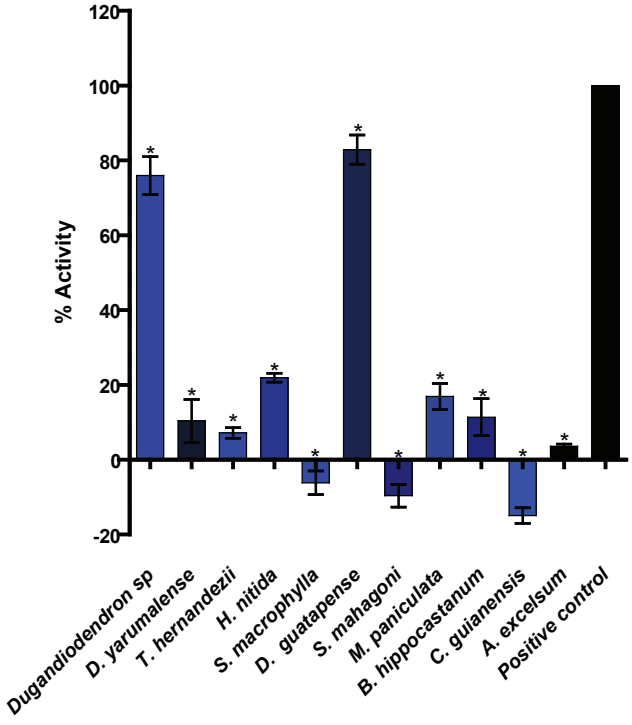

Figure 1. Cytotoxicity of plant extracts. *Represents significant difference respect to positive control (p<0.05). Results are shown as Mean±SD of three independent experiments.

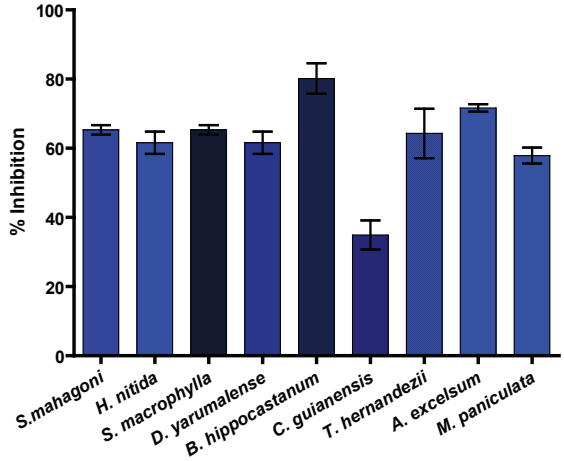

Figure 2. Inhibition of PLA_2 activity induced by Fx-V *B. asper*. Fraction alone was taken as 100% of activity (0% inhibition). Results are shown as Mean±SD of three independent experiments.

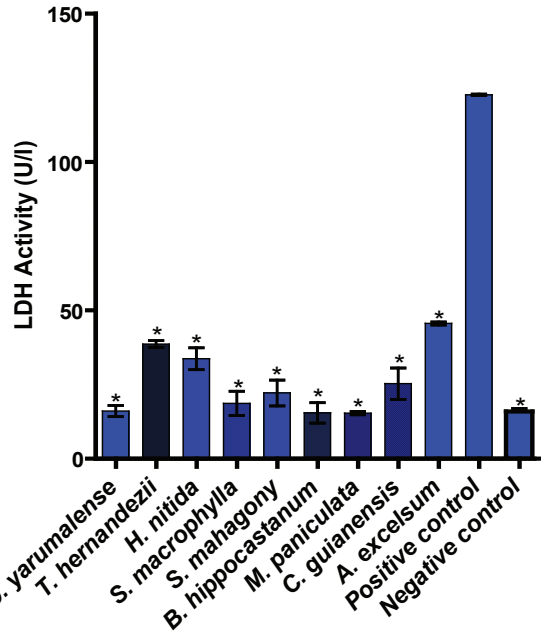

Figure 3. Inhibition of cytotoxicity induced by Fx-V *B. asper* by plant extracts. Fraction alone was taken as positive control. *Represents significant difference respect to positive control (p<0.05). Results are shown as Mean±SD of three independent experiments.

