VITAE, REVISTA DE LA FACULTAD DE CIENCIAS FARMACÉUTICAS Y ALIMENTARIAS
ISSN 0121-4004 / ISSNe 2145-2660. Volumen 23 número 2, año 2016
Universidad de Antioquia, Medellín, Colombia. págs. 124-133
DOI: http://dx.doi.org/10.17533/udea.vitae.v23n2a05

CHARACTERIZATION OF THE MOST PROMISING FRACTION OF *Swietenia macrophylla* ACTIVE AGAINST MYOTOXIC PHOSPHOLIPASES A_2: IDENTIFICATION OF CATECHIN AS ONE OF THE ACTIVE COMPOUNDS

CARACTERIZACION DE LA FRACCION MAS PROMISORIA DE *Swietenia macrophylla* ACTIVA CONTRA FOSFOLIPASAS A_2 MIOTOXICAS: IDENTIFICACIÓN DE CATEQUINA COMO UNO DE LOS COMPUESTOS ACTIVOS

Lina PRECIADO, MSc[1], Jaime Andrés PEREAÑEZ PhD[2], Vitelbina NUÑEZ PhD[2, 3.]
Tatiana LOBO-ECHEVERRI PhD[1*]

Received: February 25, 2016 Approved: August 03, 2016

ABSTRACT

Background: The pharmacological effects produced by snakebite accidents involve the actions of several enzymes, of which those of the phospholipases A_2 (PLA_2) exhibit a wide variety of effects such as edema and myotoxicity. Some plant extracts have been antagonists of crude snake venoms and toxins. Based on promising bioactivity, *Swietenia macrophylla* King was selected for further studies. **Objective:** The purpose of this study was to identify the PLA_2 inhibitors present in the crude extract of *S. macrophylla* that could be promising leads in neutralizing the local effects of ophidian accidents. **Methods:** Bioassay-guided fractionation of the ethanolic extract of the leaves of *S. macrophylla* lead to the detection of (+)-catechin, characterized through gas chromatography coupled with mass spectrometry (GC-MS), and confirmed by HPLC. The PLA_2 inhibitory activity was measured with the Dole method and a spectrophotometric assay with 4-Nitro-3-octanoyloxy-benzoic acid (4N3OBA). Cytotoxicity was done on C2C12 murine myoblast. **Results:** Fraction F5 and (+)-Catechin inhibited the PLA_2 activity of *B. asper* venom, in a dose-dependent way. In addition, (+)-Catechin showed an inhibition level of 83.1 ± 3.1 % of the enzymatic activity of one PLA_2 purified from the venom of *Crotalus durissus cumanensis* using 4N3OBA as substrate. Also the ethanolic extract and fraction F5 showed inhibition of the cytotoxicity induced by the *Bothrops atrox* venom and their Lys 49 PLA_2 (80 and 100% respectively). Molecular docking results suggested that OH from 4´ and 5' carbons of (+)-catechin could form hydrogen bonds with carboxylate moiety of residue Asp49, while OH from 5 could form a hydrogen bond with Asn 6. Additional Van der Waals interactions were also proposed. **Conclusion:** *Swietenia macrophylla* exhibited strong inhibitory activity against PLA_2s enzymes. Catechin, one of the components in the active fraction F5, is proposed as being partially responsible for the bioactivity.

Keywords: *Swietenia macrophylla*, catechin, molecular docking simulation, snakebite, Viperidae.

[1] Grupo de Química de los Productos Naturales y los Alimentos, Escuela de Química, Facultad de Ciencias, Universidad Nacional de Colombia, Calle 59 A 63-20, Medellín, Colombia.

[2] Programa de Ofidismo/Escorpionismo, Facultad de Ciencias Farmacéuticas y Alimentarias, Universidad de Antioquia, Calle 70 52-21, Medellín, Colombia.

[3] Escuela de Microbiología, Universidad de Antioquia, Calle 67 53- 108. Medellín, Colombia

