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Quercetin as an inhibitor of snake venom secretory phospholipase A2

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ABSTRACT

As polyphenolic compounds isolated from plants extracts, flavonoids have been applied to various pharmaceutical uses in recent decades due to their anti-inflammatory, cancer preventive, and cardiovascular protective activities. In this study, we evaluated the effects of the flavonoid quercetin on *Crotalus durissus terrificus* secretory phospholipase A2 (sPLA2), an important protein involved in the release of arachidonic acid from phospholipid membranes. The protein was chemically modified by treatment with quercetin, which resulted in modifications in the secondary structure as evidenced through circular dichroism. In addition, quercetin was able to inhibit the enzymatic activity and some pharmacological activities of sPLA2, including its antibacterial activity, its ability to induce platelet aggregation, and its myotoxicity by approximately 40%, but was not able to reduce the inflammatory and neurotoxic activities of sPLA2. These results suggest the existence of two pharmacological sites in the protein, one that is correlated with the enzymatic site and another that is distinct from it. We also performed molecular docking to better understand the possible interactions between quercetin and sPLA2. Our docking data showed the existence of hydrogen-bonded, polar interactions and hydrophobic interactions, suggesting that other flavonoids with similar structures could bind to sPLA2. Further research is warranted to investigate the potential use of flavonoids as sPLA2 inhibitors.

1. Introduction

Phospholipases A2 (PLA2, EC 3.1.1.4) are small proteins that catalyze the hydrolysis of glycerophospholipids at the sn-2 position in a Ca^{2+}-dependent reaction, releasing lysophospholipids and fatty acids [1–3]. These enzymes are the main component of snake venom and have been investigated not only because they have a wide range of biological effects, but also due to their similarity to mammalian phospholipases [4,5]. However, in contrast to their mammalian counterparts, several snake venom PLA2s are toxins that induce pharmacological effects [6] through arachidonic acid metabolism leading to the production of various lipid pro-inflammatory mediators such as prostaglandins, thromboxanes and leukotrienes [7]. Recent studies have shown that inhibition of cytosolic PLA2 (cPLA2) leads to a decrease in eicosanoid levels and, reduced inflammation [8].

Due to the role of PLA2s in the inflammatory process, there is pharmacological interest in PLA2 inhibitors, and among these, the flavonoids have been successfully studied. Flavonoids are widely produced in plants tissues making them suitable targets for pharmaceutical extraction and chemical synthesis [8,9]. The inhibitory effect of flavonoids on secretory PLA2 (sPLA2) was reported by Gil et al. [10], and Lindahl and Tagesson [11]. Their results showed that inhibition of sPLA2 from different sources following incubation with various flavonoids is dependent on the 5-hydroxyl group as well as the double bond and the double-bonded oxygen in the oxane ring, and that the hydroxyl groups at the 3′- and 4′-

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position are required for selective inhibition of PLA2 [11]. However, the exact mechanism by which flavonoids inhibit PLA2 remains unclear. Iglesias et al. [12] showed that morin modifies the secondary structure of sPLA2 from *Crotalus durissus cascavella* venom, but did not significantly affect its pharmacological activities.

Although there are studies of flavonoids as PLA2 inhibitors, the mode of binding of flavonoids to PLA2 as well as their inhibitory mechanism is still not clear. The aims of this article are to investigate the effect of quercetin, a widely spread flavonoid, on the structure and function of a sPLA2 isolated from *Crotalus durissus terrificus,* to increase our understanding of the mode action of polyphenolic compounds on the snake venoms, and to evaluate therapeutic application against the symptoms caused by snake bites. In the last decades the action of quercetin on the PLA2 has been studied. In 1993 Lindahl and Tagesson [13] showed that quercetin is a potent inhibitor of PLA2 (group II) from *Vipera russuli*. In addition, Lättig et al. [8] showed through computational studies chemical interactions between humans' sPLA2 and quercetin. Due to these characteristics, quercetin has been chosen as flavonoid model. Moreover we also propose, through high-resolution three-dimensional (3D) data (molecular docking), a structural model for understanding the molecular interactions between sPLA2 and quercetin, and how it can influence enzymatic and pharmacological activities of sPLA2.

2. Materials and methods

2.1. Venom, animals and reagents

C. durissus terrificus venom was purchased from Bio-Agents Serpentarium (Batatais, São Paulo, Brazil). Analytical HPLC- and sequencing-grade solutes and solvents were purchased from various suppliers (Bio Rad, Sigma Aldrich, Boehringer Mannheim, and Applied Biosystems). Female Swiss mice (18–20 g) used in the pharmacological assays were obtained from the Multidisciplinary Center of Biological Investigations (CEMIB-UNICAMP) and male chicks were obtained from Itu farm in Campinas City. All animal experiments were approved by the Ethics Committee of State University of Campinas (São Paulo, Brazil) under the number 1916-1.

2.2. Purification of sPLA2

Whole *C. durissus terrificus* venom was first fractioned as previously described by Toyama et al. [14]. Dried venom (45 mg) was dissolved in ammonium bicarbonate buffer (1.0 M; pH 8.0) and clarified by centrifugation (4500 × *g* for 1 min). The sPLA2 from *C. durissus terrificus* was eluted using a non-linear gradient of acetonitrile 66% in 0.1% of trifluoroacetic acid (TFA) by reverse-phase chromatography using a Supelco C5 column (0.10 cm × 25 cm) with flow rate of 1 mL/min with absorbance monitoring at 280 nm. The resulting PLA2 was termed sPLA2 and its purity was evaluated by SDS-PAGE.

2.3. Incubation of sPLA2 with quercetin and purification of modified sPLA2

The incubation of sPLA2 with quercetin (mol:mol) was according to the procedure described by Zhao et al. [15]. Quercetin was dissolved in dimethyl sulfoxide (DMSO), and its concentration never exceeded 1% during incubation. Quercetin (400 μL of a 0.1 mM solution) was added to 400 μL of a homogenized, purified sPLA2 solution (1 mg/mL). The mixture was incubated for 90 min at room temperature, and 200-μL samples of this mixture were loaded onto a preparative reverse-phase column to separate the treated enzyme (sPLA2:Q) from quercetin. After column equilibration with HPLC buffer A (aqueous 0.1% TFA), samples were eluted using a discontinuous gradient of HPLC buffer B (66.6% of acetonitrile in 0.1% TFA) at a constant flow rate of 1.0 mL/min. The chromatographic run was monitored at 280 nm.

