Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 1017–1025
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Inhibitory effects of *Piper umbellatum* and *Piper peltatum* extracts towards myotoxic phospholipases A_2 from *Bothrops* snake venoms: Isolation of 4-nerolidylcatechol as active principle

Vitelbina Núñez [a,b], Víctor Castro [c], Renato Murillo [c], Luis A. Ponce-Soto [d], Irmgard Merfort [e], Bruno Lomonte [a,*]

[a] *Instituto Clodomiro Picado, Facultad de Microbiologia, Universidad de Costa Rica, San José, Costa Rica*
[b] *Programa de Ofidismo, Facultad de Medicina, Universidad de Antioquia A.A. 1226, Medellín, Colombia*
[c] *Escuela de Química and CIPRONA, Universidad de Costa Rica, San José, Costa Rica*
[d] *Departamento de Bioquimica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), SP, Brasil*
[e] *Institute of Pharmaceutical Biology, Albert-Ludwigs-Universität Freiburg, Germany*

Received 3 December 2004; received in revised form 27 January 2005
Available online 27 April 2005

Abstract

Phospholipases A_2 (PLA_2) are important constituents of snake venoms, being responsible for several of their toxic actions. Extracts from plants used in folk medicine were screened for inhibition of the enzymatic activity of myotoxin I, a PLA_2 from *Bothrops asper*. *Piper umbellatum* and *Piper peltatum* extracts tested positive, and their fractionation resulted in the isolation of 4-nerolidylcatechol. Its inhibitory effects towards toxic activities of two *Bothrops* myotoxins, representing catalytically active (Asp49) and catalytically inactive (Lys49) types of group II PLA_2s, respectively, were characterized. The enzyme activity of *B. asper* myotoxin I was completely inhibited by 4-nerolidylcatechol at an inhibitor:toxin ratio of 10:1 (wt/wt) with an IC50 of ~1 mM. In addition, 4-nerolidylcatechol inhibited representatives of groups I and III of PLA_2s. Its preincubation with *Bothrops* myotoxins significantly reduced their myotoxic and edema-inducing activities in animal experiments. However, when 4-nerolidylcatechol was administered in situ, immediately after toxin injection, its inhibitory ability was substantially lower or negligible. This might be explained by the rapid action of these toxins in vivo, together with the slow inactivation of PLA_2 activity observed in vitro. Electrophoretic and chromatographic analyses of myotoxins ruled out major changes in protein charge, hydrophobicity, or gross molecular mass being involved in the inhibition mechanism. Mass spectrometry determinations are consistent with the covalent modification of myotoxin by one molecule of 4-nerolidylcatechol. Finally, a novel compound was isolated from both *Piper* species, sharing the nerolidyl skeleton, but nevertheless not being inhibitory towards the PLA_2s studied.

Keywords: *Piper umbellatum*; *Piper peltatum*; Piperaceae; 4-Nerolidylcatechol; Myotoxin; Phospholipase A_2; Snake venoms; *Bothrops*

1. Introduction

Snakebites constitute a health problem in many tropical and subtropical countries, with an estimated 2.5 million people envenomed each year on a global basis (Chippaux, 1998). These envenomations are associated with a variety of pathophysiological manifestations, frequently including a severe local tissue damage with myonecrosis, edema and hemorrhage, which may result in irreversible lesions and even amputation of the affected limb (Otero et al., 2002).

The most important and abundant muscle-damaging components in snake venoms are phospholipases A_2

* Corresponding author. Tel.: +506 229 0344; fax: +506 292 0485.
E-mail address: blomonte@cariari.ucr.ac.cr (B. Lomonte).

0031-9422/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2005.03.026

(PLA_2; EC 3.1.1.4). These enzymes hydrolyze the sn-2 ester bond of 3-sn membrane glycerophospholipids, generating fatty acids, e.g., arachidonic acid, and lysophospholipids (Rosenberg, 1997). They are classified into groups I or II, based on their sequence and mode of disulphide pairings. Group I PLA_2s are found in the venoms of Elapidae snakes, whereas group II PLA_2s are present in the venoms of Viperidae snakes (Six and Dennis, 2000). The group II is further divided into two main subgroups: Asp49 and Lys49 variants. In the latter, the aspartic acid residue at position 49, critically involved in calcium binding and essential for catalytic activity, is replaced by lysine. Due to this and other critical substitutions, the Lys49 PLA_2s cannot bind calcium efficiently and are considered to be enzymatically inactive (Scott et al., 1992; Lomonte et al., 2003). Although catalytic activity has been shown to play a role in the toxic actions of some venom PLA_2s, it is not essential in the case of Lys49 PLA_2s, which utilize non-enzymatic mechanisms to alter membrane homeostasis (Lomonte et al., 2003).

Intravenous administration of equine or ovine immunoglobulins constitutes the main treatment against snakebite envenomations (Warrel, 1995). However, it has been demonstrated that such antivenoms generally have a limited efficacy against the local tissue damaging activities of venoms (Gutiérrez et al., 1998). Thus, there is a need to search for additional inhibitors and approaches that may be useful to complement conventional antivenom therapy.

Plant extracts constitute rich sources of pharmacologically active compounds, and some of them have been reported to antagonize the activities of various crude venoms and purified toxins (Mors et al., 2000; Otero et al., 2000a). Preparations of plants from the Piperaceae family have been widely used in folk medicine as diuretic, febrifuge, laxative and anti-inflammatory remedies, as well as for the treatment of liver diseases (Zamora-Martinez and Pola, 1992; Akendengue and Louis, 1994). Furthermore, they have been described to have snake venom-neutralizing properties (Otero et al., 2000b). The present study reports the isolation and identification of 4-nerolidylcatechol as a component responsible for the inhibitory effect of *Piper umbellatum* L. and *Piper peltatum* L. extracts towards PLA_2 myotoxins isolated from *Bothrops asper* and *Bothrops atrox* venoms. In addition, a novel compound for plants of the *Piper* genus is described which has no PLA_2 inhibitory property.