[*] Corresponding author: tloboech@unal.edu.co,

RESUMEN

Antecedentes: Los efectos farmacológicos producidos en el accidente ofídico implican la acción de varias enzimas, como las fosfolipasas A_2 (PLA_2), que exhiben una amplia variedad de efectos como edema y miotoxicidad. Algunos extractos de plantas han demostrado ser antagonistas de los venenos crudos y sus toxinas. En base a una bioactividad promisoria previa, *Swietenia macrophylla* King fue seleccionada para estudios posteriores. **Objetivo:** El propósito de este estudio fue identificar metabolitos inhibidores de PLA_2 presentes en el extracto crudo de *S. macrophylla* que podrían ser prometedores en la neutralización de los efectos locales del accidente ofídico. **Métodos**: Un fraccionamiento biodirigido del extracto etanólico de hojas de *S. macrophylla* llevó a la detección de la (+)-catequina mediante cromatografía de gases acoplada a espectrometría de masas (GC-MS), y se confirmó mediante HPLC. La actividad inhibidora de PLA_2 se determinó por el método de Dole y un ensayo espectrofotométrico con Ácido 4-Nitro-3-octanoiloxibenzoico (4N3OBA). La citotoxicidad se determinó en mioblastos C2C12 murinos. **Resultados:** La fracción F5 de *S. macrophylla*, mostró la mayor inhibición del veneno de *Bothrops asper* y PLA_2s aisladas de *B. atrox* y *Crotalus durissus cumanensis* e inhibición completa de la citotoxicidad. La (+)-catequina fue el metabolito más abundante en F5, mostró una inhibición de PLA_2 de 89,8%, 81,1% y 74,3%, a diferentes relaciones de veneno: catequina. Mediante estudios de *docking* molecular se demostró la unión de la (+)-catequina al sitio activo de la PLA_2. **Conclusión:** *Swietenia macrophylla* exhibió una fuerte actividad inhibitoria sobre las enzimas PLA_2s. La (+)-catequina, uno de los metabolitos presentes en la fracción activa F5, se propone como uno de los compuestos responsables de la bioactividad.

Palabras clave: *Swietenia macrophylla*, catequina, simulación de acoplamiento molecular, mordedura de serpiente, Viperidae.

INTRODUCTION

It has been estimated that the annual number of snakebite accidents in the world is around 5 million, among which, 20,000 to 94,000 result in the death of the patient [1-3]. In general, accidents caused by species within the Viperidae family (*Bothrops* spp., *Crotalus* spp.) are characterized by local damage to the tissues, including myonecrosis, dermonecrosis, hemorrhage, and edema [4]. Myonecrosis is one of the most prominent effects induced by these venoms, causing the loss of tissue and permanent disability. This effect is produced mainly by the enzyme phospholipase A_2 (PLA_2; EC 3.1.1.4.), which hydrolyzes membrane phospholipids generating fatty acids which participate in the inflammatory process [5]. The current main treatment for snakebite accidents is intravenous administration of equine immunoglobulins [6]. However, the antivenoms available for bothropic accidents exhibit a limited neutralization of local and rapid installation effects [7].

Natural products are a rich source of pharmacologically active phytochemicals, which are beneficial as complex mixtures (single or mixed plant extracts), or as chemical backbones for new therapeutic agents. In fact, some plant extracts have been reported as antagonists of crude snake venoms and toxins [8-11]. Likewise, different types of plant-derived compounds, such as flavonoids, coumarins, and other phenolic metabolites, have shown promise in neutralizing the PLA_2 effects [12-15]. The species *Swietenia macrophylla* King (Meliaceae), also known as mahogany, was selected as a promising lead after screening thirty-six ethanolic leaf extracts, in a search for their neutralizing activity against *Bothrops asper* venom and PLA_2 isolated from the venom of *Crotalus durissus cumanensis* [16,17]. The tree is native to tropical America [18] and numerous biological properties have been reported for its leaves and twigs, including antidiabetic, tumor inhibition, anti-inflammatory, antifungal, antimalarial and insect antifeedant activities [19].

The purpose of this study was to identify the PLA_2 inhibitors present in the crude extract of *S. macrophylla* that could be promising leads in neutralizing the local effects, and complement the traditional antivenoms. This involved the bioassay-guided fractionation of the ethanolic extract of the leaves of *S. macrophylla*, to characterize the metabolites that could be implicated in the observed bioactivity. Due to the challenges that snakebite accidents present in tropical countries, and the limited availability of antivenoms, the need for new alternatives to antagonize the activity of venoms and toxins is vital. In this way, the study of sustainable, locally available species is highly relevant.

MATERIALS AND METHODS

Reagents

All solvents used for the extraction and partition procedures were of analytical grade (Merck, Darmstadt, Germany). Reagents for the phytochemical analysis and the (+)-catechin (98% purity) used for bioassays, were from Sigma-Aldrich® (St. Louis, MO, USA). The derivatizing agent used (BSTFA+TMCS) was purchased from Supelco (Bellefonte, PA, USA).

Plant material

For the extraction and bioassay-guided fractionation, *S. macrophylla* leaves (2 kg) were collected in the tropical and pre-mountain forests of Medellin, Colombia, at 1600 m.s.l (6°15'41" N, 75°34'35.5" W). The voucher specimen, labeled as TL-103, was deposited in the Herbarium of the Universidad Nacional, Gabriel Gutiérrez Villegas (MEDEL) and identified by Professor Mauricio Sanchez Saenz (Universidad Nacional de Colombia, sede Medellin).

Extraction and bioassay-guided fractionation of *Swietenia macrophylla*

The leaf plant material was dried at room temperature, milled, and extracted by percolation at room temperature overnight with 90% ethanol (1L x 100 g). The ethanol extract was filtered and concentrated under reduced pressure, using a rotary evaporator (Büchi R-144) at a temperature below 40°C. The resultant ethanolic extract was mixed with distilled water to 10%, and then defatted with hexane. Subsequently, the ethanolic aqueous extract was evaporated and lyophilized.

