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Original article

Molecular modeling and inhibition of phospholipase A_2 by polyhydroxy phenolic compounds

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Abstract

Phospholipases A_2 are enzymes responsible for the hydrolysis of membrane phospholipids that release arachidonic acid, which serves as substrate for pro-inflammatory mediators, such as prostaglandins and leucotriens. The design of specific inhibitors for PLA_2 might help in the development of new anti-inflammatory drugs. Polyhydroxy phenolic compounds, such as flavonoids, vitamin E, rosmarinic acid and aristolochic acid, are able to inhibit PLA_2 from different sources. Herein, we have studied the kinetic behavior and the capacity of inhibiting edema formation induced by PLA_2 of five different polyhydroxy phenolic compounds (two phenolic derivatives and three acetophenone hydroxylated derivatives) extracted from the venom of *Crotalus adamanteus*. The results showed that compounds 1,3-dihydroxy benzene, 1,3,5-trihydroxy benzene and 2,4,6-trihydroxy acetophenone were the most efficient in the inhibition of the enzymatic activity and edema induction by PLA_2. It was also verified that the number of hydroxyls in each molecule is not a limiting factor for the inhibition capacity of these compounds. Molecular modeling studies indicated that the most active compounds are linked to the amino acid Asp 49 and that they destabilize the coordination of the calcium atom, which is essential to the catalytic activity. The study of potential surfaces showed that there are conditions in which the potential values must be adequate for enzyme complex formation with polyhydroxy phenolic compounds. When the potential over the hydroxyl surfaces is very high, formation of stable complexes does not occur and the enzyme does not act intensely. These results might be helpful in the design of a drug that specifically inhibits PLA_2.

Keywords: PLA_2; Phenolic compounds; Enzymatic kinetics; Molecular modeling; DFT

1. Introduction

Snake venoms are complex mixtures of enzymatic and toxic proteins, including phospholipases A_2, myotoxins, hemorrhagic metalloproteases and other proteolytic enzymes, coagulant components, neurotoxins, cytotoxins and cardiotoxins, among others. Intense local inflammation is a characteristic associated with envenomation due to the presence of PLA_2 in the snake venom [1]. Phospholipases A_2 (PLA_2; EC 3.1.1.4) are enzymes that abundantly occur in snake venoms with crucial action in the hydrolysis of phospholipids. PLA_2 can also induce several pharmacological effects such as edema, hemorrhage and inhibition of platelet aggregation, as well as neurotoxic, anticoagulant and myotoxic effects [2,3].

The action of PLA_2 over membrane phospholipids releases, among others, arachidonic acid (AA), which is a precursor of pro-inflammatory eicosanoids [4,5]. Usually, the arachidonic acid released is converted to prostaglandins and leukotrienes by cyclooxygenases (COX1/COX2) and 5-lypoxygenase (5-LO), respectively [6]. Non-steroidal anti-inflammatory drugs

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(NSAIDs) reduce the conversion of AA to prostaglandins (PGs), inhibiting COX1/COX2, but not 5-LO. The excess of AA produced is converted to leucotriens (LTs) by 5-LO, which in high levels are directly linked to adverse effects in the gastric and renal tracts, as observed in patients that use NSAIDs [7]. An efficient anti-inflammatory drug should selectively inhibit PLA_2 and avoid not only PG but also LT formation and all their side effects [7,8].

In the development of new anti-inflammatory drugs, the PLA2 inhibition capacity has been tested in many chemical substances [9–12]. Polyphenolic compounds, such as flavonoids, have already been reported as presenting great capacity of PLA_2 inhibition, as other enzymes [13–17]. Other polyphenols, like rosmarinic acid, aristolochic acid and α-tocoferol (vitamin E), have also inhibited PLA_2 from snake venoms [18–20].

Hence, in order to increase the knowledge about the inhibition mechanism of PLA_2 by polyphenolic compounds and help in the development of new specific inhibitors for this compound, we have tested the *in vitro* and *in vivo* behavior of PLA_2 from *Crotalus adamanteus* (eastern diamondback rattlesnake) venom against five polyphenolic compounds (1,3,5-trihydroxy benzene, 1,3-dihydroxy benzene, 2,4,6-trihydroxy acetophenone, 2,4-dihydroxy acethophenone and 2,6-dihydroxy acetophenone – Fig. 1). The molecular modeling studies and the quantum-chemical calculations through DFT (density functional theory), together with the experimental results obtained, make the proposition of an enzyme inhibition mechanism possible.

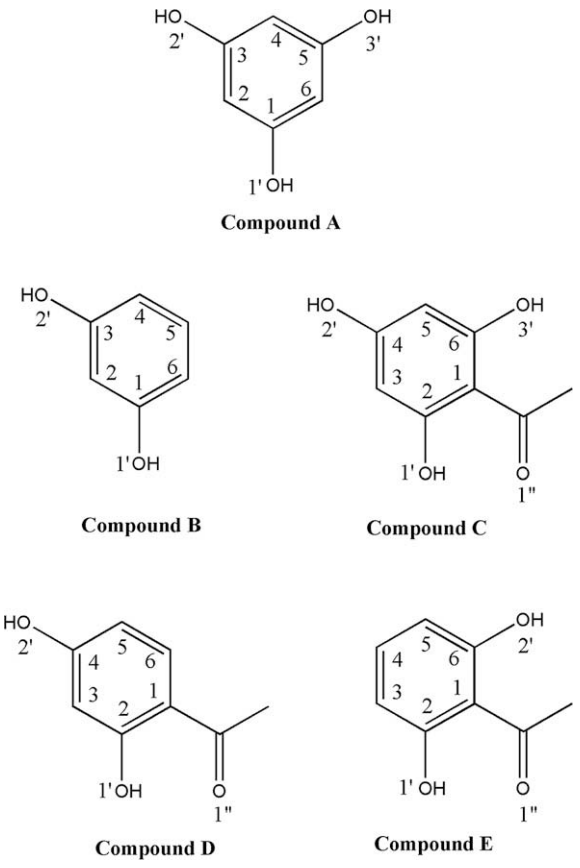


Fig. 1. Structures of 1,3,5-trihydroxy benzene (**A**), 1,3-dihydroxy benzene (**B**), 2,4,6-trihydroxy acetophenone (**C**), 2,4-dihydroxy acetophenone (**D**) and 2,6-dihydroxy acetophenone (**E**).