2. Results

The crude extracts of both *P. umbellatum* and *P. peltatum* completely inhibited the PLA_2 activity of *B. asper* myotoxin I in screening assays, at an extract/toxin ratio of 20:1 (wt/wt), corresponding to a final extract concentration of 2 mg/ml. After fractionation of both extracts, two compounds were obtained (Fig. 1). Compound **1** was clearly identified as 4-nerolidylcatechol by comparison of its spectroscopic data (Kijjoa et al., 1980), and exhibited PLA_2 inhibitory activity in subsequent experiments. Compound **2** did not inhibit this activity, but nevertheless is of phytochemical interest, as it corresponds to a compound that has not been previously reported. The molecular formula $C_{23}H_{30}O_4$ followed from its HREIMS, which showed a $[M]^+$ at *m/z* 370.21441. Its structure was deduced from its 1D and 2D NMR spectra (^1H NMR, ^{13}C NMR, H,H COSY, gHSQC, gHMBC) as summarized in Table 1. The

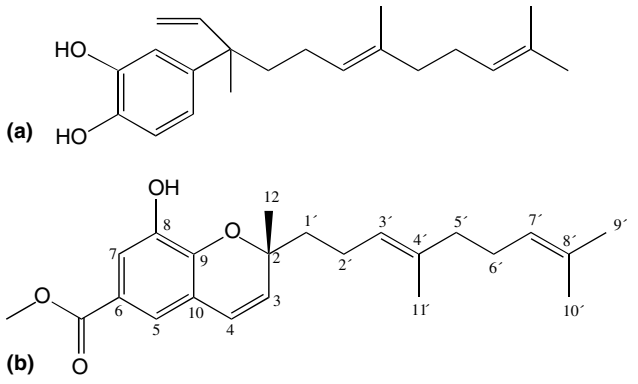

Fig. 1. Compounds isolated from *Piper peltatum* and *Piper umbellatum*. (a) 4-Nerolidylcatechol (compound **1**). (b) 2-(4′,8′-Dimethyl-nona-3′,7′-dienyl)-8-hydroxy-2-methyl-2H-chromene-6-carboxylic acid methyl ester (compound **2**).

Table 1
^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compound **2** in $CDCl_3$

Position	δ_H	*m*	*J* (Hz)	δ_C	*m*
2				81.0	*s*
3	5.61	*d*	10.4	129.7	*d*
4	6.39	*d*	10.4	122.2	*d*
5	7.31	*d*	1.6	119.9	*d*
6				122.6	*s*
7	7.48	*d*	1.6	116.3	*d*
8				143.9	*s*
9				143.7	*s*
10				120.3	*s*
11				166.8	*s*
12	1.45	*s*		27.0	*q*
1′	1.70–1.82	*m*		41.5	*t*
2′	2.1	*m*		22.6	*t*
3′	5.08	*m*		123.4	*d*
4′				135.8	*s*
5′	1.95	*m*		39.6	*t*
6′	2.03	*m*		26.6	*t*
7′	5.08	*m*		124.2	*d*
8′				131.4	*s*
9′	1.67	*brs*		25.7	*q*
10′	1.59	*brs*		17.7	*q*
11′	1.55	*brs*		16.0	*q*
MeO	3.87	*s*		51.9	*q*

^{13}C and ^{1}H NMR spectroscopic data were similar to those recently published for piperochromenoic acid (Ampofo et al., 1987) indicating the occurrence of a chromenoic acid nucleus with a 4,8-dimethyl-3,4-nonadienyl group. However, the carboxylic acid was esterified which was deduced from the upfield shift of the carbonyl resonance (δ_C 166.8 ppm), the singlet at δ_H 3.87 and the J3 between these signals. Furthermore, differences in the aromatic resonances suggested A ring modifications relative to piperochromenoic acid. A meta-coupling doublet (1H at δ_H 7.48 pmm) was assigned to the hydrogen ortho to the carboxy methyl group. The molecular formula obtained from the HREIMS as well as the signals at *m/z* 370 and 220 in the EIMS required the presence of a hydroxyl group at C-8 which was confirmed by the downfield shift of C-8 (δ_C 143.9 ppm) and the upfield shifts of C-7 and C-9 (δ_C 116 and 143.7 ppm) compared to piperochromenoic acid, which was isolated from *Piper auritum*. Thus, compound **2** can be assigned as 8-hydroxy-piperochromenoic acid methyl ester or as 2-(4′,8′-dimethylnona-3′,7′-dienyl)-8-hydroxy-2-methyl-2H-chromene-6-carboxylic methyl ester (Fig. 1).

The PLA_2 inhibitory ability of 4-nerolidylcatechol **1** was time- and concentration-dependent, with a maximal effect observed at an inhibitor/enzyme ratio of 10:1 (wt/wt) and 30 min incubation (Fig. 2). On this basis, all subsequent inhibition experiments were performed at this ratio and incubation time. In molar terms, the half-maximal PLA_2 inhibitory concentration (IC_{50}) of 4-nerolidylcatechol **1** towards *B. asper* myotoxin I corresponded to 987 μM, or roughly 1 mM (Fig. 2). The catalytic activities of purified bee venom PLA_2, crotoxin B, pseudexin, and of whole *Heloderma horridum* and *Micrurus mipartitus* venoms, were also inhibited completely or partially at the inhibitor/enzyme ratio of 10:1 (wt/wt) (Fig. 3). Additionally, 4-nerolidylcatechol **1** inhibited by 75% the proteolytic activity of trypsin upon casein, and abolished the in vitro procoagulant action of a serine proteinase isolated from *Bothrops jararacussu* venom, under the conditions described. However, it did not inhibit other enzymes, such as alkaline phosphatase or *B. asper* metalloproteinase BaP1. Accordingly, the hemorrhagic activities of BaP1 or crude venom from *B. atrox* were not inhibited by 4-nerolidylcatechol **1**.

Myotoxins I from *B. asper* and *B. atrox* induce myonecrosis and increments in plasma CK activity in a dose-dependent manner (Gutiérrez et al., 1984; Núñez et al., 2004). Preincubation of these toxins with 4-nerolidylcatechol **1** reduced their myotoxic activity by approximately 50% (Fig. 4). However, in animal experiments where this compound was rapidly administered after toxin injection, at the same site, no statistically significant inhibitions were recorded for both myotoxins (Fig. 5).