For the fractionation of the resulting *S. macrophylla* extract (140 g), silica gel 60 F_{254} open column chromatography was carried out, using a gradient of dichloromethane-acetone-methanol, beginning with the less polar solvent and ending with methanol. According to the chromatographic profiles obtained by thin layer chromatography (TLC), twenty-five initial fractions were combined to afford eight final fractions that were evaluated in the bioassays described below.

Characterization of compounds using gas chromatography coupled with mass spectrometry (GC-MS)

The chemical profiles of the ethanolic extract and the most promising biologically active fractions of *S. macrophylla* were analyzed using a gas chromatograph (Agilent 6890), coupled with a mass spectrometer (Agilent 5973), employing a capillary column of fused silica (Agilent HP-5, 0.25 mm x 30 m x 0.25 μm) covered with 5% phenyl methyl siloxane. All of the samples (extract and fractions) were derivatized before injection according to the method described by Silici and Kutluca [20]. One mg of each sample was diluted in 50 μL of pyridine and a mixture of 100 μL of BSTFA (*N,O*-bis(trimethylsilyl) trifluoroacetamide) with 1% of TMCS (trimethylchlorosilane) was added. The mixture was heated for 30 min at 100°C. For each sample, 5.0 μL was injected, using helium gas grade 5 (AGA Fano S.A., UAP 99.999 %) at a flux of 1.0 mL/min (linear velocity 37 cm/s). The injection used the split-less mode with an initial temperature of 200°C for 3 min, which was raised to 250°C and maintained for 1 min. Finally, the temperature was raised by 2°C per minute to a maximum of 350°C for 60 min. To obtain the mass data, the detector was fixed at 350°C. The run was made using the SCAN mode between *m/z* 30-800.

The chromatograms were analyzed with AMDIS software (Automated Mass Spectral Deconvolution and Identification System), and the spectral database NIST 98 (2001). The identification of compounds was done through comparison of the mass spectral fragmentation patterns of each compound with the databases mentioned.

Determination of (+)-catechin in *Swietenia macrophylla*

The confirmation and quantification of (+)-catechin in the ethanolic extract and active fractions of *S. macrophylla* was performed through HPLC analysis. The separation of the compound was done using an ultra-aqueous C_{18} column with a particle size of 5 μm (250 mm x 4.6 mm, Merck). As a mobile phase, methanol (A), and formic acid (0.1%) were used in a gradient system of elution of 0.01 min 60% of A; 5-12 min 80% of A; 13-14 min 60% of A. The mobile phase flux was 1.0 mL/min. The identification was done by comparison with a standard of (+)-catechin (Sigma).

Venoms and toxins

Crotalus durissus cumanensis (Colombian rattlesnake), and *Bothrops atrox* venom was obtained from specimens from Meta (southeast region of Colombia) and *B. asper* venom was obtain from

specimens from Antioquia and Chocó (western region of Colombia). Venoms were obtained by manual extraction of the specimens that are kept in captivity at the Serpentarium (Universidad de Antioquia, Colombia). Venoms were centrifuged at 800 g for 15 min, and supernatants were lyophilized and stored at -20 °C until used.

Purification of PLA_2s

The Lys 49 PLA_2 was obtained from *B. atrox*, and Asp 49 from *C. d. cumanensis*. Each venom was initially chromatographed by cationic interchange on CM Sephadex and molecular exclusion on Sephadex G-75 respectively. Peak of interest were purified by reverse-phase HPLC on C_{18} column eluted at 1 mL/min with a gradient from 0% to 100% acetonitrile in 0.1% trifluoroacetic acid (v/v). Absorbance in the effluent solution was recorded at 280 nm [21]. Snake venom PLA_2 are enzymes that are able to resist extreme chemical conditions, such as low pH, higher ion concentrations and temperature, among others [21, 22]. In addition, several studies have demonstrated that snake venom PLA_2 can be purified by HPLC using acetonitrile without changes in their enzyme and biological activities [21, 23, 24].

Inhibitory activity of PLA_2 using e*gg yolk as substrate*

The inhibitory activity of the extract, fractions and compounds against the PLA_2 present in the venom of *B. asper* was assayed using egg yolk phospholipids suspended in 1% Triton X-100, 0.1 M Tris HCl, 0.01 M $CaCl_2$, buffer (pH 8.5) according to the method reported by Dole [25]. Measurement of the inhibition of the PLA_2 activity was conducted at different concentrations (w/w toxin:fraction) with 10 μg of venom in the presence of the substrate. Samples were incubated for 30 min at 37°C before calculation of the inhibition percentage, where 0 % is the activity induced by PLA_2 alone.