2. Materials and methods

2.1. Chemicals

C. adamanteus venom, 1,3,5-trihydroxy benzene (compound **A**), 1,3-dihydroxy benzene (compound **B**), 2,4,6-trihydroxy acetophenone (compound **C**), 2,4-dihydroxy acetophenone (compound **D**) and 2,6-dihydroxy acetophenone (compound **E**) were provided by Sigma Co. The micellar substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP) was furnished by Molecular Probes (USA). All solvents, chemicals and reagents used in this work were of highest purity available from Sigma, Aldrich Chemicals and Merck.

2.2. Purification of *C. adamanteus* PLA_2

The phospholipase A_2 used in this study of polyhydroxyphenol action was extracted from the venom of *C. adamanteus* (eastern diamondback rattlesnake). This enzyme has already been isolated and well characterized in several works [21–24] and herein, we have utilized two chromatographic steps to isolate it.

Liquid chromatography molecular exclusion. The whole *C. adamanteus* venom (45 mg) was dissolved in 0.2 M ammonium bicarbonate buffer (pH 7.9) until complete homogenization followed by a clarification step, using high-speed centrifugation (4500*g* for 2 min). The supernatant obtained was covered and applied on the liquid chromatography molecular exclusion column (1 × 60 cm, Pharmacia) previously equilibrated with the same buffer used for the venom dissolution. The main fractions of the venom were purified by constant flow rate of 0.3 ml/min; the chromatography run was monitored at 280 nm. The fractions that showed phospholipasic activity were pooled, lyophilized and stored at −20 °C until purification by reverse phase HPLC (RP-HPLC).

Reverse phase HPLC. The fractions were dissolved in 200 μL of TFA 0.1% (buffer A) until complete dissolution, followed by clarification, using high-speed centrifugation (4500*g* for 3 min). The supernatant was then loaded on a μ-Bondapack C18 RP-HPLC column (0.3 × 30 cm). The elution of PLA_2 was done using a non-linear gradient of buffer B (66.6% of acetonitrile in TFA 0.1%) at constant flow rate of 1.0 ml/min. The chromatographic run was monitored at 214 nm and the fraction with phospholipasic activity obtained was then lyophilized and stored at −20 °C until its utilization on the enzymatic activity assays with DIP and PMP inhibitors. The purity degree of *C. adamanteus* PLA_2 was evaluated by Tricine SDS-PAGE, according to Schagger and Von Jagow (1987) [25], and mass spectrometry, using MALDI TOF mass spectrometer.

2.3. Measurement of PLA_2 activity

The measurements of enzymatic activity using the micellar substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP) was done through the microtiter plate assay [26,27]. In each well of a 96-well microtiter plate, 100 μL of solution A in assay buffer (27 μM bovine serum albumin, 50 mM KCl, 1 mM $CaCl_2$, 50 mM Tris–HCl, pH 8.0) was added, followed by addition of 1 μL of each polyphenolic compound dissolved in DMSO (dimethyl sulfoxide) or 1 μL of DMSO only for control reactions. Solution B had the same composition as Solution A, however with PLA_2 (0.5 μg/mL), and it was delivered in 100 μL portions to all seven wells except for the first one. Instead of Solution B, an additional 100 μL portion of Solution A was added to the first of the seven wells in the assay. Solution B was prepared immediately prior to each set of assays to avoid loss of enzymatic activity. Quickly after the addition of Solution B, the assay was initiated by the addition of 100 μL of Solution C (4.2 mM 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol vesicles in assay buffer) with a repeating pipettor to the seven wells. The fluorescence (excitation = 342 nm, emission = 395 nm) was read with a microtiter plate spectrophotometer (Fluorocount, Packard Instruments). Control reactions without enzyme or inhibitor were run to all assays and the initial velocity was calculated from the initial slopes of fluorescence versus time for each concentration of the substrate used. The significance of differences between groups was evaluated using the Student's *t*-test. P value <0.05 was considered to be significant.

2.4. Edema-inducing activity

Edema was induced by i.d. injection (20 μL) of purified *C. adamanteus* PLA_2 (10 μg) in the right footpad of male Swiss mice (18–22 g). The left footpad received 20 μL of saline with ethanol 1% as control. Inhibition studies were performed by preincubating each inhibitor with *C. adamanteus* PLA_2 for 15 min at 37 °C (50 μg of inhibitor + 10 μg of PLA_2). After injection of 20 μL of mixture (inhibitor-PLA_2), the progression of edema was evaluated with a low pressure pachymeter (Mitutoyo, Japan) at intervals of 0.5, 1, 3, 6, 24 h and expressed in millimeters. Three independent experiments were performed in groups of six animals [27].