2.4. Electrophoresis

Electrophoresis was carried out following the Laemmli method [16]. The degree of purity of fractions was assessed by discontinuous electrophoresis using a final acrylamide concentration of 12.5% in the resolving gels (1.0 M Tris–HCl, pH 8.8) and 5% in the stacking gel (0.5 M Tris–HCl, pH 6.8). Electrophoretic separation was carried out in a 250 Mighty Small (Hoefer Scientific Instruments) for SDS-PAGE. All samples and the molecular marker were treated with SDS and 1.0 M dithiothreitol (DTT), and the run was conducted at 60 mA for stacking gel and 90 mA for running gel. After electrophoresis, samples were stained with Coomassie brilliant blue R-250.

2.5. Circular dichroism spectroscopy

sPLA2 and sPLA2:Q (sPLA2 + quercetin) were dissolved in 10 mM sodium phosphate buffer (pH 7.4) and final protein concentrations were adjusted to 8.7 mM. After centrifugation at 4000 × *g* for 5 min, samples were transferred to a 1-mm path length quartz cuvette. Circular dichroism spectra in the wavelength range 185–300 nm were acquired in-house with a J720 spectropolarimeter (Jasco Corp., Japan) using a bandwidth of 1 nm and a response time of 1 s. Data collection was performed at room temperature, with a scanning speed of 100 nm/min. Nine scans were obtained for each sample, and all spectra were corrected by subtracting buffer blanks.

2.6. Intrinsic fluorescence.

The relative intrinsic fluorescence intensities of sPLA2 and sPLA2:Q were monitored with a Varian Cary Eclipse. The proteins were solubilized in water at room temperature. The measurements were performed in a 1.5-mL 1-cm path length quartz cuvette. Fluorescence was measured between 300 and 450 nm after excitation at 280 nm.

2.7. Mass spectrometry

The molecular mass of sPLA2 and sPLA2:Q were determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems). One microliter of samples (sPLA2 and sPLA2:Q) in 0.1% TFA was mixed with 2 μL of the matrix α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% TFA (v/v). The matrix was prepared with 30% acetonitrile and 0.1% TFA (v/v). Ion masses were determined with an acceleration voltage of 25 kV, the laser operated at 2890 μJ/com2, a 300-ns delay, and the linear analysis mode.

2.8. Measurement of sPLA2 activity

sPLA2 activity was measured following the protocol described by Lee et al. [17] and modified by Toyama et al. [14] in 96-well plates, using 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA, BIOMOL, USA) as substrate. Enzyme activity, expressed as the initial velocity of the reaction (Vo), was calculated based on the increase in absorbance after 20 min. All assays were performed with absorbance at 425 nm using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA). After the addition of native or treated sPLA2 (20 μg), the reaction mixture was incubated for up to 40 min at 37 °C and the absorbance read at 5-min intervals.

2.9. Antibacterial activity

The antibacterial activity was assayed as described by Santi-Gadelha et al. [18] and the structural modification was done following the method of Toyama et al. [19]. *Clavibacter michiganensis michiganensis* cells were harvested from fresh agar plates and suspended in sterile distilled water ($A_{600\,nm} = 3 \times 10^8$ CFU/mL). Aliquots of bacterial suspension were diluted to 10^3 colony-forming units/mL (CFU/mL) and incubated with sPLA2 or sPLA2:Q samples (75 μg/mL) for 60 min at 28 °C. Survival was assayed on nutrient agar (Difco) plates ($n = 5$). For both antibacterial assays, electron microscopy assessments of morphologic alterations were performed in the presence of saline (negative control), quercetin (100 μM, quercetin control), sPLA2, and sPLA2:Q.

2.10. Paw edema assay

Paw edema assays were performed with the aim of evaluate the inflammatory activity induced by sPLA2. For this assay female Swiss mice were used, since they show a lower aggressive behavior than male mice. Paw edema was induced by a single subplantar injection of 25 μL sPLA2 or sPLA2:Q (25 μg/paw). Paw volume was measured immediately before the injection and at selected time intervals thereafter (30, 60, 120, 180, and 360 min) using a hydroplethysmometer (model 7150, Ugo Basile, Italy). All samples were dissolved in sterile saline solution (0.9%). Results were expressed as the increase in paw volume (μL) and calculated by subtracting the basal volume from the volume following treatment.

2.11. Neurotoxic effect assay

Male chicks (4–8 days old) were killed with ether, and the biventer cervicis muscle was removed [20] and mounted under a resting tension of 1 g in a 4 mL organ bath containing aerated (95% O_2 + 5% CO_2) Krebs solution (118.7 mM NaCl, 4.7 mM KCl, 1.88 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25.0 mM $NaHCO_2$ and 11.65 mM glucose, pH 7.5) at 37 °C. A bipolar platinum ring electrode was placed around the tendon, which ran the length of the nerve trunk supplying the muscle. Indirect stimulation was applied with a Grass S4 stimulator (0.1 Hz, 0.2 ms, 3–4 mV). Muscle contractions and contractures were recorded by connecting the preparation to a force displacement transducer (Narco Biosystems Inc.) coupled to a Gould RS 3400 recorder. Contractures to exogenous acetylcholine (ACh, 55 or 110 μM for 60 s) and KCl (5 mM for 120–130 s) were obtained in the absence of nerve stimulation prior to the addition of sPLA2 or sPLA2:Q (10 μg/mL) and at the end of the experiment. The preparations were allowed to stabilize for at least 20 min before the addition of Ach, KCl, or a single concentration (10 μg/mL) of the compounds.