Inhibitory activity of PLA_2 using *4- Nitro-3-octanoyloxy-benzoic acid (4N3OBA) as substrate*

The measurements of enzymatic activity using the monodispersed substrate 4N3OBA were performed according to the method described by Holzer and Mackessy [26], and adapted for a 96-well ELISA plate. The Asp 49 PLA2 isolated from *C. d. cumanensis* was used in this assay. The standard assay contained 200 μL of buffer (10 mM Tris–HCl, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0), 20 μL of 10 mM of substrate (4NO3BA), 20 μL of sample (20 μg PLA_2 or 20 μg PLA_2 + several concentrations of active fraction or (+)-catechin) and 20 μL of water. The negative control was only the buffer solution. The inhibitory effect of the active fraction F5 and (+)-catechin on PLA_2 activity was determined through co-incubation of the enzyme with each concentration of the compound for 30 min at 37°C. 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 released 3-hydroxy-4-nitrobenzoic acid was proportional to the enzymatic activity, and the initial velocity (V_o) was calculated considering the absorbance measured at 20 min.

Inhibition of cytotoxic activity induced by PLA_2

The inhibitory ability of crude extract and fraction F5 on the cytotoxicity caused by *B. asper* venom or a Lys 49 PLA_2 isolated from *B. atrox* venom were evaluated using murine skeletal muscle C2C12 myoblasts (ATCC CRL-1772), as reported [27]. For the assay, 100 μg of toxin or 20μg of *B. asper* were incubated for 30 min at 37°C with *S. macrophylla* or their F5 fraction and diluted in 150 μL of medium DME (Dulbecco's Modified Eagle's Medium supplemented with 1% fetal calf serum). After this period, the mixture was added to the plates (150 μL/plate) and incubated for 3 h at 37°C. An aliquot of the supernatant was collected to detect the activity of the enzyme lactate dehydrogenase (LDH; EC 1.1.1.27) through a kinetic method (Wiener LDH-P UV). Reference controls to determine the 0 and 100 % of cytotoxicity were the medium and the medium with 0.1% (v/v) of Triton X-100, respectively. Additional controls consisted of incubated cells with samples and without the toxins. The results are expressed as inhibition percentages, considering the toxin and the culture media as 100 and 0 % of activity, respectively.

Molecular docking studies using (+)-catechin as ligand

The program Avogadro 1.1.0 [28] was used to build the (+)-catechin molecule showed in Figure 1. The same software was used to improve its overall structure using an energy minimization process based on the MMF94 force field by means of a steepest-descent algorithm in 500 steps. Molecular

docking was carried out on a personal computer using Autodock Vina [29]. The PLA_2 (PDB code 2QOG) from *C. d. terrificus* showed 57% homology in the *N*-terminal with the Asp49 PLA_2 used in these studies [21]. Protein was used with the exclusion of water molecules. The structure of the protein was prepared using the Protein Preparation module implemented in the Maestro program. First, hydrogen atoms were automatically added to each protein according to the chemical nature of each amino acid, on the basis of the ionized form expected in physiological conditions. This module also controls the atomic charges assignment. Second, each 3D structure of the protein was relaxed through constrained local minimization using the OPLS (Optimized Potentials for Liquid Simulations) force fields in order to remove possible structural mismatches due to the automatic procedure employed to add the hydrogen atoms. When necessary, bonds, bond orders, hybridizations, and hydrogen atoms were added, charges were assigned (a formal charge of +2 for the Calcium ion) and flexible torsions of ligands were detected. The α-carbon of His48 was used as the center of the grid (X = 44.981, Y = 27.889 and Z = 46.392), whose size was 24 $Å^3$. Exhaustiveness = 20. Then, the ligand poses with the best affinity were chosen, and a visual inspection of the interactions at the active site was performed and recorded. Molegro Molecular Viewer (MMV 2.5.0, http://www.clcbio.com/products/molegro/#molecular-viewer) and UCSF Chimera (www.cgl.ucsf.edu/chimera/) were used to generate docking images.

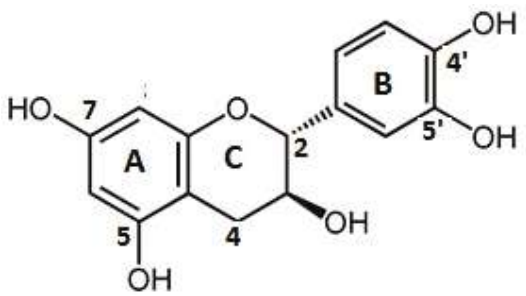

Figure 1. Structure of (+)-catechin

Statistical analysis

To identify the variation in the biological activity, a one-way analysis of variance (ANOVA) was carried out. When significant differences were detected ($\alpha \leq 0.05$), a Tukey's range test, with a confidence level of 95%, was done to establish the differences in each level of activity. All tests were done in triplicate and are expressed as the mean ± standard deviation using R, version 2.15 [30].