2.5. Molecular modeling

From the experimental results obtained, a possible model explaining the way by which the polyhydroxy phenolic derivatives studied might be inhibiting PLA_2 was proposed. The molecular models developed in this work utilized a crystalline structure of *Crotalus atrox* PLA_2 homodimer developed by Brunie et al., 1985 [28] (code 1PP2-RCSB – protein data bank (PDB)). Since there is no crystalline structure of *C. adamanteus* available, we have utilized PLA_2 from *C. atrox* due to its high homology degree with *C. adamanteus* (94%). The catalytic site region is identical and well characterized in both PLA_2. In the active site, the Asp 99 and His 48 amino acids activate a water molecule and, through basic general catalysis, they hydrolyze the phospholipid. Calcium is coordinated by the Tyr 28, Gly 30, Gly 32 and Asp 49 amino acids and is responsible for polarization and correct positioning of the phospholipid sn2 ester carbonyl, providing an adequate region for the nucleophilic attack from the water molecule [29,30].

Using crystalline structures, it was shown that polyphenolic compounds, as aristolochic acid and α-tocoferol (vitamin E), inhibit PLA_2 by their connection to the active site of the enzyme [18–20]. Thus, in this work, we have inserted inhibitor molecules (compounds **A**–**E** – Fig. 1) inside the active site of PLA_2 crystallized structure developed by Brunie et al., 1985 [28] and the geometry of a spherical region of 16 $Å^3$ containing the inhibitor and all the active site was optimized through the molecular mechanics method OPLS (optimized potential for liquid simulations) up to an RMS gradient of 10^{-5} kcal $Å^{-1}$ mol^{-1}. OPLS united-atom force fields (frequently designated as OPLS-UA) are used to describe interactions between molecular groups (segments) separated by more than three bonds or belonging to different molecules [31]. The final geometry optimization by application of the OPLS force fields methodology and visualization of the model structures were carried out utilizing the Hyperchem 7.51 package [32].

2.6. Quantum calculations

The final geometry optimization and the partial charge calculation over the atoms were carried out using the Gaussian03 package [33] by application of the DFT methodology with the use of UB3LYP functional [34] with 6-31G* basis set, developed by Hariharan and Pople [35].

2.7. Statistical analysis

All results are presented as the mean value ± SD obtained from three independent experiments with the indicated number of animals tested or replicate assay. The statistical significance of differences between groups was evaluated using Student's *t*-test. A P value <0.05 was considered to be significant.

3. Results and discussion

3.1. Isolation of PLA2 from C. adamanteus venom

The chromatographic profile obtained from LC molecular exclusion chromatography of *C. adamanteus* is shown in Fig. 2a and the peak region detached was the one that displayed PLA_2 activity. The fractions corresponding to this peak were pooled, lyophilized and purified utilizing reverse phase high performance liquid chromatography (RP-HPLC). The chromatographic profile utilizing RP-HPLC is shown in Fig. 2b and the detached peak corresponds to PLA_2 herein utilized. The PLA_2 M_r estimated was 15 kDa and the amino acid analysis showed a high content of basic amino acids (Lys, Arg) and the presence of 14.5 half-cysteines in PLA_2; the

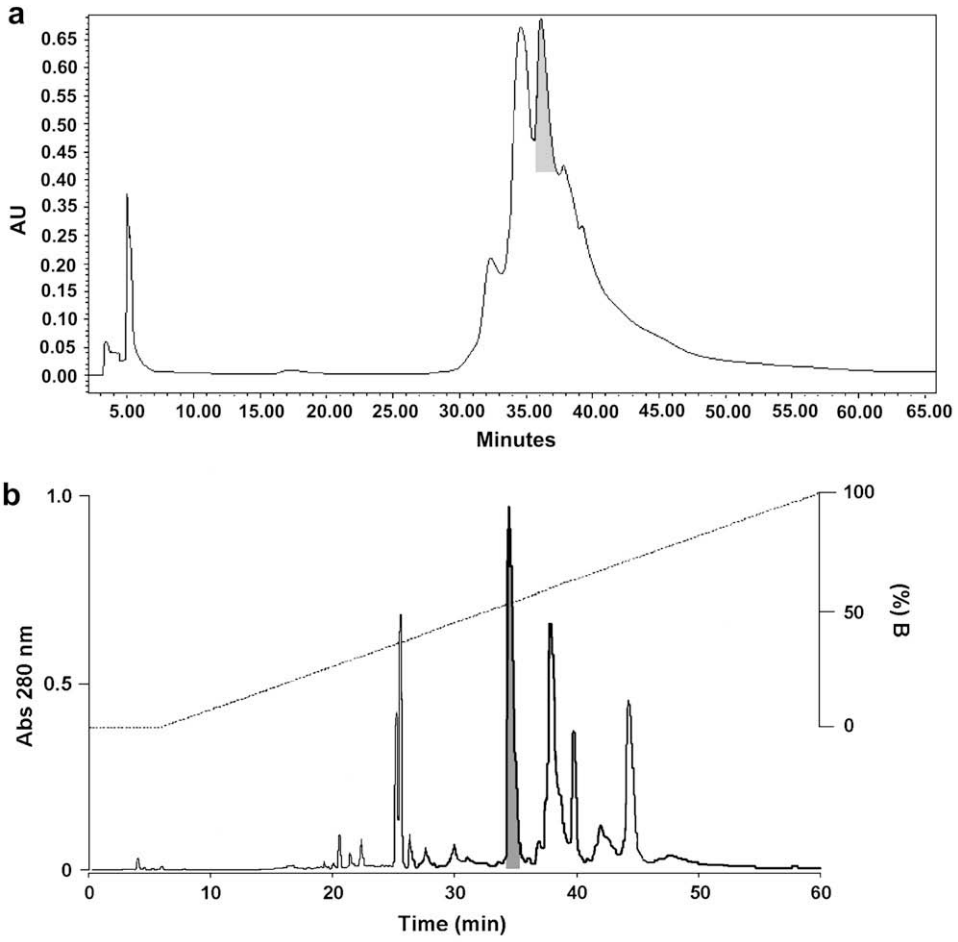


Fig. 2. a) Chromatographic profile of the liquid chromatography of *C. adamanteus* venom molecular exclusion. The detached peak shows the fractions that presented PLA_2 activity. (b) Chromatographic profile of the RP-HPLC chromatography. The detached peak corresponds to PLA_2 herein utilized.

last value indicates the presence of seven disulfide bridges. As expected, this is characteristic of the PLA_2 enzyme group II.