2.12. Platelet aggregation studies

Platelet aggregation activities were assayed as described by Toyama et al. [19]. Briefly, venous blood was collected with informed consent from healthy volunteers who denied taking any medication in the previous 14 days. Collected blood was immediately transferred into polypropylene tubes containing one-tenth of final volume of acid citrate dextrose (ACD-C; citric acid 3%, trisodium citrate 4%, glucose 2%; 1:9 v/v). Platelet-rich plasma (PRP) was obtained by centrifuging whole blood at 200 × *g* for 15 min. PRP was washed in a wash buffer solution (NaCl 140 mM, KCl 5 mM, sodium citrate 12 mM, glucose 10 mM and saccharose 12 mM; pH 6; 5:7 v/v) and centrifuged at 800 × *g* for 12 min at 20 °C. The platelet pellet was gently resuspended in Krebs–Ringer solution and counts were performed on a Neubauer chamber. The final platelet suspension was adjusted to 1.2×10^8 platelets/mL. Platelet aggregation was carried out using 400 μL of the washed platelet solution in a cuvette and incubated at 37 °C with constant stirring. The desired concentration of protein was added 3 min prior to the addition of a platelet aggregation inducer (thrombin). Aggregation was subsequently recorded for 5–10 min with an aggregometer (Chrono-log Lumi-Aggregometer model 560-Ca, Havertown, PA, USA). Aggregation experiments were performed with a concentration of 15 μg/mL of sPLA2 and sPLA2:Q.

2.13. Myotoxic activity

The liberation of creatine kinase (CK) from damaged muscle cells was followed by use of the CK-NAc kit (Laborlab) to measure the enzyme activity in mice plasma. Five groups of animals (18–22 g) were injected in the right gastrocnemius muscle with 25 μL of 1.0 mg/mL of sPLA2, sPLA2:Q, or quercetin ($n = 4$) while the control group received an equal volume of 0.15 M NaCl. Blood was collected from the tail after 1 h into tubes containing heparin. The amount of CK was determined using 4 μL plasma, which was incubated for 3 min at 37 °C with 1.0 mL of the reagent according to the kit protocol. Activity was expressed in units per liter (U/L).

2.14. Molecular modeling (docking)

The structural optimization of the quercetin ligand was initially achieved using the quantum chemical AM1 method [21] implemented in the BioMedCache program (BioMedCache, 1989) with default values for the convergence criteria. Docking calculations were performed with the GOLD 4.0 program [22] to obtain the *in silico* affinity of quercetin to the Crotoxin B target, a basic sPLA2 from *C. durissus terrificus* venom. This sPLA2 structure was taken from the RCSB Protein Data Bank [PDB] under the PDB code 2QOG. The structure of the B chain was chosen for calculations, maintaining the Ca^{2+} ion and the water molecule number 188, located 3.16 Å from Ca^{2+}.

Docking calculations were performed to consider the flexibility of the quercetin ligand in such a way that torsions were considered active during the calculation. The active site was defined as all atoms within a 10-Å radius from His48, an important residue according to the literature [23,24].

2.15. Statistical analyses

Results were reported as means ± SEM of replicate experiments. The significance of differences between means was assessed by an analysis of variance, followed by a Dunnett's test when several experimental groups were compared to the control group. The confidence limit for significance was 5%.

3. Results

3.1. Purification of sPLA2 and Incubation of sPLA2 with quercetin

sPLA2 was isolated from *C. durissus terrificus* venom through reverse-phase chromatography (Fig. 1a) and its enzymatic activity was evaluated using 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA) as a substrate. SDS-PAGE revealed the presence of one protein band with a molecular mass of 14 kDa (Fig. 1c), corresponding to sPLA2. After incubation with quercetin, sPLA2:Q (sPLA2 + quercetin) eluted at 23.7 min whereas sPLA2 had a retention time of 24.3 min (Fig. 1b). This difference indicates an interaction between sPLA2 and quercetin, which changed the hydrophobicity of sPLA2.

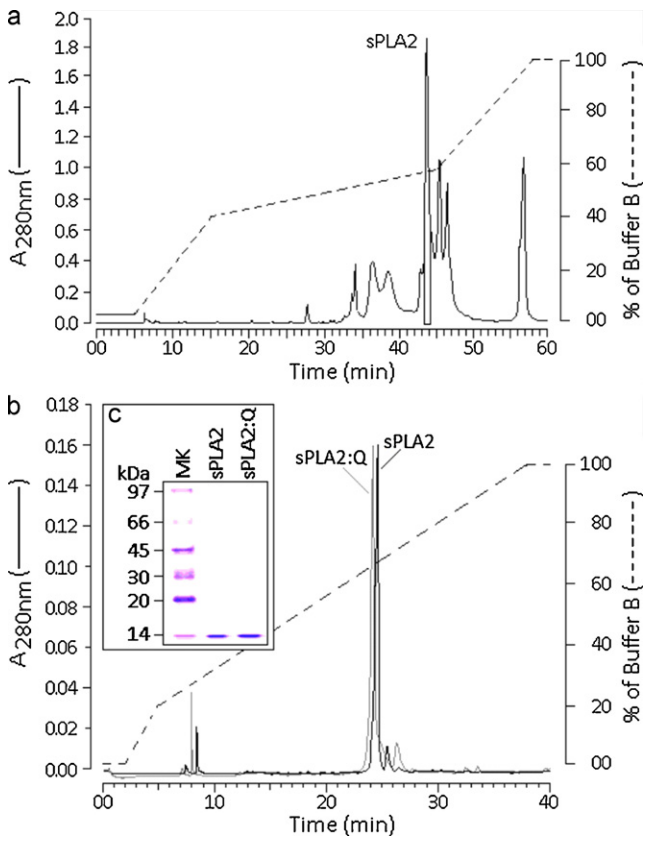

Fig. 1. Purification and chemical modification of secretory phospholipase A2 (sPLA2). (a) Fractionation of whole venom was performed by reverse-phase HPLC (C5 column 0.10 cm × 25 cm) using a non-linear concentration gradient of buffer to obtain a high purity protein. (b) Reverse phase HPLC profile of sPLA2 before and after modification with quercetin. (c) Tricine SDS-PAGE of sPLA2 from *Crotalus durissus terrificus*.

3.2. Circular dichroism spectroscopy

The effect of quercetin on the sPLA2 structure was evaluated by absorption spectra of sPLA2 and sPLA2:Q, circular dichroism, and fluorescence spectroscopy. As shown in Fig. 2a, few modifications were observed in the 270–280 nm wavelength region. However, some changes were observed on CD and fluorescence spectra after treatment with quercetin. CD spectra analysis showed modifications mainly in the region corresponding to the α-helices, suggesting that quercetin is able to induce changes in the secondary structure of this enzyme (Fig. 2b).