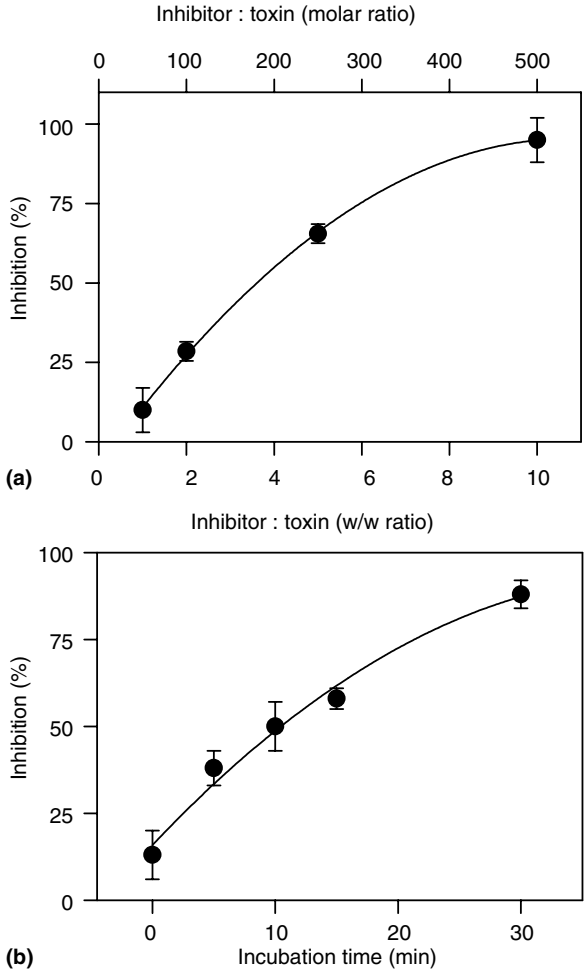

Fig. 2. Inhibition of phospholipase A_2 activity of *Bothrops asper* myotoxin I by 4-nerolidylcatechol. (a) 10 μg of toxin were preincubated with the compound at different ratios for 30 min at 37 °C, and then enzyme activity was assayed as described in Section 4. The activity of untreated toxin was considered as 100%, corresponding to 36 ± 2 μEq/mg min. (b) Time-course of the inhibition of phospholipase A_2 activity by 4-nerolidylcatechol, at an inhibitor:toxin ratio of 10 (wt/wt). All points represent mean ± SD of duplicate assays.

Myotoxins induced an edema of 30% in the mouse footpad, at a dose of 16 μg. When toxins were preincubated with 4-nerolidylcatechol **1** (100 or 200 μg), this inflammatory response was significantly reduced in both cases, with a slightly higher inhibition observed for *B. atrox* myotoxin I (Fig. 5). Moreover, when mice were pretreated with 0.4 mg of this compound by the intraperitoneal route, 1 h before toxin injection in the footpad, edema was reduced approximately by one-third, for both the *B. asper* and *B. atrox* proteins.

Electrophoretic analyses were utilized to assess possible changes occurring in *B. atrox* myotoxin I after its incubation with 4-nerolidylcatechol **1**. The untreated toxin migrated as a sharp band of ~15 kD under reducing conditions, corresponding to the mass of its monomeric subunit, whereas it formed a typical smear

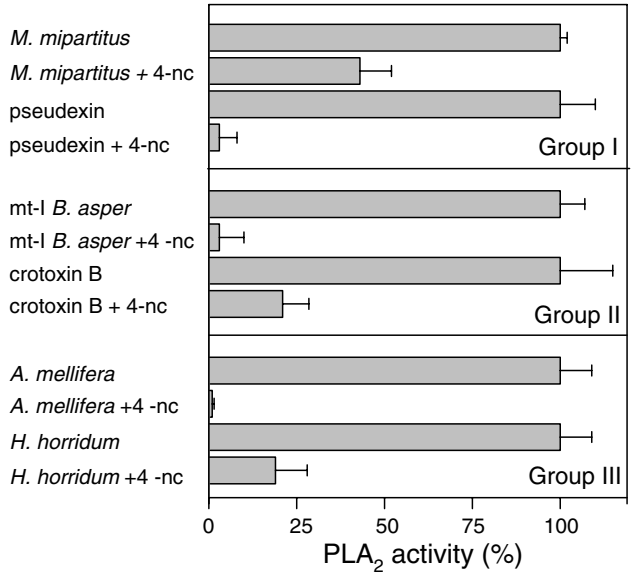

Fig. 3. Inhibitory ability of 4-nerolidylcatechol (**1**) towards the enzymatic activity of venom phospholipases A_2 of group I (*Pseudechis porphyriacus* pseudexin, 2.5 μg and *Micrurus mipartitus* whole venom, 2.5 μg), group II (*B. asper* myotoxin I, 10 μg; and crotoxin B from *Crotalus durissus collilineatus*, 10 μg), and group III (bee venom PLA_2, 2.5 μg; and *Heloderma horridum* whole venom, 2.5 μg). Each sample was incubated with the compound at an inhibitor:toxin ratio of 10 (wt/wt) for 30 min at 37 °C, and then enzyme activity was assayed as described in Section 4. Untreated samples were considered as having 100% activity, which corresponded to 115 ± 2, 216 ± 21, 36 ± 2, 33 ± 5, 245 ± 21, 108 ± 10 μEq/mg min, respectively. Bars represent mean ± SD of duplicate assays.

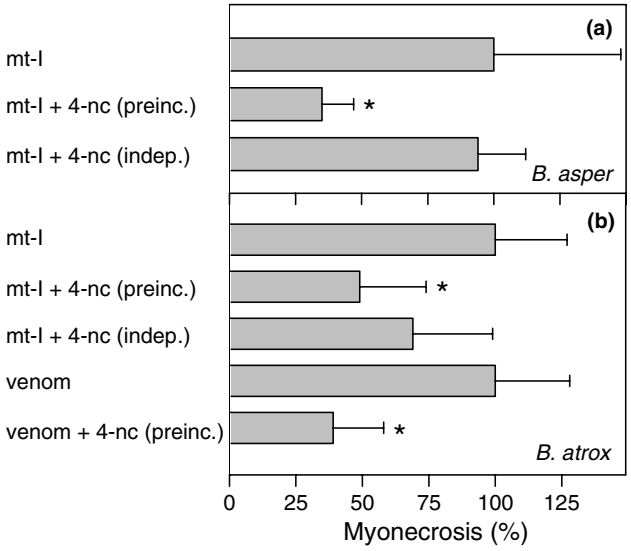

Fig. 4. Inhibition of myonecrosis induced by *Bothrops asper* (a) or *Bothrops atrox* (b) myotoxins by 4-nerolidylcatechol (4-nc) in mice. In preincubation experiments, either toxin or crude venom (50 μg) were incubated with 500 μg of inhibitor, and the mixture was then injected into groups of mice, by the i.m. route. In independent administration experiments, inhibitor (500 μg) was administered in situ immediately after i.m. toxin injection (50 μg). After 2 h, plasma creatine kinase (CK) was determined as described in Section 4. Basal CK activity in the control groups was 224 ± 16 U/L (vehicle alone) and 261 ± 146 U/L (inhibitor alone), respectively. Asterisks indicate a statistically significant ($p < 0.05$) reduction in myonecrosis, in comparison to the corresponding 100% controls, by Student's t test. All bars represent mean ± SD of five animals.