3.2. Measurement of PLA_2 activity

The kinetic behavior study of PLA_2 from *C. adamanteus* in the presence and absence of polyphenolic compounds is shown in Fig. 3. All the curves shown indicate that, in the substrate concentration range used in this study (0–10 mM), the enzyme action over the micellar substrate 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol (HPGP) reflects a classical behavior of a Michaelian enzyme, not only in the presence of small concentrations of the compounds **A** and **E** (0.15 μM and 0.30 μM), but also in their absence.

Fig. 3 also shows that all tested compounds are able to inhibit PLA_2 enzymatic activity. However, compounds **A**, **B** and **C** (1,3,5-trihydroxy benzene, 1,3-dihydroxy benzene and 2,4,6-trihydroxy acetophenone, respectively) were more efficient in inhibiting PLA_2 from *C. adamanteus* than compounds **D** and **E** (2,4-dihydroxy acetophenone and 2,6-dihydroxy acetophenone, respectively). These observations were confirmed by kinetic parameters, shown in Tables 1 and 2.

Observing the values described in Tables 1 and 2, it is possible to verify that, in all the tests, the maximum velocity of the enzyme (V_{max}) has not varied with respect to the presence of compounds **A**–**E** and presented a value around 18 nmols/min. On the other hand, the inhibitor's presence has induced an increase in K_M values. In the presence of mounting concentrations of compounds **A**, **B** and **C**, the K_M values of PLA_2 increased from around 0.7 mM to around 38.2 mM. In compounds **D** and **E**, the K_M values increased from 0.7 mM to around 7.78 mM. In addition, the inhibition constant values (K_I) for compounds **A**, **B** and **C** are around 8.8 nM and for compounds **D** and **E**, around 31 nM. These results show that the enzymatic inhibition is competitive and that compounds **A**, **B** and **C** present similar capacity of inhibiting PLA_2, being superior to compounds **D** and **E**.

3.3. Edema-inducing activity

Edema induction is a complex pharmacological activity that depends on many factors related with several toxins and, at least apparently, the enzymatic activity is not necessary to make a toxin able to induce edema formation [20,27]. Nevertheless, the effects observed in the inhibition of edema