3.3. Intrinsic fluorescence

The presence of aromatic amino acids such as tryptophan and tyrosine in the protein chain allows the use of fluorescence spectra, which is sensitive for the investigation of protein conformation and ligand binding. Fig. 3a shows an increase in the intensity of fluorescence emission spectra after treatment with quercetin, suggesting that this flavonoid is able to change the structure of the protein at the tertiary structure level, however, fluorescence spectrum of quercetin has shown that this compound has a peak of fluorescence near the region of the tryptophan and possibly contributes to increase the fluorescence observed.

3.4. Mass spectrometry

Mass spectrometry by MALDI-TOF indicated that the exact molecular mass of the native protein is 14,425.56 Da while that of sPLA2:Q is 14,727.79 (Fig. 3b), an increase of 302.23 Da in sPLA2 treated with quercetin, suggesting that one molecule of quercetin is bound to the protein structure.

3.5. Measurement of sPLA2 activity and antibacterial activity

The effect of quercetin on sPLA2 enzymatic activity was evaluated. Both enzymes, sPLA2 and sPLA2:Q exhibit allosteric behavior (Fig. 4a), and quercetin strongly inhibits sPLA2 activity. The maximum velocity after 20 min for sPLA2 was 0.330 ± 0.04 vo/mol whereas for sPLA2:Q was 0.196 ± 0.02 vo/mol, showing a significant decrease of 40%. To analyze possible correlations between the enzymatic activity of sPLA2 and its antibacterial activity, bacterial viability was tested by CFU counting. The assay was performed using *C. michiganensis michiganensis* (Gram-positive). As shown in Fig. 4b, sPLA2 has a higher inhibitory potential on bacterial growth than sPLA2:Q, since sPLA2 decreased CFU levels to 9.8% (90.2% inhibition) compared to 63.5% (only 36.5% inhibition) for sPLA2:Q.

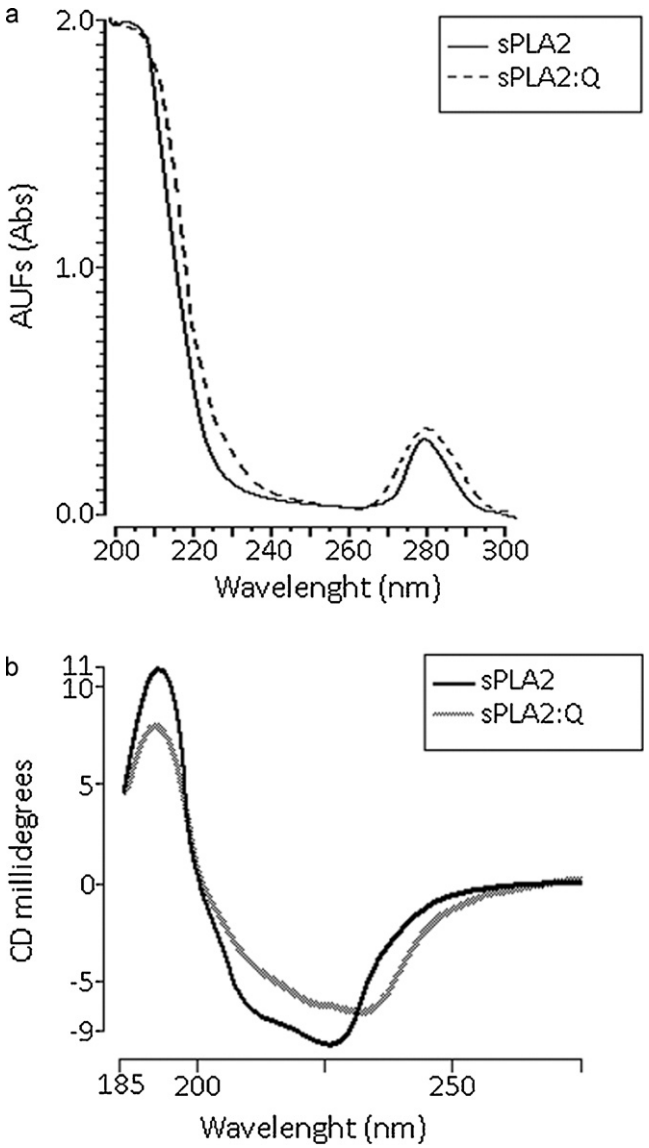

Fig. 2. UV/vis absorption and circular dichroism (CD) spectra. (a) Absorption spectrum of sPLA2 and sPLA2 after treatment with quercetin (sPLA2:Q) at wavelength intervals 200–300 nm. The proteins were analyzed at 280 nm. (b) CD spectra of native sPLA2 and sPLA2:Q. Data over the range 185–280 nm are shown. The CD spectra are expressed in theta machine units in millidegrees.