under non-reducing conditions, spanning from about 28 kD (homodimer) to 15 kD (monomer) (Fig. 6). Under non-reducing conditions, 4-nerolidylcatechol **1** altered the electrophoretic behaviour of the protein in a time-dependent manner, inducing a shift in the upper position of the smear (Fig. 6). Nevertheless, reduced samples evidenced that the protein subunit essentially retained its normal molecular mass, with the appearance of only trace amounts of higher mass bands (Fig. 6). On the other hand, two-dimensional electrophoresis indicated that 4-nerolidylcatechol **1** did not alter the pI of myotoxin (Fig. 7). This analysis also detected the formation of traces of higher molecular mass protein products, as observed in one-dimensional SDS–PAGE, which nevertheless migrated with an identical pI value as the untreated protein (Fig. 7).

Analytical RP-HPLC of myotoxin after incubation with 4-nerolidylcatechol **1** also evidenced that most of the protein maintained its normal retention time on a C4 column, despite the appearance of two smaller peaks with higher retention times (Fig. 8). Finally, mass spectrometry analyses of myotoxin after its incubation with 4-nerolidylcatechol **1**, revealed two main signals of 14,121 and 14,103 daltons, in comparison with 13,825 daltons for the untreated myotoxin control.

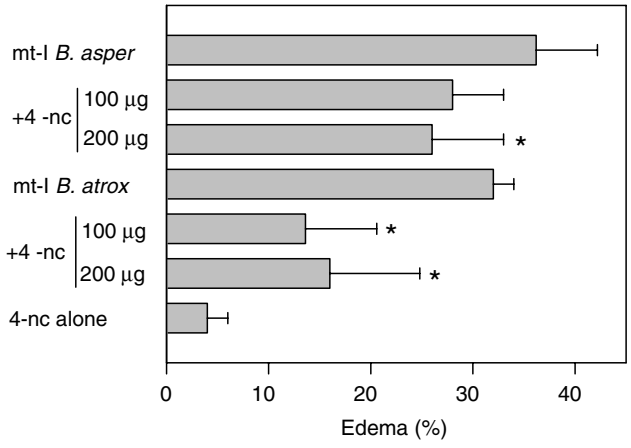

Fig. 5. Inhibition of the edema-forming activity of *Bothrops asper* and *B. atrox* myotoxins (16 μg) by 4-nerolidylcatechol **1** (4-nc), after preincubation for 30 min at 37 °C. Asterisks indicate a statistically significant ($p < 0.05$) reduction in edema, in comparison the corresponding 100% controls, by Student's t test. All bars represent mean ± SD of five animals.

3. Discussion

While quite a number of reports, from different geographical areas, mention plants reputed to neutralize the action of snake venoms, only few attribute such

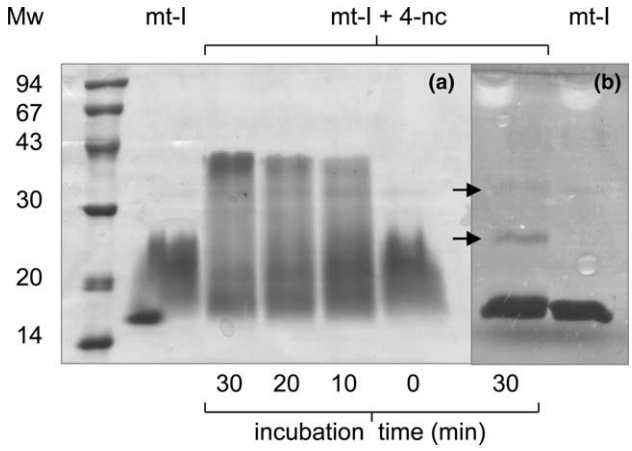

Fig. 6. SDS–PAGE analysis of *Bothrops atrox* myotoxin I, either untreated or after its incubation with 4-nerolidylcatechol **1** (1:10 wt/wt) at the indicated time periods. Samples were run under non-reducing (a) or reducing (b) conditions, as described in Section 4. Molecular mass markers are indicated to the left. Arrows point to two faint bands observed in the reduced toxin sample that was treated with the inhibitor.

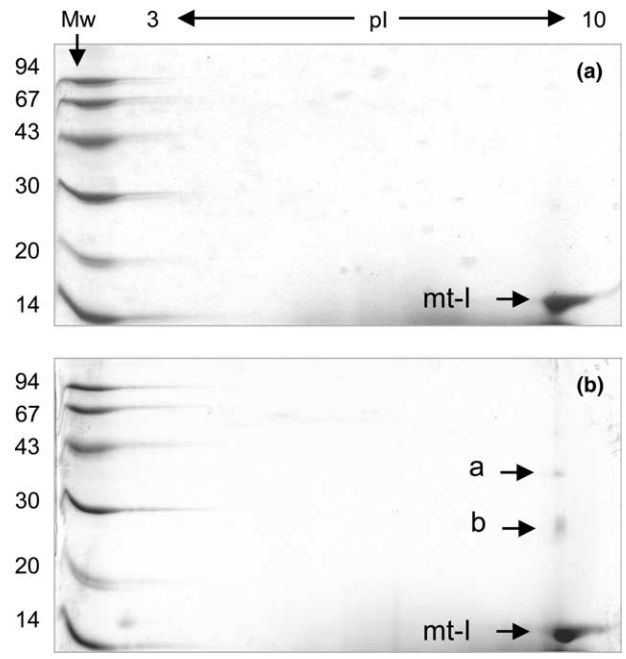

Fig. 7. Two-dimensional gel electrophoresis of *Bothrops atrox* myotoxin I, either untreated (a), or after its incubation with 4-nerolidylcatechol for 30 min at 37 °C (b). The first dimension (horizontal) corresponds to isolectric focusing on an immobilized pH gradient from 3 to 10. The second dimension (vertical) is SDS–PAGE, with molecular mass markers indicated to the left. Arrows (a and b) point to two faint spots observed in the reduced toxin sample that was treated with the inhibitor.