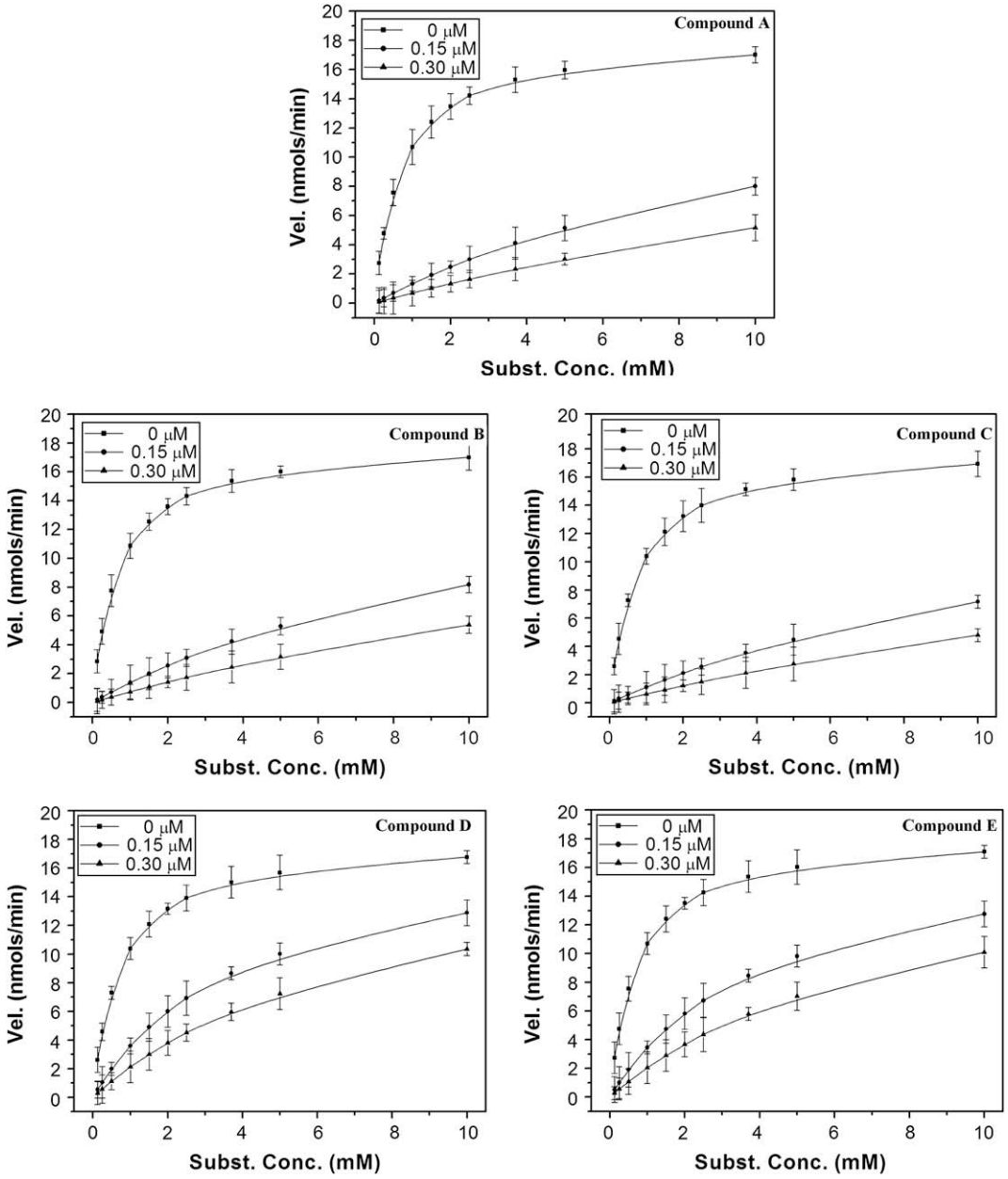


Fig. 3. Effect of substrate concentration on the kinetics of *C. adamanteus* PLA_2 activity. The enzymatic kinetics was studied under the influence of different concentrations of the compounds 1,3,5-trihydroxy benzene (**A**), 1,3-dihydroxy benzene (**B**), 2,4,6-trihydroxy acetophenone (**C**), 2,4-dihydroxy acetophenone (**D**) and 2,6-dihydroxy acetophenone (**E**). Results are expressed by the mean ± S.D of three independent experiments in six replicates ($n = 6$).

Table 1
Kinetic parameters[a] obtained on the *C. adamanteus* PLA_2 reaction in the presence and absence of 1,3,5-trihydroxy benzene (**A**), 1,3-dihydroxy benzene (**B**) inhibitors

	1,3,5-Trihydroxy benzene			1,3-Dihydroxy benzene		
	0 μM	0.15 μM	0.30 μM	0 μM	0.15 μM	0.30 μM
V_{max}	18.14 ± 1.09	18.10 ± 1.03	17.98 ± 0.94	18.20 ± 0.85	18.13 ± 1.00	18.00 ± 0.67
K_M	0.67 ± 0.14	12.06 ± 0.53	23.21 ± 0.27	0.70 ± 0.31	12.62 ± 0.37	24.82 ± 0.37
K_I		8.77 ± 0.19	8.86 ± 0.28		8.83 ± 0.29	8.72 ± 0.42

The V_{max} values are given in nmol/min. K_M values are given in mM. K_I values are given in nM.
[a] Average of three independent determinations; five replicates; values are mean ± SD.

Table 2
Kinetic parameters[a] obtained on the *C. adamanteus* PLA_2 reaction in the presence and absence of 2,4,6-trihydroxy acetophenone (**C**), 2,4-dihydroxy acetophenone (**D**) and 2,6-dihydroxy acetophenone (**E**) inhibitors

	2,4,6-Trihydroxy acetophenone			2,4-Dihydroxy acetophenone			2,6-Dihydroxy acetophenone		
	0 μM	0.15 μM	0.30 μM	0 μM	0.15 μM	0.30 μM	0 μM	0.15 μM	0.30 μM
V_{max}	18.21 ± 0.99	17.97 ± 1.01	18.32 ± 0.43	17.98 ± 1.15	18.08 ± 1.06	18.20 ± 0.81	18.31 ± 1.03	18.19 ± 1.07	17.97 ± 0.91
K_M	0.75 ± 0.18	15.05 ± 0.17	28.17 ± 0.23	0.73 ± 0.29	4.03 ± 0.14	7.57 ± 0.56	0.71 ± 0.26	4.25 ± 0.72	7.78 ± 0.11
K_I		8.90 ± 0.31	8.74 ± 0.17		33.31 ± 0.39	32.10 ± 0.71		30.11 ± 0.11	31.20 ± 0.59

The V_{max} values are given in nmols/min. K_M values are given in mM. K_I values are given in nM.
[a] Average of three independent determinations; five replicates; values are mean ± SD.

formation by PLA_2 from *C. adamanteus* venom, due to the presence of compounds **A**–**E** (Fig. 1), were similar to the ones found in the enzymatic activity inhibition tests of PLA_2.

Compounds **A** and **B** were effectively more successful in the inhibition of edema induced by PLA_2 when compared to the other compounds (Fig. 4a). Compound **C** was slightly less capable than compounds **A** and **B**, but it could also inhibit the edema formation in a significant manner (Fig. 4a and b). Compounds **D** and **E** have shown the lowest inhibition capacity of inducing edema formation by PLA_2 (Fig. 4b).

3.4. Molecular modeling

Molecular modeling facilitates the understanding of the differences among the inhibitions provoked by the five polyhydroxyphenol derivatives used in this work. Once the catalytic site is well characterized and known [29,30], a very consistent molecular model was obtained when compounds **A**–**E** were inserted inside the crystalline structure of PLA_2 from *C. atrox*. When the models were optimized through molecular mechanics utilizing the OPLS method, the results shown in Fig. 5 were obtained.

All compounds could be perfectly adjusted inside the active site of the enzyme. The aromatic rings occupy the hydrophobic region formed by the amino acids Leu 2, Phe 5 and Phe 106 (Fig. 5). Analyzing the models shown in Fig. 5, it is possible to observe that compounds **A**, **B** and **C** interact more effectively with PLA_2 since they form hydrogen bonds with Asp 49. Compounds **D** and **E** interact with the enzyme just through hydrophobic bonds and consequently, they are less intensely connected to PLA_2.