RESULTS

Biological activity of extract and fractions

The ethanolic extract of *S. macrophylla* leaves exhibited an 87.2 ± 11.0% effect in neutralizing the phospholipase enzymatic activity of the *B. asper* venom using egg yolk as substrate. Furthermore, as mentioned Table 1, there was a differential in the activity between the eight fractions derived from the leaf extract, with the most significant activity found in fraction F5.

Table 1. Inhibition of the enzymatic activity of PLA_2 from *Bothrops asper* venom by fractions of *Swietenia macrophylla* leaf extract using egg yolk as substrate

Fraction	Percentage of Inhibition of PLA_2 1:10 (w/w venom:fraction ratio) *	Tukey significant differences ($\alpha \leq 0.05$)**
F1	55.6 ± 11.7	a
F2	58.8 ± 17.6	a
F3	58.1 ± 12.9	a
F4	71.4 ± 8.4	b
F5	80.3 ± 10.6	b
F6	63.3 ± 4.0	ab
F7	63.3 ± 12.5	ab
F8	64.3 ± 12.3	ab

*Results expressed as the mean ± standard deviation (n=3)

**The different letters represent significant differences among the results.

Because of the promising results obtained with fraction F5, it was selected for further study. As described Table 2, Fraction F5 and (+)-Catechin inhibited the PLA_2 activity of *B. asper* venom, in a dose-dependent way. Also the ethanolic extract and fraction F5 showed inhibition of the cytotoxicity induced by the *B. atrox* venom and their Lys 49 PLA_2 (80 and 100% respectively).

Table 2. Inhibition of the enzymatic activity from the of *B. asper* venom by fraction F5 of *Swietenia macrophylla* leaf extract and (+)-catechin, using egg yolk as substrate

Sample	Percentage of Inhibition of PLA_2 (w/w venom:fraction ratio)*		
	1:10	1:5	1:1
Fraction F5	80.3 ± 10.6	59.1 ± 7.8	27.9 ± 6.8
(+)-Catechin	89.8 ± 12.0	61.1 ± 6,3	34.3 ± 7.5

*Results expressed as the mean ± standard deviation (n=3)

Characterization of fraction F5 of Swietenia macrophylla

In the mass spectra of the ethanolic extract and fraction F5 of *S. macrophylla* the fragmentation patterns of phenolic compounds were seen. Catechin was detected at the end of the elution period as shown in Figure 2B. A compilation of the proposed identifications of the compounds found in the extract and fraction F5 through mass spectral analysis are shown in Table 3. Through HPLC, it was possible to confirm that the isomer of catechin in fraction F5 was (+)-catechin, at a concentration of 0.184 mg/mL.

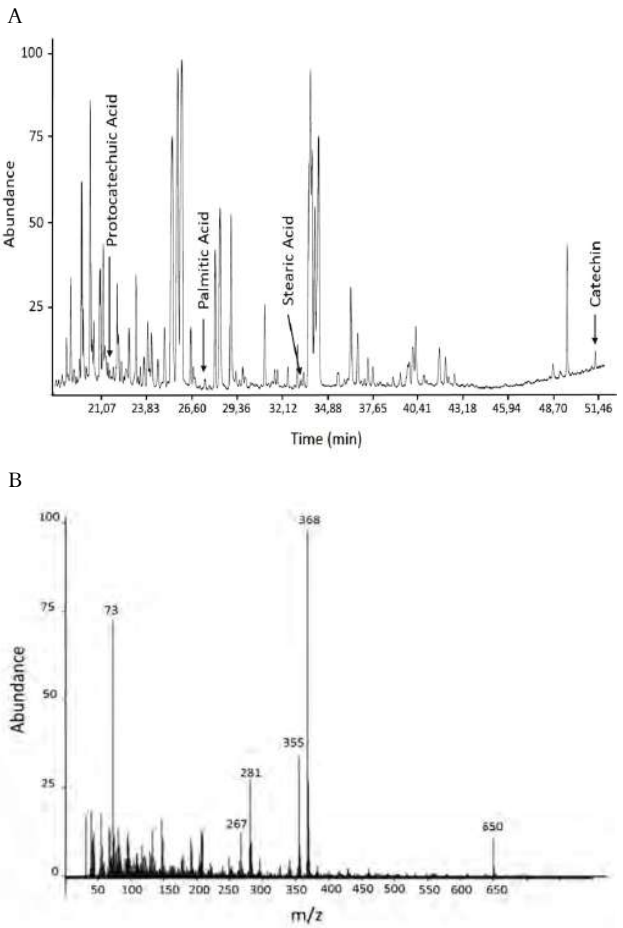

Figure 2. GC-MS of fraction F5. A. Gas chromatogram of F5. B. Mass spectrum of catechin eluted at 51.46 minutes.