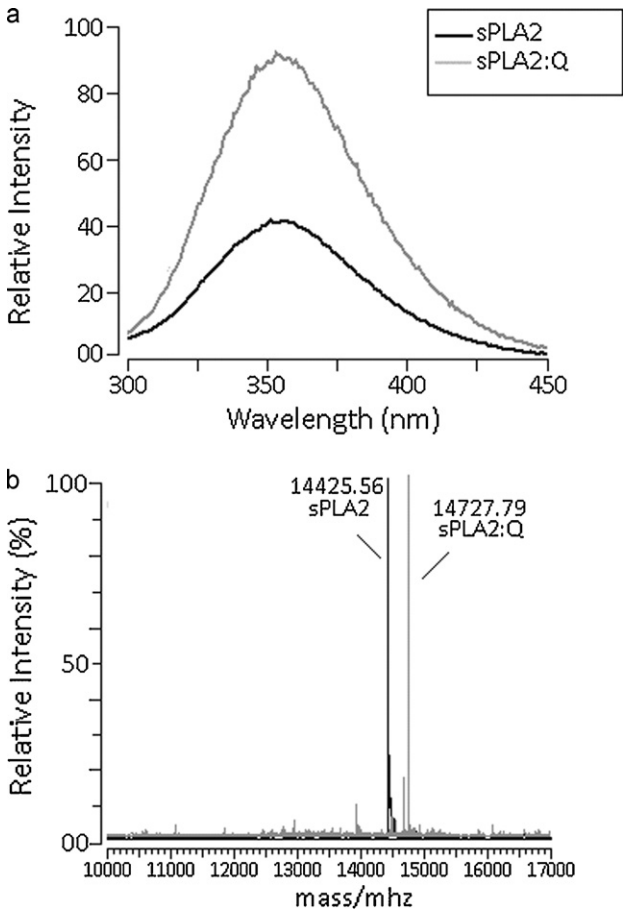

Fig. 3. Intrinsic fluorescence and mass spectrometry. (a) Intrinsic fluorescence of native sPLA2 and sPLA2:Q was measured with excitation at 280 nm and emission monitoring between 300 and 450 nm. (b) MALDI-TOF mass spectrometry analysis of native sPLA2 and sPLA2:Q shows a difference between the molecular masses corresponding to one molecule of bound quercetin.

3.6. Paw edema assay

Since flavonoids have shown a good capacity to inhibit sPLA2 and consequently decrease its pro-inflammatory activity [13,25], the effect of quercetin on sPLA2 was evaluated. Following subplantar injections on Swiss mice, sPLA2 had a huge potential to induce edema after 60 min (Fig. 5a). Under the same experimental conditions, sPLA2:Q did not show a decrease in edema effect, but the maximum edema was observed after 30 min.

3.7. Neurotoxic effect assay

When the neurotoxic activity was evaluated, both sPLA2 and sPLA2:Q induced neuromuscular blockage, but sPLA2 induced a faster effect at 80 min, whereas sPLA2:Q had a similar behavior at 100 min (Fig. 5b), suggesting that quercetin changed the velocity of native sPLA2 binding to the neurotoxic site of chick biventer muscle. However, this change is not able to abolish the neurotoxic effect of sPLA2.

3.8. Platelet aggregation studies

In order to evaluate the anti-coagulant potential induced by sPLA2 were performed platelet aggregation assays. The results have shown that sPLA2 from *C. durissus terrificus* has a moderate ability to cause aggregation of washed platelets. While sPLA2 from *C. durissus cascavella* induces about 85% aggregation at 3 μg/μL [26], sPLA2 from *C. durissus terrificus* induced 70% aggregation at a concentration of 20 μg/μL. Treatment with quercetin decrease this effect, with 32% aggregation induced by sPLA2:Q at the same concentration (Fig. 5c).

3.9. Myotoxic activity

The ability of sPLA2 to cause myonecrosis was also evaluated through measurement of released creatine kinase. Fig. 5d shows that sPLA2 induces an increase in plasma creatine kinase levels of 821.39 ± 107.8 U/L, indicating its ability to cause muscle damage. Treatment with quercetin significantly decreased the creatine kinase levels measured to 492.28 ± 71.5 U/L, a 40% decrease.

3.10. Molecular modeling (docking)

In order to analyze possible intermolecular interactions between sPLA2 and quercetin, we performed *in silico* studies using molecular modeling (docking). The best docking solution for the quercetin ligand is shown in Fig. 6a. The GOLD score for this result was 43.94, showing good affinity for the target. The presence of several important intermolecular interactions such as (i) hydrogen bonds with residues Cys45 and His48 at 2.69 and 3.00 Å, respectively; (ii) hydrogen bond with water molecule 188 at 3.16 Å; (iii) one polar contact with Ca^{2+} ion at 3.19 Å; and (iv) hydrophobic interactions with residue Phe5 can account for the stability of the quercetin–sPLA2 complex (Fig. 6a and b).

4. Discussion and conclusions

In this study, sPLA2 from *C. durissus terrificus* was modified by quercetin, a flavonoid known for its anti-inflammatory activ-

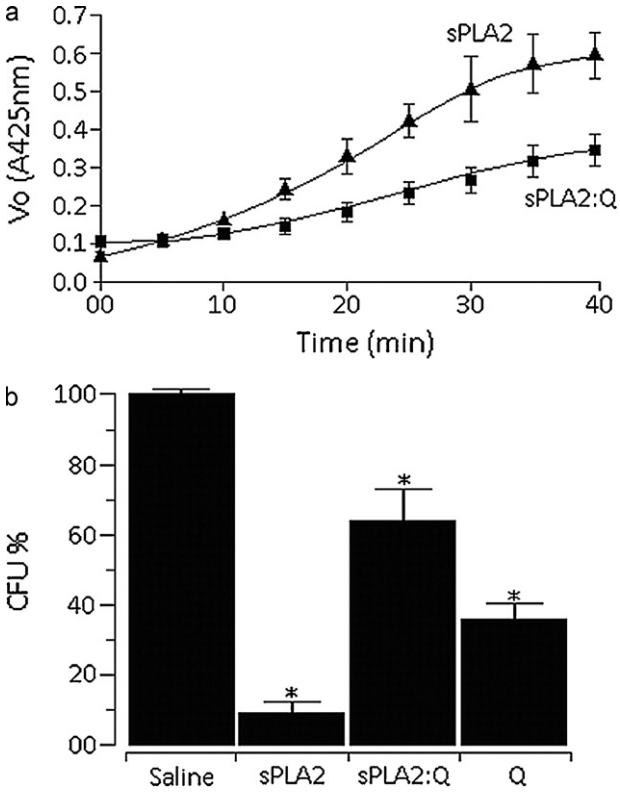

Fig. 4. Enzymatic and antibacterial assays. (a) Enzymatic activity was analyzed using 4N3OBA as substrate and monitored at wavelength 425 nm. sPLA2:Q shows a significant decrease compared to native sPLA2. (b) Effect of native sPLA2 and sPLA2:Q against Gram-positive bacteria. Quercetin decreases the ability of sPLA2 to inhibit Gram-positive bacteria. Error bars indicate SEM $^*P < 0.05$ compared to saline control.