3.5. Quantum calculations

The ESP (electrostatic surface potential) data calculated for the compounds **A**–**E** are shown in Fig. 6. Molecular modeling (Fig. 5) showed that compounds **A**, **B** and **C** form a hydrogen bond through H2′ with the amino acid Asp 49 of PLA_2 and produce a stable complex. On the other hand, the structures **D** and **E** form less stable complexes with PLA_2 since their connections with the enzyme are exclusively through hydrophobic interactions.

The molecules with hydroxyls that formed hydrogen bonds (compounds **A**, **B** and **C**) showed a potential around 0.7 eV, what seems to be an adequate value for interaction with the enzyme. Compound **D** showed one hydroxyl (position 4) that could form a hydrogen bond with PLA_2, but its molecular potential is much higher (0.912 eV) than the ones showed by compounds **A**, **B** and **C**. This value seems to be too high to allow the complex formation between the compound **D** and the enzyme. Compound **E** has a potential value of 0.753 eV that could allow the formation of hydrogen bond; probably it does not occur due to the absence of hydroxyls in position 4, while the hydroxyls in positions 2 and 6 are sterically blocked by the acetyl group. In addition, Fig. 6 shows that

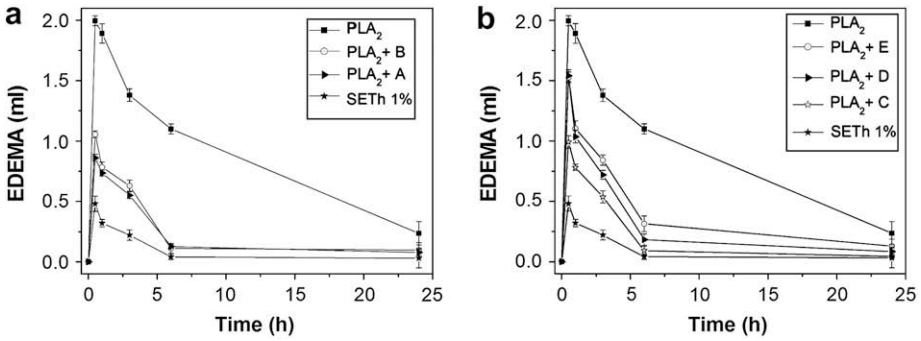
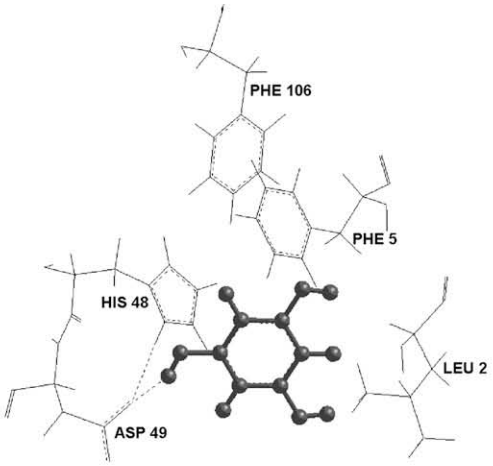
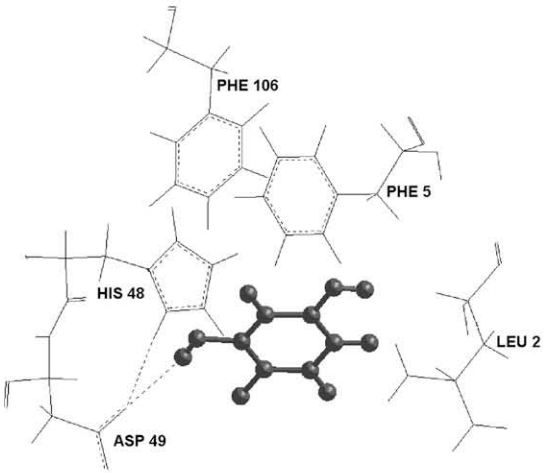


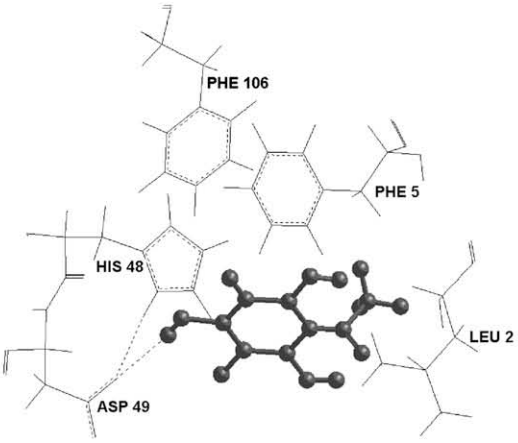
Fig. 4. Inhibition of the edema-inducing activity (a) by polyhydroxy phenolics: 1,3,5-trihydroxy benzene (**A**) and 1,3-dihydroxy benzene (**B**); (b) by polyhydroxy phenolic acetophenone derivatives: 2,4,6-trihydroxy acetophenone (**C**), 2,4-dihydroxy acetophenone (**D**) and 2,6-dihydroxy acetophenone (**E**). All points represent mean ± SD of three independent experiments performed in groups of six animals.