activity to specific chemical compounds identified in them (Vishwanath et al., 1987; Melo and Ownby, 1999; Batina et al., 2000; Bernard et al., 2001). The present study demonstrates that *P. peltatum* and *P. umbellatum* contain components that inhibit the enzymatic

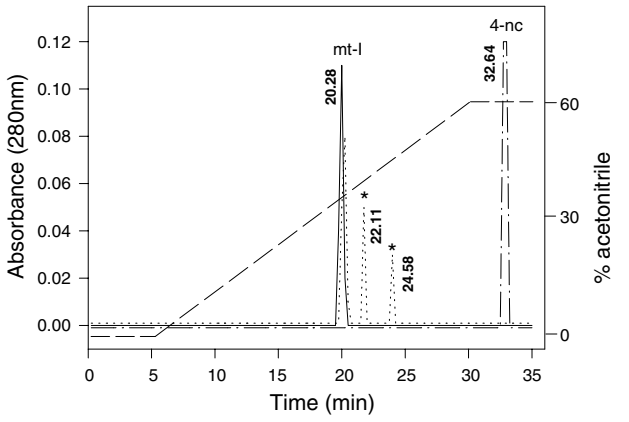

Fig. 8. RP-HPLC analysis of *Bothrops atrox* myotoxin I on a C4 column, either untreated (continuous line), or after its incubation with 4-nerolidylcatechol for 30 min at 37 °C (dotted line). The compound alone (4-nc) is represented by the dash-dot line, eluting at 32.64 min. Elution was carried out with a 0–60% acetonitrile linear gradient in 0.1% trifluoroacetic acid, at 1 ml/min, and monitored at 280 nm, as described in Section 4.

and toxic activities exerted by venom PLA_2s. After purification, 4-nerolidylcatechol **1** was shown to contribute to such inhibitory effect in both plant species.

Bothrops venoms are known to induce prominent tissue damage at the site of injection, and PLA_2s are the main group of toxins that damage skeletal muscle in these venoms (Gutiérrez and Lomonte, 1995; Gutiérrez and Ownby, 2003). 4-Nerolidylcatechol (**1**) inhibited, albeit to a partial extent, myonecrosis induced by both Asp49 (*B. asper* myotoxin I) and Lys49 (*B. atrox* myotoxin I) PLA_2s in mice. Thus, inhibition of enzymatic activity does not necessarily imply total inhibition of myotoxicity, suggesting that other molecular regions besides the catalytic site are involved in the myotoxic action of PLA_2s, as previously proposed (Gutiérrez and Lomonte, 1995; Gutiérrez and Ownby, 2003). In addition to myotoxicity, the compound inhibited the edema-forming effect of myotoxins. Previous studies had reported the ability of *P. peltatum* to prevent edema induced by carragenin, if administered before its injection (Desmarchelier et al., 2000).

Interestingly, 4-nerolidylcatechol (**1**) inhibited not only the PLA_2 activity of enzymes classified within group II, such as the *Bothrops* toxins, but also of enzymes belonging to groups I (pseudexin and *Micrurus mipartitus* venom) or III (bee PLA_2 and lizard venom). Since the catalytic mechanism of all these enzymes is conserved, in spite of their structural differences (Berg et al., 2001), 4-nerolidylcatechol (**1**) could be affecting this process via binding to the active site or its proximity, or via modification of conserved residues that are critical to catalysis.

The observation that 4-nerolidylcatechol (**1**) was also able to inhibit a serine proteinase, but not other enzymes such as a metalloproteinase or a phosphatase, suggests

that this compound probably does not act by causing a general chemical modification of proteins, but that it may have at least some specificity for certain protein types. The possible mechanism of myotoxin inhibition was explored using electrophoretic and chromatographic techniques. These analyses indicated that 4-nerolidylcatechol (**1**) did not induce major changes in isoelectric point or gross molecular mass (degradation, aggregation) of the inhibited PLA_2 toxin. Although formation of two minor, but stable (under reducing conditions) bands of higher molecular mass of the toxin was observed, both in one- and two-dimensional gels, most of the protein migrated identically to the uninhibited PLA_2. The two smaller protein peaks eluting with retention times different from that of untreated toxin in HPLC, most likely correspond to the two higher molecular mass toxin bands observed by electrophoresis. Results from the two-dimensional electrophoresis suggest that charged amino acids are not modified by the inhibitor.

The presence of at least one hydroxy group has been reported to be relevant for the PLA_2 inhibitory activity demonstrated by other compounds. Thus, Chandra et al. (2002) showed that aristolochic acid inhibits *Daboia russelli pulchella* venom PLA_2 by its binding oriented in such a way that its only OH group forms two hydrogen bonds with active site residues His48 and Asp49. Docking of betulinic acid and bovine pancreatic PLA_2 showed that an anionic group is involved in the inhibition mechanism (Bernard et al., 2001). Results of mass spectrometry suggest that one molecule of inhibitor is covalently bound to one molecule of toxin, consistent with a possible mechanism of action based on the esterification of one or two hydroxy groups, with the consequent loss of one (mass signal of 14.121), or two (mass signal of 14.103) water molecules, respectively. Nevertheless, the presence of an anionic group in the molecule is probably not the only requirement for inhibition, since compound **2** (Fig. 1(b)) presents a hydroxyl group, but did not inhibit PLA_2 activity. Whether the large aliphatic tail may also be an important, but not essential structural element, has to be elucidated in further experiments. Interestingly, α-tocopherol which resembles compound **2** by possessing a chroman skeleton, as well as an aliphatic saturated tail, exerts PLA_2s inhibitory activity (Takeda et al., 2004).