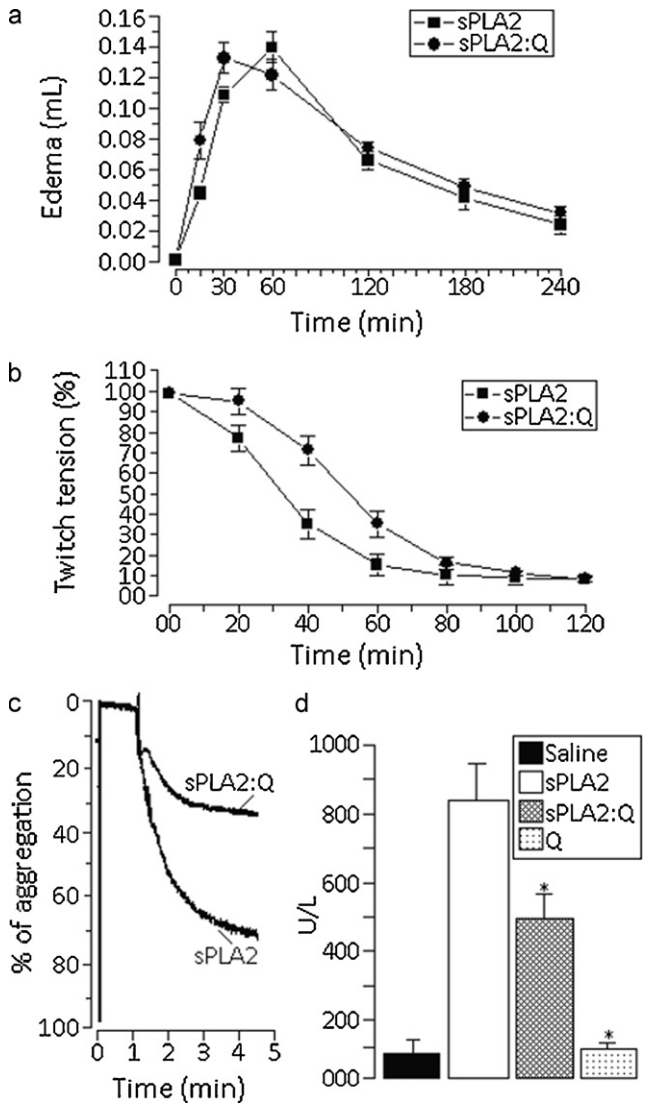

Fig. 5. Pharmacological assays. (a) Paw edema induced after the injection of sPLA2 and sPLA2:Q (25 μg/paw) into the right paw of Swiss mice. Measurements were done after 30, 60, 120, 180 and 240 min, with no differences observed after treatment with quercetin. (b) Neurotoxic effect of sPLA2 and sPLA2:Q on chick biventer cervicis muscle. Results were expressed as the percentage change in twitch tension. (c) Percent platelet aggregation caused by native sPLA2 and sPLA2:Q. The washed platelets assay was performed with venous blood collected from healthy volunteers and the concentration of both proteins was 15 μg/mL (d) Myonecrosis was assayed based on the creatine kinase levels in Swiss mice. Twenty-five micrograms of native sPLA2, sPLA2:Q, and pure quercetin were injected into the gastrocnemius muscle. Results were expressed as units of enzymatic activity per liter (U/L). Error bars indicate the SEM. $^{*}P < 0.05$ compared to sPLA2 activity.

ities. Treatment with quercetin resulted in modification of the protein secondary structure as observed in circular dichroism assay (Fig. 2b). Similar results were observed by Iglesias et al. [12] with sPLA2 from *C. durissus cascavella* after modification with the flavonoid morin. Although a secondary structure modification could be observed, the results do not allow concluding about tertiary structure modification, once the fluorescence spectrum of quercetin has shown that this compound has a fluorescent peak near the region of the tryptophan, suggesting that the increase in the total fluorescence of the protein might be caused due to the bonding of quercetin.

Treatment with quercetin led to a decrease of about 40% in sPLA2 enzymatic activity, similar to p-bromophenacyl bromide (p-BPB), a PLA2 inhibitor. Docking studies suggest that quercetin binds in the vicinity of the His48 residue, leading to inhibition of enzymatic activity. As shown by Verheij et al. [23], and Scott and Sigler [24], these amino acids are fundamental to enzymatic activity; His48 is involved in the first step of the enzymatic mechanism, and Asp49 is important for binding to Ca^{2+}, a cofactor. It seems that quercetin bound to sPLA2 interferes with the binding between the protein and its substrate. Soares et al. [27] suggested that enzymatic activity is not required for antibacterial activity after chemical modification with BPB and EDTA in a *C. durissus terrificus* sPLA2 isoform, and the same was observed by Diz Filho et al. [28] for *Crotalus durissus ruruima*. However, our results show correlation between the enzymatic and antibacterial activities against Gram-positive bacteria. Treatment of sPLA2 with quercetin decreased both these activities. According to Buckland and Wilton [29], mammalian PLA2 phospholipid hydrolysis is correlated with antibacterial activity because both are calcium dependent. We believe this isoform behaves as a mammalian PLA2 in this respect.

Several studies have investigated the anti-inflammatory effects of flavonoids as well as their effect on both catalytic and pharmacological activities of PLA2 [8,11,12,30,31]. According to these models, quercetin molecules would interact with the enzymatic site thus affecting enzymatic and pharmacological activities. Our results have showed that some pharmacological activities such as inflammatory and neurotoxicity were not inhibited after treatment with quercetin. This result corroborates the proposal by Ohno et al. [32], who suggested the existence of a distinct pharmacological site