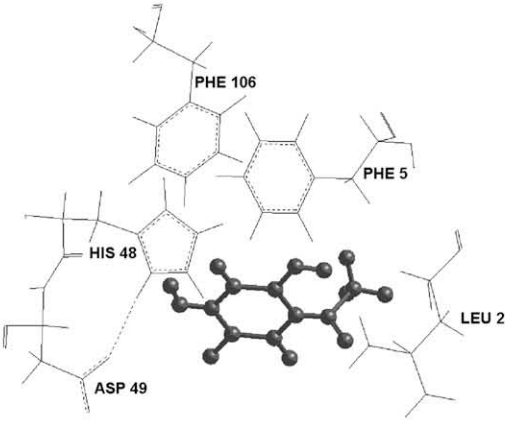
PLA2 + Compound A



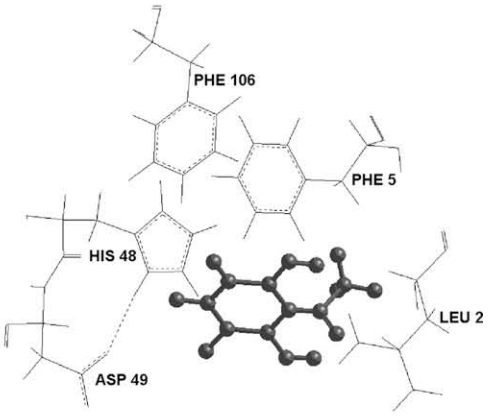
PLA2 + Compound B



PLA2 + Compound C



PLA2 + Compound D



PLA2 + Compound E

Fig. 5. Molecular models obtained by molecular mechanics (OPLS) with 1,3,5-trihydroxy benzene (**A**), 1,3-dihydroxy benzene (**B**), 2,4,6-trihydroxy acetophenone (**C**), 2,4-dihydroxy acetophenone (**D**) and 2,6-dihydroxy acetophenone (**E**) inside the crystalline structure of *C. atrox*. The highest difference observed is that compounds **A**, **B** and **C** form hydrogen bond with Asp 49 in the catalytic site region.

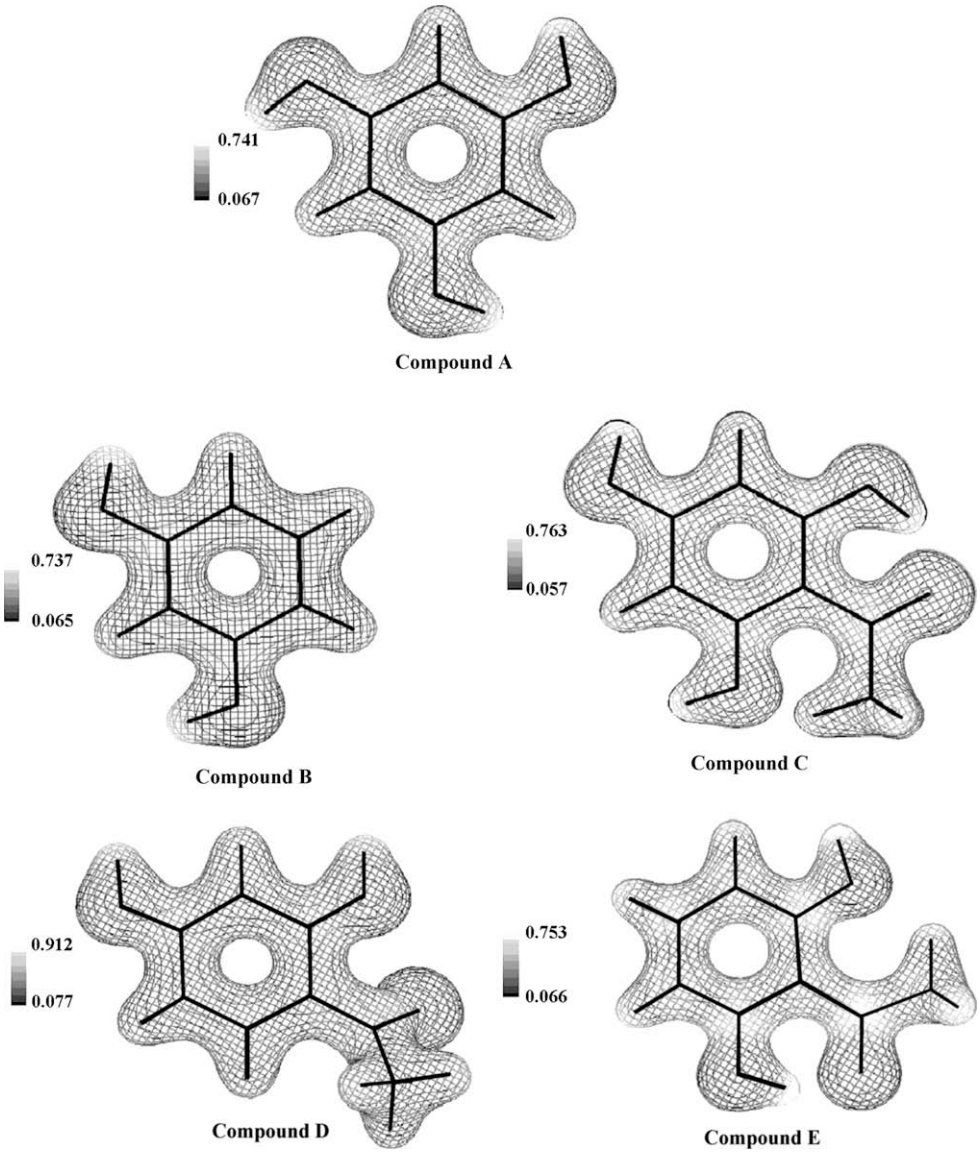


Fig. 6. Electrostatic surface potential (ESP) calculated for compounds **A**–**E**. The more efficient compounds on inhibiting either the enzymatic activity or edema formation (compounds **A**, **B** and **C**) showed favorable potentials (around 0.7 eV) for formation of PLA_2 inhibitor complex formation. Compound **D** presents a very high electrostatic potential (0.912 eV), favoring the complex formation. Compound **E**, even showing a potential around 0.7 eV, has sterically hindered hydroxyls by the acetyl group that avoid the PLA_2 inhibitor complex formation. Both the compounds **C** and **E** form internal hydrogen bond between the hydroxyl from position 2 and the carbonyl from the ketone grouping.

both compounds **C** and **E** form an intramolecular hydrogen bond between the hydroxyl from position 2 and the acetyl group. This connection improves the electron distribution through the molecular surface and might be responsible for the similarity among their electrostatic potential values.