The best inhibition of 4-nerolidylcatechol (**1**) towards PLA_2 enzymatic and toxic effects was observed in preincubation experiments, possibly because the interaction between this compound and toxin is slow, as observed in time-course PLA_2 inhibition assays, and also since these toxic effects have an extremely rapid onset once the toxins, or venoms, are injected in mice. Thus, its potential usefulness against snakebite envenomations is likely to be limited, knowing that the effects of myotoxins are acute and extremely fast (Gutiérrez and Lomonte, 1995). Nevertheless, further characterization of the mechanism of interaction between 4-nerolidylcatechol and these toxic enzymes, i.e., identification of the specific amino acid residue(s) that are modified, could improve our understanding of the structure–function relationships of venom PLA_2s, and possibly of other PLA_2s that play important roles in pathological or inflammatory processes. In addition, the broad range of secreted PLA_2s that were inhibited by 4-nerolidylcatechol (**1**) suggests that it might be useful as a lead structure for developing novel PLA_2 inhibitors.

4. Experimental

4.1. Venoms, toxins and animals

Venoms of *B. asper* and *B. atrox* were obtained from Instituto Clodomiro Picado, University of Costa Rica, and Antioquia University Serpentarium, Colombia, respectively. *B. asper* myotoxin I (Asp49) and *B. atrox* myotoxin I (Lys49), were purifed as previously described (Gutiérrez et al., 1984; Núñez et al., 2004). CD1 mice of 20–22 g body weight were utilized for determination of toxic activities, in accordance with guidelines of the Institutional Committee for the Use and Care of Research Animals, University of Costa Rica.

4.1.1. Plant material

Branches of *P. umbellatum* and *P. peltatum* were collected in Upala and Guápiles (Costa Rica), respectively, and identified by Luis Poveda (Universidad Nacional, Costa Rica). Voucher specimens (JVR 9449 and JVR 9425) were deposited in Herbario Juvenal Valerio, Universidad Nacional.

4.1.2. General experimental procedures

NMR spectra were recorded in $CDCl_3$ using a Varian Mercury 400 instrument. EIMS were taken with a MAT 8200 (Finnigan), HREIMS with a Mat 95 SL (Finnigan). Mass spectra of *B. atrox* myotoxin I before and after its incubation with inhibitor were analyzed by LC-ESI Q-TOF.

4.1.3. Extraction and isolation

After drying and grinding the plant material (*P. umbellatum*: 1800 g; *P. peltatum*: 950 g), it was percolated with methyl-terbutyl ether (METB) for two days. A semisolid material was finally obtained using a rotavapor. After removal of fat with methanol at −20 °C for 24 h, the amount of residues were 15 and 5 g, respectively. PLA_2 inhibition screening of the extracts was performed as described below.

4.1.4. Purification of compounds

Five g of each extract were applied to a silica gel column, initially eluted with a mixture of hexane/METB (8:2, 1:1, 3:7, 0:10) and then with mixtures of METB/MeOH (9:1, 8:2). Resulting fractions were labeled 1–8, evaporated, and then evaluated for PLA_2 inhibition. Fractions 3 (~2 g), found to be active in both extracts, were rechromatographed on a medium pressure reverse phase column (C8) using MeOH/H_2O (7:3, 8:2, 9:1, 10:0). Four new fractions were collected, evaporated, labeled 3a–3d, and again evaluated for PLA_2 inhibitory activity. Fraction 3c (220 mg) from *P. umbellatum* tested positive, was further separated on a silica gel plate using CH_2Cl_2/benzene/ether (4.5:4.5:1). Two compounds were finally obtained, 120 mg of a yellow oily residue (compound 1) and 12 mg of a brown residue (compound 2), respectively. In the case of *P. peltatum*, fraction 3b (440 mg) was similarly separated on silica plates, resulting in 80 mg of compound **1** and 10 mg of compound **2**, that were also analyzed by NMR.

Homogeneity of the fractions of interest was assessed using thin-layer chromatography (TLC) on silica gel 60 F254 (Merck). Detection was carried out using a UV lamp or by developing with a 1% (w/v) potassium permanganate solution.

4.1.5. 2-(4′,8′-Dimethylnona-3′,7′-dienyl)-8-hydroxy-2-methyl-2H-chromene-6-carboxylic methyl ester (compound 2)

Brown amorphous solid; EIMS *m/z* (rel. Int.): 370 (17), 339 (5) $[M - CH_3O]^+$, 283 (20), 259 (8), 220 (80), 181 (15), 160 (15), 107 (15), 81 (25), 69 (100) $[C_5H_9]^+$; HREIMS *m/z*: 370.214410 (calcd for $C_{23}H_{30}O_4$, 370.214359); for ^1H and ^{13}C NMR spectroscopic data, see Table 1.

4.1.6. Inhibition of PLA_2 activity

PLA_2 activity was assayed by titration of free fatty acids released from egg yolk phospholipids, suspended in 1% Triton X-100, 0.1 M Tris–HCl, 0.01 M $CaCl_2$, pH 8.5 buffer (Dole, 1956), using 10 μg of *B. asper* myotoxin I (Asp49 PLA_2), and a reaction time of 15 min at 37 °C. This protein amount was selected from the linear region of activity curves. For inhibition experiments, the toxin was mixed with variable amounts of crude extracts, fractions, or pure compounds, and incubated for 30 min at 37 °C before PLA_2 determination.

In order to evaluate the specificity of 4-nerolidylcatechol (**1**) towards different types of enzymes, inhibition assays were also performed with the following PLA_2s: pseudexin, purified from the venom of *Pseudechis porphyriacus* (group I); B subunit of crotoxin, from *Crotalus durissus collilineatus* (group II); bee venom PLA_2, from *Apis mellifera* (group III); and the crude venoms of the coral snake *M. mipartitus* (containing group I PLA_2s), and the Gila monster *H. horridum* (containing group III PLA_2s).

4.1.7. Inhibition of alkaline phosphatase and trypsin activity

4-Nerolidylcatechol (**1**) was incubated with each enzyme (Sigma) at a ratio of 10:1 (wt/wt), and the mixtures were assayed on their corresponding substrates (*p*-nitrophenyl phosphate or casein, respectively), in comparison to controls from which the compound was omitted.

4.1.8. Inhibition of B. atrox and B. asper venom, as well as jararacussin-I activity

4-Nerolidylcatechol (**1**) was incubated with whole *B. atrox* venom or BaP1 (a metalloproteinase hemorrhagin purified from *B. asper* venom; (Gutiérrez et al., 1995), at an inhibitor/protein ratio of 10:1 (wt/wt). Then, haemorrhagic activity was evaluated in mice, using previously described methods (Gutiérrez et al., 1995).