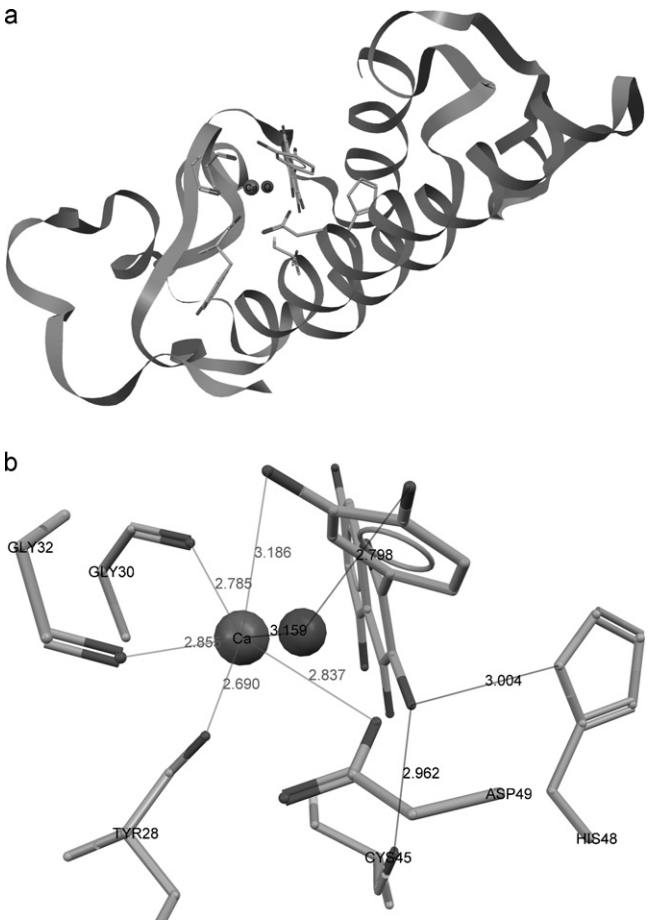

Fig. 6. Molecular docking. (a) Best docking solution for quercetin (stick model) and important interacting residues of the Crotoxin B target, besides Ca^{2+} ion and water oxygen. (b) Detailed view, with hydrogen bonds (distances in Å) shown as lines not involving the Ca^{2+} ion. Hydrogen atoms were omitted for clarity and the lines linked to the Ca^{2+} ion represent the coordination polyhedron.

near the C-terminal region of PLA2 away from the catalytic site. After modifications with 7-hydroxycoumarin, Toyama et al. [31] observed that decreasing the enzymatic activity had not suppressed the pharmacological effect of PLA2, suggesting the presence of a distinct site. Although the inflammatory and neurotoxic effects were unchanged after treatment with quercetin, the myotoxic and platelet aggregation effects were decreased by 40% and 55%, respectively, indicating dependency on the enzymatic activity. We believe that perhaps both the myotoxic and the platelet aggregation sites are located in a second pharmacological site near the calcium binding loop as proposed by Valentin and Lambeau [33]. Thus, after binding to the protein near that region, quercetin affects this site more directly leading to considerable changes in these activities.

Molecular docking has been used during the last decades due to its importance in elucidating, through computational approaches, the best matches for protein–ligand interactions [34]. In this study, molecular docking was an important tool in evaluating the interactions between sPLA2 and quercetin. Our result agrees with that observed by mass spectrometry, showing that one molecule of quercetin is binding to the protein. Additionally, molecular docking was useful in elucidating some important interactions between sPLA2 and quercetin, which allowed better understanding of the molecular reasons for the effects of quercetin as well as other structurally similar flavonoids that could be studied in the future.

The results shown here led us to conclude that quercetin binding to the protein decreased the catalytic activity of sPLA2 from *C. durissus terrificus*. This sPLA2 probably has two distinct pharmacological sites: one responsible for effects such as platelet aggregation, myotoxicity, and antibacterial activities; and another responsible for inflammatory and neurotoxic effects. We believe the first one is located near the calcium-binding loop region and consequently near the catalytic site where quercetin binds to the protein, thus leading to a loss of activities dependent on this site. The second one, we believe is located near C-terminal region, away from where quercetin binds, and is not directly or indirectly affected by the quercetin binding to the catalytic site. Other flavonoids with very similar chemical structures have a potential to interact with sPLA2 from serpents. Further docking studies with new molecules of this class could be useful in predicting how potent these compounds could be as sPLA2 inhibitors. Although our study clarifies some aspects of the chemical and pharmacological interactions between a flavonoid and a sPLA2, further studies are necessary to better understand the action of flavonoids on sPLA2 activities.

Conflict of interest statement

The authors have no conflict of interest to disclose.