4. Conclusions

The results achieved in this work showed that all the polyphenolic compounds used herein were able to reduce not only the intensity of the enzymatic activity but also the edema formation induced by PLA_2 from *C. adamanteus*. The presence of phenolic hydroxyl, the influence of its quantity and the presence or not of acetyl groups were the main characteristics analyzed in this study. Some works showed that the presence of, at least, one phenolic hydroxyl is a possible structural requirement for the inhibition of PLA_2 and phenolic compounds, such as aristolochic acid, vitamin E and rosmarinic acid, can inhibit enzymatic activity of PLA_2 derived from different sources [18–20,36,37].

The experiments indicated that the presence of phenolic hydroxyls might be really important in the inhibition of PLA_2. Apparently, the number of hydroxyls does not influence the inhibition intensity. Compounds **A** and **B** (three and two hydroxyls, respectively) were the most efficient in the enzyme inhibition and presented very similar behavior in all the biological assays tested (Figs. 3 and 4). Moreover, for both compounds the kinetic parameters were extremely alike (Table 1).

In the experiments done with all the inhibitors, the V_{max} values were held constant while the K_M values increased and hence we inferred that compounds **A**–**E** inhibit PLA_2 from *C. adamanteus* in a competitive manner. Despite the presence of an additional acetyl group in compound **C**, the inhibition constant (K_I) as well as V_{max} and K_M indicate that this structure presents a capacity of PLA_2 inhibition similar to compounds **A** and **B** (Tables 1 and 2). Thus, it was verified that the presence of an acetyl group in the polyhydroxy phenolic compounds can reduce the efficiency in inhibition of the PLA_2 enzymatic activity, as in compounds **D** and **E**, or it does not influence, as in compound **C** (Table 2).

In order to propose a possible action mechanism of the polyhydroxy phenolic compounds over PLA_2, as well as to verify the influence of the acetyl group on the inhibition of the enzymatic activity, we tried to analyze how the inhibitors **A**–**E** interact with the enzyme. Since the enzymatic inhibition process was of the competitive type, the inhibitor structures were inserted in the interior of the enzyme active site and, then, the complex enzyme–inhibitor was optimized using molecular mechanics through the OPLS method. The molecular models showed that the most active compounds (**A**, **B**, **C**) are able to interact with the enzyme through the formation of a hydrogen bond with the amino acid Asp 49, while the less active ones (**D** and **E**) interact only with hydrophobic bounds (Fig. 5). In the calcium-dependent phospholipases A_2, the amino acid Asp 49 is one of the residues that coordinates with the calcium atom, which is essential for the enzymatic activity, since it helps to polarize the *sn2* ligation of the glycerophospholipids that will be hydrolyzed [29,30]. The binding of compounds **A**, **B** and **C** to the amino acid Asp 49 promotes the destabilization of the calcium coordination in the active site and decreases the catalytic efficiency of the enzyme. In the case of the compounds **D** and **E**, the inhibition occurs only through the occupation of the enzyme active site by the inhibitors and, apparently, there is no significant alteration in the calcium atom coordination. The molecular model has also shown that the acetyl group assumes a position inside the active site of the enzyme that interacts through hydrophobic bonds with the amino acid Leu 2, with no significant sterical impediments (Fig. 5).

The quantum calculations indicate that the main effect caused by the acetyl group is related to the electronic aspects of the inhibitors. It is interesting to note that the derivative compounds of acetophenone which formed intramolecular hydrogen bonds between the hydroxyl in position 2 and the carbonyl group of acetone (**C** and **E**) were those that presented electrostatic potential with values close to the ones presented by compounds **A** and **B** (Fig. 6). Compound **D** was unable to form the intramolecular bonds and it seems that the acetyl group influenced, taking off electrons from the hydroxyl from position 4 and increasing the molecular potential. It did not benefit the formation of a complex between compound **D** and PLA_2. Furthermore, observing Fig. 6, it is possible to see that the acetyl from compound **D** is positioned almost perpendicularly in comparison to the acetyl groups from compounds **C** and **E**, and thus, it is not possible to discharge the possibility of the existence of a steric hindrance effect of this acetyl group inside the enzyme active site. In addition, despite presenting electrostatic potential similar to compounds **A**, **B** and **C**, compound **E** cannot form a hydrogen bond with the enzyme due to the absence of a hydroxyl in position 4 and, moreover, the hydroxyls in positions 2 and 6 are sterically blocked by the acetyl group (Fig. 6).

Briefly, the results herein obtained reinforce the idea that the polyphenolic compounds present a significant capacity of inhibiting the enzymatic activity of PLA_2. Possibly, the phenolic hydroxyls are bound to Asp 49 and influence the capacity of this amino acid to participate in the coordination of the calcium atom that is essential to the catalytic enzyme. Apparently, the number of hydroxyls is not a limiting factor for the efficiency in the capacity of enzymatic inhibition. It is possible that chemical groups might be bound in the polyphenolic structure (acetyl, for instance), however some conformations of these groups might influence the electron availability in the phenolic hydroxyls and increase the electrostatic potential of the molecule. This increased electrostatic potential avoids the formation of hydrogen bonds between the polyphenolic compounds and Asp 49 and decreases the inhibitory efficiency of these compounds.

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