Bioactivity of (+)-Catechin

Due to the high concentration of (+)-catechin in fraction F5, additional biological evaluation was carried out on the pure compound. As described Table 2, (+)-catechin inhibited the PLA_2 enzymatic activity of the *B. asper* venom, in a dose dependent way. Moreover, to support the hypothesis that the biological activity observed for fraction F5 is mainly due to the presence of (+)-catechin, a spectrophotometric assay was done to quantify the inhibition on the enzymatic activity of Asp49 PLA_2 purified from the venom of *C. d. cumanensis.* As substrate, 4N3OBA was used and 18% of (+)-catechin, in an attempt to reflect the same percentage of this compound present in fraction F5, as determined by the GC-MS analyses. In this case, the inhibition of the PLA_2 by (+)-catechin was 83.1 ± 3.1%. In addition, (+)-catechin inhibited by 83.3 ± 11.2% the liberation of lactate dehydrogenase induced by Lys 49 PLA_2 from *B. atrox* in cytotoxicity assay.

Molecular docking

To support the results obtained with the bioassays of (+)-catechin, and in an attempt to explain the mechanism of neutralization of the PLA_2 by this metabolite, molecular docking studies of (+)-catechin were performed. The observed binding free energy of (+)-catechin was -8.6 kcal/mol. Docked solution with the lowest binding energy was selected and described. Docking results are displayed in Figure 3 an they suggested that OH from 4´ and 5' carbons of (+)-catechin could form hydrogen bonds with carboxylate moiety of residue Asp49, while OH from 5 could form a hydrogen bond with Asn6 (Figure 2). In addition, our results suggested a π-π stacking interaction between rings A of (+)-catechin with that of the residues Phe5 and Tyr22 of the toxin; and ring B of the flavonoid and Tyr52 of the enzyme. Additional Van der Waals interactions with Ala18, Ala23, Cys29, Gly30, His48 and Lys69 were also detected (distance lower than 3.5 Å). As shown in Figure 4, the effect of (+)-catechin in blocking the hydrophobic channel of the enzyme may be observed.

Table 3. Mayor compounds detected through GC-MS in the ethanolic leaf extract and fraction F5 of *Swietenia macrophylla*

Compound	Abundance in F5	Fragments of the trimethylsilyl derivatives (*m/z*)
Palmitic acid	10.2%	73 (99.9), 117 (77.1), 75 (73.0), 313 (63.9), 132 (49.4), 129 (33.1), 43 (33.1), 145 (31.6), 55 (26.0), 41 (24.2)
Stearic acid	6.68%	73 (99.9), 117 (73.8), 75 (70.0), 132 (50.2), 341 (42.0), 145 (32.9), 129 (30.2), 55 (29.6), 41 (27.3)
Protocatechuic acid	14.3%	193 (99.9), 73 (47.1), 370 (42.0), 355 (25.9), 311 (15.1), 194 (14.7), 371 (13.1), 281 (9.3), 356 (9.0), 223 (7.7)
(+)-Catechin	17.9%	368 (99.9), 73 (72.0), 355 (35.0), 369 (34.0), 370 (16.0), 650 (13.0), 267 (12.0), 356 (12.0), 383 (10.0), 179 (8.0)

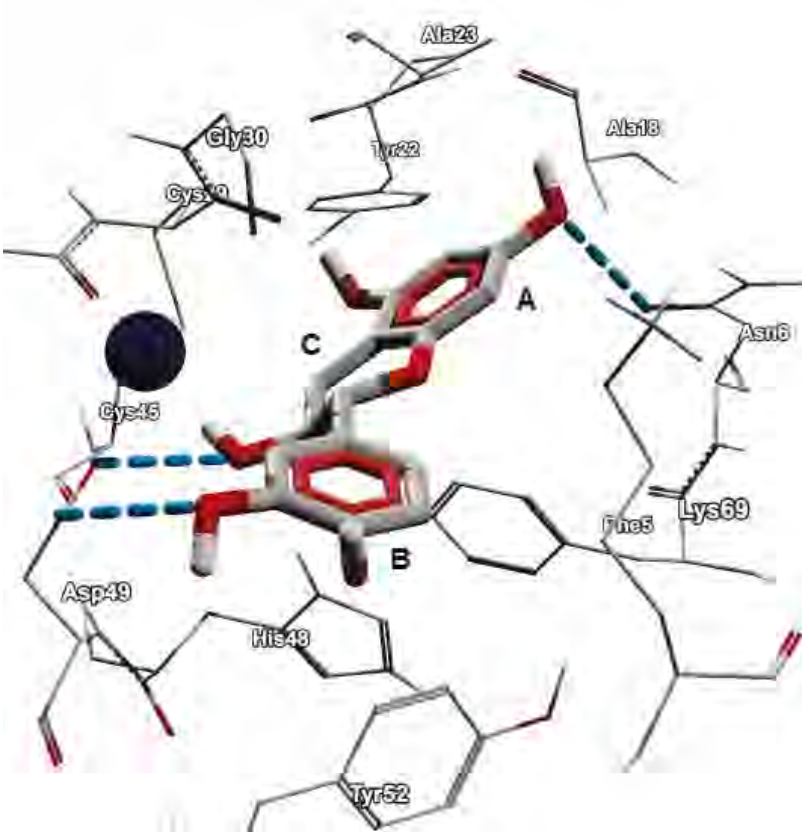

Figure 3. Molecular docking of (+)-catechin with a PLA_2 from the venom of *Crotalus durissus terrificus*. The blue sphere represents Ca^{2+}. The dotted blue lines represent the hydrogen bonds. All the amino acids of the enzyme at a maximum distance of 3 Å are shown.