Inhibition of the procoagulant activity of jararacussin-I, a serine proteinase purified from *B. jararacussu* venom (Bortoleto et al., 2002), was evaluated by incubating 4-nerolidylcatechol (**1**) with this enzyme at a 10:1 ratio (wt/wt), and then adding 3 μg/100 μl of the mixture (or enzyme alone) to 0.2 ml of citrated human plasma, in order to record coagulation times (Theakston and Reid, 1983).

4.1.9. Myotoxic activity

Groups of five mice received an intramuscular (i.m.) injection, in the right gastrocnemius, of solutions containing either *B. atrox* myotoxin I or *B. asper* myotoxin I (50 μg) alone, or mixtures of 4-nerolidylcatechol (**1**) and toxin, at a 10:1 ratio (wt/wt), previously incubated for 30 min at 37 °C. Control mice received phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate, pH 7.2) alone, or the inhibitor alone. Two hr after injection, blood was collected from the tail into heparinized capillary tubes, and the plasma creatine kinase (CK) activity was determined by a kinetic assay (Sigma 47-UV). These experiments were also performed using whole venom of *B. atrox*, under the same conditions.

In another set of experiments, the ability of 4-nerolidylcatechol (**1**) to inhibit myotoxicity by independent in situ administration was evaluated. Mice received an i.m. myotoxin injection (50 μg), followed 20 s later by 500 μg of the compound (in 50 μl of PBS) at the same site. Myonecrosis was estimated as described. In all experiments, the inhibitor was initially dissolved in PBS containing 4% (v/v) dimethylsulfoxide (DMSO).

4.1.10. Edema-inducing activity

Edema was evaluated after the subcutaneous injection of *B. atrox* myotoxin I or *B. asper* myotoxin I (16 μg in 50 μl of PBS), into the right footpad of mice.

The left footpad received 50 μl PBS as a control. Inhibition assays were performed by preincubating 4-nerolidylcatechol with toxins for 30 min at 37 °C. Three hr after injection, mice were sacrificed by CO_2 inhalation and both feet were cut and weighed, in order to determine the percent increase due to edema (Yamakawa et al., 1976).

In another set of experiments, mice were pretreated with 0.4 mg/0.2 ml of 4-nerolidylcatechol by intraperitoneal route, 1 h before toxin injection in the footpad (16 μg/50 μl), and edema was quantified as described above.

4.1.11. High-performance liquid chromatography

Inhibitor (4-nerolidylcatechol (**1**)), *B. atrox* myotoxin I, or a mixture of inhibitor:toxin (10:1 wt/wt ratio) were incubated for 30 min at 37 °C, and then analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) on a C4 column (150 × 10 mm; Vydac). Elution was done with a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid, developed at 1 ml/min, on an Agilent 1100 system. Absorbance was monitored at 280 nm.

4.1.12. Electrophoretic analyses

For one-dimensional electrophoresis, toxin alone or mixtures of 4-nerolidylcatechol (**1**) and toxin (10:1 wt/wt ratio) were incubated for 0, 10, 20, or 30 min, and then analyzed on SDS–polyacrylamide 12% gels, under non-reducing or reducing (2-mercaptoethanol, 5% v/v) conditions (Laemmli, 1970).

For two-dimensional (2-D) electrophoresis, the first dimension (isoelectric focusing, IEF) was performed with 10 μl of samples containing either toxin or a mixture of 4-nerolidylcatechol (**1**) and toxin (10:1 wt/wt ratio) incubated for 30 min 37 °C, and then mixed with 115 μl of sample buffer (8 M urea, 1% ditiothreitol (DTT), 0.5% Triton X-100, and 2% ampholytes (Amersham Bioscience) in the pH 3–10 range). IEF strips (7 cm; Amersham Bioscience) were rehydrated with this solution for 12 h prior to focusing, which was performed at 200 v for 1 min, and 3500 v for 2.5 h. Then, strips were equilibrated for 30 min in 10 ml of equilibrium buffer (6 M urea, 1% SDS, 30% glycerol, 0.5% DTT, 0.002% bromophenol blue, and 50 mM Tris–HCl, pH 6.8) and placed onto 12% SDS–polyacrylamide gels for the second dimension electrophoresis. Proteins were stained with Coomassie blue R-250.

Acknowledgements

We thank Drs. Ivan Kaiser, Lourival Possani, Andreimar Soares, and Alexandra Rucavado for their generous gift of toxins. Thanks are also due to Dr. Jürgen Wört and Christoph Warth (University of Freiburg) for taking the HREIMS, Luis Poveda for botanical identification, Juan José Araya, Max Chavarría, Raúl Arce, and Teresa Escalante for valuable collaborations, and Dr. José María Gutiérrez for critical reading of the manuscript. A scholarship from Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología (COLCIENCIAS) to V. Núnez, and the financial support from Vicerrectoría de Investigación-UCR (VI-741-99269), the Volkswagen Stiftung, the Embassy of Japan in Costa Rica, and UNESCO (883.701-3) are also acknowledged. This study was performed as partial requirement for the Ph.D. degree of V. Núñez at the University of Costa Rica.

References

Akendengue, B., Louis, A.M., 1994. Medicinal plants used by Masango people in Gabon. J. Ethnopharmacol. 41, 193–200.

Ampofo, S.A., Roussis, V., Wiemer, D.F., 1987. New prenylated phenolics from *Piper auritum*. Phytochemistry 26, 2367–2370.

Batina, M.F., Cintra, A.C., Veronese, E.L., Lavrador, M.A., Giglio, J.R., Pereira, P.S., 2000. Inhibition of the lethal and myotoxic activities of *Crotalus durissus terrificus* venom by *Tabernaemontana catharinensis*: identification of one of the active components. Planta Medica 66, 424–428.

Berg, O., Gelb, M.H., Tsai, M.D., Jain, M.K., 2001. Interfacial enzymology: the secreted phospholipase A_2-paradigm. Chem. Rev. 101, 2613–2653.

Bernard, P., Scior, T., Didier, B., Hibert, M., Berthon, J.-Y., 2001. Ethnopharmacology and bioinformatic combination for leads discovery: application to phospholipase A_2 inhibitors. Phytochemistry 58, 865–874.