DISCUSSION

In sight of the health problem that snakebite accidents still represent in many tropical countries, and due to the fact that conventional therapy has a limited efficacy against damage in local tissue (Gutiérrez et al., 1998), there is a need to find approaches that can optimize the discovery of promising leads. In this regard, plant extracts have become an attractive research material as a complement to the antiserum therapy (Martz, 1992; Mors et al., 2000). In the present study nine promising plant extracts that inhibited *B. asper`s* venom $PLA_2$s with none cytotoxic effect were identified. The $PLA_2$s present in the *B. asper`s* venom included two major subgroups, namely, the Asp49 and Lys49 variants. The latter proteins lack phospholipolytic activity, but nevertheless exert cytotoxicity and other membrane-damaging actions (Gutiérrez & Lomonte, 1997; Lomonte et al., 2003). In this way, the fraction Fx-V *B. asper* used in the assays contained both, active enzymatic and non-enzymatic PLA_2. Consequently, the active extracts inhibited both protein subgroups by having an effect over the enzymatic and cytotoxic activity. The Fx-V *B. asper* fraction induced a conspicuous damage on myoblast, but when it was

preincubated with the extracts before the bioassay with the cells, the damage was reduced significantly. These results imply a therapeutic potential of these plants against necrosis in snakebite victims, by inhibiting the myotoxic effect induce by PLA_2 present in the venoms.

Due to abundance of the PLA_2 in the venom of viperids/crotalids, and to the large amounts of venoms frequently injected in such accidents, these myotoxins are undoubtedly central to the development of myonecrosis. Their predominant role in the myotoxicity of the corresponding crude venoms has been demonstrated by using specific neutralizers. When the PLA_2 myotoxins are selectively neutralized, most of the muscle-damaging effect of whole venoms is prevented. Moreover, venoms that contain these PLA_2 myotoxins induce significantly higher muscle damage than venoms that lack of them (Lomonte et al., 2003).

In addition, some of the plant extracts evidenced antioxidant activity, in accordance to previous reports which demonstrated that antioxidant compounds are group II PLA_2 inhibitors (Chandra et al., 2002; Lindahl et al., 1993, 1997). Our results were supported by the significant correlation between both activities (Table 1). Furthermore, the quantification of total phenols present in the plant extracts indicated the presence of a higher concentration of this class of compounds in active extracts, suggesting that these metabolites could be responsible for the in vitro inhibition of the toxic effects of PLA_2s in most of the cases. In accordance to this evidence, similar correlations have been reported with extracts of different plant parts of *Pentace burmanica, Pithecellobium dulce, Areca catechu*, and *Quercus infectoria*, which inhibited almost completely the lethal, necrotizing, and acetylcholinesterase activities of *Naja kaouthia* venom (Pithayanukul et al., 2005). Additionally, the ethanolic extract of seed kernels of *Mangifera indica* and its major compound, the phenol pentagalloyl glucopyranose, exhibited dose-dependent inhibitory effects on PLA_2 enzymatic activities of *Calloselasma rhodostoma* (CR) and Naja naja kaouthia (NK) venoms. In this case, through molecular docking studies the authors evidenced how phenolic molecules of *M. indica* could selectively bind to the active sites or modify conserved residues that are critical for the catalysis of PLA_2 (Leanpolchareanchai et al., 2009). On the other hand, vitamin E (α-tocopherol, an antioxidant molecule) decreased both enzymatic and inflammatory activities of an isolated PLA_2. In this direction, it was also suggested that vitamin E has the ability to bind to the hydrophobic pocket of PLA_2, inhibiting free access of substrate to the catalytic site (Takeda et al., 2004). In relation to the mode of action, several studies have concluded that the inhibition 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 results in the formation of a stable complex (Chandra et al., 2002; Da Silva et al., 2009; Lindahl et al., 1997; Toyama et al., 2009). 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.

The type of preliminary search done in this work can have potential to select a set of promising leads against PLA_2s. However, the detailed mechanism of venom neutralization by these extracts is yet to be fully understood and plant derived compounds responsible for the activity have to be identified to propose molecular modeling studies to support the mechanism hypothesis.

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