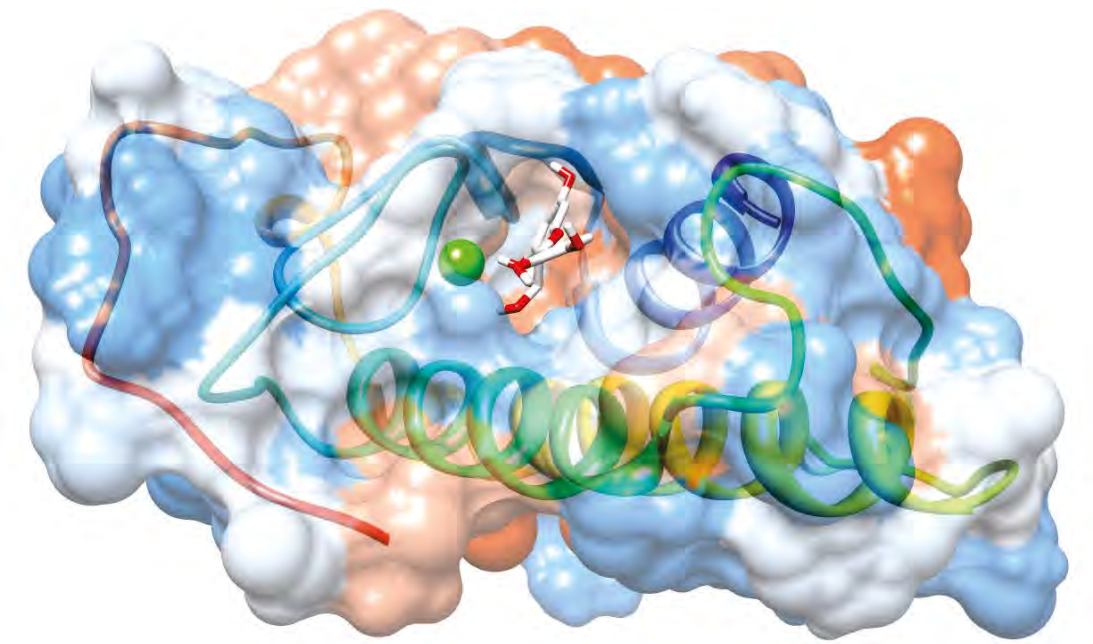

Figure 4. Molecular surface of PLA_2 and interaction of (+)-catechin with the hydrophobic channel and the active site of the protein. The red areas of the surface represent the acid regions; the white areas represent the neutral and the blue areas the basic regions. The green sphere represents Ca^{2+}. (+)-Catechin is shown as white (carbon and hydrogen atoms) and red sticks (oxygen atoms).

DISCUSSION

In this study, the inhibitory activity of 87.2 ± 11.0% for the extract led to bioassay-guided fractionation, wherein all the fractions exhibited activity, but the most significant inhibitory potential was observed for fractions F4 and F5. Fraction F5 was also evaluated against PLA_2s present in *B. asper* venom, and dose-dependent activity was exhibited. These results are comparable to those reported by Pereañez et al. [16], in which an inhibition level of 59.3 ± 3.5% was found for the crude extract of the leaves of *S. macrophylla*. Similarly, the total inhibition of the cytotoxic activity caused by a Lys49 PLA_2, by fraction F5 was evidenced. Pereañez et al. [17] had reported inhibition of the edema induction, myotoxic and anticoagulant activities induced by an Asp49 PLA_2, from *C. d. cumanensis* venom. Moreover, given the promising bioactivity of fraction F5, in the characterization of the major compounds by GC-MS it was relevant to find two phenolic compounds in the derivatized fraction F5, namely protocatechuic acid, and catechin. Protocatechuic acid is an antioxidant that also has inhibitory capacity against the PLA_2 of snake venoms [31]. Likewise, various isomers of catechin have been studied because of their inhibitory activity of PLA_2 present in snake venoms [12, 32]. The presence of these two compounds was proposed based on their mass spectral fragmentation patterns, and confirmed by comparison with the spectral database NIST 98. The data obtained with GC-MS for these compounds was in accordance with the literature [33]. In this way, the TMS derivative proposed as protocatechuic acid presented a molecular ion with *m/z* 370 and a base peak at *m/z* 193. For the flavan-3-ol, catechin, the derivatized sample exhibited a molecular ion with *m/z* 650 and a characteristic base peak of *m/z* 368 from the excision of the heterocyclic ring through a Retro-Diels Alder fragmentation were the keys to identify this compound [34].