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References

[1] R. Kini, Excitement ahead: structure, function and mechanism of snake venom phospholipase A2 enzymes, Toxicon 42 (8) (2003) 827–840.
[2] T. Nevalainen, G. Graham, K. Scott, Antibacterial actions of secreted phospholipases A2. Review, Biochim. Biophys. Acta 1781 (1–2) (2008) 1–9.
[3] J. Burke, E. Dennis, Phospholipase A2 biochemistry, Cardiovasc. Drugs Ther. 23 (1) (2009) 49–59.
[4] J. Evangelista, A. Martins, N. Nascimento, C. Sousa, R. Alves, D. Toyama, M. Toyama, J. Evangelista, D. Menezes, M. Fonteles, M. Moraes, H. Monteiro, Renal and vascular effects of the natriuretic peptide isolated from Crotalus durissus cascavella venom, Toxicon 52 (7) (2008) 737–744.
[5] A. Soares, J. Giglio, Chemical modifications of phospholipases A2 from snake venoms: effects on catalytic and pharmacological properties, Toxicon 42 (8) (2003) 855–868.
[6] R. Kini, Y. Chan, Accelerated evolution and molecular surface of venom phospholipase A2 enzymes, J. Mol. Evol. 48 (2) (1999) 125–132.
[7] S. Chakraborti, Phospholipase A(2) isoforms: a perspective, Cell Signal. 15 (7) (2003) 637–665.
[8] J. Lättig, M. Böhl, P. Fischer, S. Tischer, C. Tietböhl, M. Menschikowski, H. Gutzeit, P. Metz, M. Pisabarro, Mechanism of inhibition of human secretory phospholipase A2 by flavonoids: rationale for lead design, J. Comput. Aided Mol. Des. 21 (8) (2007) 473–483.
[9] G. Di Carlo, N. Mascolo, A. Izzo, F. Capasso, Flavonoids: old and new aspects of a class of natural therapeutic drugs, Life Sci. 65 (4) (1999) 337–353.
[10] B. Gil, M. Sanz, M. Terencio, R. Gunasegaran, M. Paya, M. Alcaraz, Morelloflavone, a novel biflavonoid inhibitor of human secretory phospholipase A(2) with anti-inflammatory activity, Biochem. Pharmacol. 53 (1997) 733.
[11] M. Lindahl, C. Tagesson, Flavonoids as phospholipase A2 inhibitors: importance of their structure for selective inhibition of group II phospholipase A2, Inflammation 21 (3) (1997) 347–356.
[12] C. Iglesias, R. Aparicio, L. Rodrigues-Simioni, E. Camargo, E. Antunes, S. Marangoni, D. de Oliveira Toyama, L. Beriam, H. Monteiro, M. Toyama, Effects of morin on snake venom phospholipase A2 (PLA2), Toxicon 46 (7) (2005) 751–758.
[13] M. Lindahl, C. Tagesson, Selective inhibition of group II phospholipase A2 by quercetin, Inflammation 17 (5) (1993) 573–582.
[14] M. Toyama, D. de Oliveira, L. Beriam, J. Novello, L. Rodrigues-Simioni, S. Marangoni, Structural, enzymatic and biological properties of new PLA(2) isoform from Crotalus durissus terrificus venom, Toxicon 41 (8) (2003) 1033–1038.
[15] H Zhao, L. Tang, X. Wang, Y. Zhou, Z. Lin, Structure of a snake venom phospholipase A2 modified by p-bromo-phenacyl-bromide, Toxicon 36 (6) (1998) 875–886.
[16] U. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (5259) (1970) 680–685.
[17] W. Lee, M. Toyama, A. Soares, J. Giglio, S. Marangoni, I. Polikarpov, Crystallization and preliminary X-ray diffraction studies of piratoxin III, a D-49 phospholipase A2 from the venom of Bothrops pirajai, Acta Crystallogr. D: Biol. Crystallogr. 55 (Pt. 6) (1999) 1229–1230.
[18] T. Santi-Gadelha, B. Rocha, C. Oliveira, K. Aragão, E. Marinho, C. Gadelha, M. Toyama, V. Pinto, C. Nagano, P. Delatorre, J. Martins, F. Galvani, A. Sampaio, H. Debray, B. Cavada, Purification of a PHA-like chitin-binding protein from Acacia farnesiana seeds: a time-dependent oligomerization protein, Appl. Biochem. Biotechnol. 150 (1) (2008) 97–111.
[19] M. Toyama, D.O. Toyama, L. Passero, M. Laurenti, C. Corbett, T. Tomokane, F. Fonseca, E. Antunes, P. Joazeiro, L. Beriam, M. Martins, H. Monteiro, M. Fonteles, Isolation of a new L-amino acid oxidase from Crotalus durissus cascavella venom, Toxicon 47 (1) (2006) 47–57.
[20] B. Ginsborg, J. Warriner, The isolated chick biventer cervicis nerve-muscle preparation, Br. J. Pharmacol. Chemother. 15 (1960) 410–411.
[21] M.J.S. Dewar, E.G. Zoebisch, E.F. Healy, J.J.P. Stewart, Development and use of quantum mechanical molecular models. 76. AM1: a new general purpose quantum mechanical molecular model, J. Am. Chem. Soc. 107 (13) (1985) 8.
[22] G. Jones, P. Willett, R. Glen, A. Leach, R. Taylor, Development and validation of a genetic algorithm for flexible docking, J. Mol. Biol. 267 (3) (1997) 727–748.
[23] H. Verheij, J. Volwerk, E. Jansen, W. Puyk, B. Dijkstra, J. Drenth, G. de Haas, Methylation of histidine-48 in pancreatic phospholipase A2. Role of histidine and calcium ion in the catalytic mechanism, Biochemistry 19 (4) (1980) 743–750.
[24] D. Scott, P. Sigler, Structure and catalytic mechanism of secretory phospholipases A2, Adv. Protein Chem. 45 (1994) 53–88.
[25] B. Gil, M. Sanz, M. Terencio, M. Ferrándiz, G. Bustos, M. Payá, R. Gunasegaran, M. Alcaraz, Effects of flavonoids on Naja naja and human recombinant synovial phospholipases A2 and inflammatory responses in mice, Life Sci. 54 (20) (1994) PL333–338.
[26] F. Fonseca, E. Antunes, R. Morganti, H. Monteiro, A. Martins, D. Toyama, S. Marangoni, M. Toyama, Characterization of a new platelet aggregating factor from crotoxin Crotalus durissus cascavella venom, Protein J. 25 (3) (2006) 183–192.
[27] A. Soares, A. Mancin, A. Cecchini, E. Arantes, S. França, J. Gutiérrez, J. Giglio, Effects of chemical modifications of crotoxin B, the phospholipase A(2) subunit of crotoxin from Crotalus durissus terrificus snake venom, on its enzymatic and pharmacological activities, Int. J. Biochem. Cell Biol. 33 (9) (2001) 877–888.
[28] E. Diz Filho, S. Marangoni, D. Toyama, F. Fagundes, S. Oliveira, F. Fonseca, A. Calgarotto, P. Joazeiro, M. Toyama, Enzymatic and structural characterization of new PLA2 isoform isolated from white venom of Crotalus durissus ruruima, Toxicon 53 (1) (2009) 104–114.
[29] A. Buckland, D. Wilton, The antibacterial properties of secreted phospholipases A(2), Biochim. Biophys. Acta 1488 (1–2) (2000) 71–82.
[30] H. Kim, H. Pham, V. Ziboh, Flavonoids differentially inhibit guinea pig epidermal cytosolic phospholipase A2, Prostaglandins Leukot. Essent. Fatty Acids 65 (5–6) (2001) 281–286.
[31] D. Toyama, S. Marangoni, E. Diz-Filho, S. Oliveira, M. Toyama, Effect of umbelliferone (7-hydroxycoumarin, 7-HOC) on the enzymatic, edemato-

genic and necrotic activities of secretory phospholipase A2 (sPLA2) isolated from Crotalus durissus collilineatus venom, Toxicon 53 (4) (2009) 417–426.
[32] M. Ohno, T. Chijiwa, N. Oda-Ueda, T. Ogawa, S. Hattori, Molecular evolution of myotoxic phospholipases A2 from snake venom, Toxicon 42 (8) (2003) 841–854.
[33] E. Valentin, G. Lambeau, Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins, Biochim. Biophys. Acta 1488 (1–2) (2000) 59–70.
[34] I. Halperin, B. Ma, H. Wolfson, R. Nussinov, Principles of docking: an overview of search algorithms and a guide to scoring functions, Proteins 47 (4) (2002) 409–443.