The fact that fraction F5 had the most promising activity, and since catechin was a major compound within this fraction (Table 3), led to a study of the activity of catechin against PLA_2. For the biological activity, a commercial sample of (+)-catechin was used, and the determination of the isomer and its purity was made through HPLC. As observed in Table 3, (+)-catechin was a promising compound with dose-dependent activity. Additionally, in the spectrophotometric assay using the monodispersed substrate 4N3OBA, (+)-catechin presented inhibition of PLA_2. For this assay, the same concentration of (+)-catechin present in the fraction F5 was used to keep a ratio of 1:10. In this way, it was suggested that the percent inhibition of the enzyme PLA_2 by (+)-catechin is similar to that in the fractions, using the same ratio venom: compound. This was done to determine if (+)-catechin was the main compound responsible for the bioactivity of fraction F5, which can be positively suggested based on the results obtained and displayed in Table 2.

Another significant finding was the total inhibition of the cytotoxicity induced by Lys49 PLA_2s from *B. atrox*, observed by fraction F5 suggesting that it reduces the myonecrosis induced by this type of toxins. The Lys49 PLA_2 in relation with Asp49 showed several substitutions in amino acids residues, but the replacement of amino acid Asp49 by the amino acid Lys, is the principal cause that leads to a loss of the enzyme activity, especially because it damages the loop that binds to calcium [35, 36]. Nevertheless, it is reported that the Lys49 PLA_2 induces local myotoxicity and edema through a mechanism that is not well established, in which a participation of the *C*-terminal region has been proposed [35]. In this way, to find a compound that inhibits this kind of toxins is a relevant outcome. The polyphenol rosmarinic acid isolated from diverse species of the Boraginaceae and Lamiaceae, reduced 80% and 90 % the muscle damage and the neuromuscular blockade induced by PrTX-I (a PLA_2 Lys 49 from the venom of Bothrops pirajai) on mice neuromuscular (phrenic-diaphragm) preparations. X-ray crystallographic studies between two molecules demonstrated that rosmarinic acid obstruct the entrance of the hydrophobic channel in PrTX-I affecting the interaction with membrane [37].

The molecular docking experiment performed with an Asp49 PLA_2 enlightens how (+)-catechin can bind to the PLA_2 active site. In this way, this metabolite can interact by hydrogen bonds mediated by the hydroxyl at carbons-4´and 5´of ring B and the hydroxyl of carbon-3 of ring C with Asp49, which, in this case, is one of the residues implicated in the coordination of calcium required for the enzymatic catalysis. As a result, this interaction of (+)-catechin-enzyme could destabilize the cofactor binding. In the same way, it is evident that (+)-catechin could form Van der Waals interactions with residues on the interfacial binding surface (Asn6, Ala 18, and Lys69),

which is the region of the PLA_2 that allows adsorption of the enzyme onto the lipid-water interface of the phospholipids membrane bilayer. Thus, these interactions could block the recognition of aggregated phospholipids. All of the interactions mentioned, suggest the mode of interaction of (+)-catechin with PLA_2s. Lindahl and Tagesson [38] informed that hydroxyl groups in 3´and 4´-position in the B-ring, 5-hydroxyl group in the A-ring, unsaturation and the 4-oxy in the C-ring appear to be important for the overall ability of flavonoids to inhibit PLA_2 activity. (+)-Catechin has some of these structural characteristics, which could support the inhibitory activity of the compound. In addition, it is important to mention that these authors also stablished that removal or addition of one hydroxyl group in the B-ring retains a strong PLA_2 inhibition but leads to a decreased selectivity towards PLA_2 from group II [38]. This information is in agreement to that reported by Lättig and collaborators [39], which points out the significance of hydroxyl groups in flavonoids for the inhibition of PLA_2 type II.

CONCLUSIONS

The crude extract of the leaves of S*wietenia macrophylla* exhibited strong inhibitory activity against the PLA_2s enzymes present in the venoms of *B. asper, B atrox* and *C. d. cumanensis*,. Catechin, is one of the components of the most active fraction, F5. In molecular docking studies, (+)-catechin was shown to bind to the active site, and interacts through hydrogen bonds with the amino acid Asp49, which is implicated in the calcium chelation (enzyme cofactor). It is also suggested that (+)-catechin blocks the recognition of aggregated phospholipids, while the aromatic rings A and B can bind through π-π interactions to the aromatic rings of residues Tyr22, Phe5, and Tyr52.

ACKNOWLEDGMENTS

Authors are thankful to Professor Emeritus Geoffrey Cordell, University of Florida, for his suggestions and for improving the text. L. Preciado acknowledges the School of Chemistry for teaching assistantships during the period of her graduate student training. The authors thank the Comité para el Desarrollo de la Investigación and Sostenibilidad 2015–2016 and Project CIQF-217, Universidad de Antioquia and Universidad Nacional Colombia, sede Medellín.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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