Bortoleto, R.K., Murakami, M.T., Watanabe, L., Soares, A.M., Arni, R.K., 2002. Purification, characterization and crystallization of jararacussin-I, a fibrinogen-clotting enzyme isolated from the venom of *Bothrops jararacussu*. Toxicon 40, 1307–1312.

Chandra, V., Jasti, J., Kaur, P., Srinivasan, A., Betzel, Ch., Singh, T.P., 2002. Structural basis of phospholipase A_2 inhibition for the synthesis of prostaglandins by the plant alkaloid aristolochic acid from 1.7 Å crystal structure. Biochemistry 41, 10914–10919.

Chippaux, J.P., 1998. Snake-bites: appraisal of the global situation. Bull. World Health Org. 75, 515–524.

Desmarchelier, C., Slowing, K., Ciccia, G., 2000. Anti-inflammatory activity of *Pothomorphe peltata* leaf methanol extract. Fitoterapia 71, 556–558.

Dole, V.P., 1956. Relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. Clin. Invest. 35, 150–154.

Gutiérrez, J.M., Lomonte, B., 1995. Phospholipase A_2 myotoxins from *Bothrops* snake venoms. Toxicon 33, 1405–1424.

Gutiérrez, J.M., Ownby, C.L., 2003. Skeletal muscle degeneration induced by venom phospholipases A_2: insights into the mechanisms of local and systemic myotoxicity. Toxicon 42, 915–931.

Gutiérrez, J.M., Ownby, C.L., Odell, G.V., 1984. Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. Toxicon 22, 115–128.

Gutiérrez, J.M., Romero, M., Díaz, C., Borkow, G., Ovadia, M., 1995. Isolation and characterization of a metalloproteinase with weak hemorrhagic activity from the venom of the snake *Bothrops asper* (terciopelo). Toxicon 33, 19–29.

Gutiérrez, J.M., León, G., Rojas, G., Lomonte, B., Rucavado, A., Chaves, F., 1998. Neutralization of local tissue damage induced by *Bothrops asper* (terciopelo) snake venom. Toxicon 36, 1529–1538.

Kijjoa, A., Giesbrecht, A.M., Akisue, M.K., Gottlieb, O.R., Gottlieb, H.E., 1980. 4-Nerolidylcatechol from *Potomorphe umbellata*. Planta Medica 39, 85–87.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lomonte, B., Angulo, Y., Calderón, L., 2003. An overview of lysine-49 phospholipase A_2 myotoxins from crotalid snake venoms and their structural determinants of myotoxic action. Toxicon 42, 885–901.

Melo, P.A., Ownby, C.L., 1999. Ability of wedelolactone, heparin and *p*-bromophenacyl bromide to antagonize the myotoxic effects of two crotalid venoms and their PLA_2 myotoxins. Toxicon 37, 199–215.

Mors, W.B., Nascimento, M.C., Ruppelt, B.M., Alvares, N., 2000. Plant natural products active against snake bite – the molecular approach. Phytochemistry 55, 627–642.

Núñez, V., Arce, V., Gutiérrez, J.M., Lomonte, B., 2004. Structural and functional characterization of myotoxin I, a Lys49 phospholipase A_2 homologue from the venom of the snake *Bothrops atrox*. Toxicon 44, 91–101.

Otero, R., Núñez, V., Jiménez, S., Fonnegra, R., Osorio, R.G., García, M.E., Díaz, A., 2000a. Snakebites and ethnobotany in the northwest region of Colombia. Part II: Neutralization of lethal and enzymatic effects of *Bothrops atrox* venom. J. Ethnopharmacol. 71, 505–511.

Otero, R., Fonnegra, R., Jiménez, S.L., Núñez, V., Evans, N., Alzate, S.P., García, M.E., Saldarriaga, M., Del Valle, G., Osorio, R.G., Daíz, A., Valderrama, R., Duque, A., Vélez, H.N., 2000b. Snakebites and ethnobotany in the Northwest region of Colombia. Part I: Traditional use of plants. J. Ethnopharmacol. 71, 493–504.

Otero, R., Gutiérrez, J., Mesa, M.B., Duque, E., Rodríguez, O., Arango, J.L., Gómez, F., Toro, A., Cano, F., Rodríguez, L.M., Caro, E., Martínez, J., Cornejo, W., Gómez, L.M., Uribe, F.L., Cárdenas, S., Núñez, V., Díaz, A., 2002. Complications of *Bothrops*, *Porthidium*, and *Bothriechis* snakebites in Colombia. A clinical and epidemiology study of 39 cases attended in a university hospital. Toxicon 40, 1107–1114.

Rosenberg, P., 1997. Lethal potency of snake venom phospholipase A_2 enzymes. Pitfalls to avoid in the study of correlations between enzymatic activity and pharmacological properties of phospholipase A_2 enzymes. In: Kini, R.M. (Ed.), Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism. John Wiley & Sons, New York, pp. 129–154.

Scott, D.L, Achari, A., Vidal, J.C., Sigler, P.B., 1992. Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. J. Biol. Chem. 267, 22645–22657.

Six, D.A., Dennis, E.A., 2000. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. Biochim. Biophys. Acta 1488, 1–19.

Takeda, A.A.S., Dos Santos, J.I., Marcussi, S., Silveira, L.B., Soares, A.M., Fontes, M.R.M., 2004. Crystallization and preliminary X-ray diffraction analysis of an acidic phospholipase A_2 complexed with *p*-bromophenacyl bromide and α-tocopherol inhibitors at 1.9- and 1.45-Å resolution. Biochim. Biophys. Acta 1699, 281–284.

Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. Bull. World Health Org. 61, 949–956.

Vishwanath, B.S., Appu Rao, A.G., Gowda, T.V., 1987. Interaction of phospholipase A_2 from *Vipera russelli* venom with aristolochic acid: a circular dichroism study. Toxicon 25, 939–946.

Warrel, D.A., 1995. Clinical toxicology of snakebite in Africa and the Middle East/Arabian Peninsula. In: Meier, J., White, J. (Eds.), Handbook of Clinical Toxicology of Animal Venoms and Poisons. CRC Press, Boca Raton, pp. 433–492.

Yamakawa, M., Nozaki, M., Hokama, Z., 1976. Fractionation of Sakishima-habu (*Trimeresurus elegans*) venom, and lethal, hemorrhagic, and edema-forming activities of the fractions. In: Ohsaka, A., Hayashi, K., Sawai, Y. (Eds.), Animal, Plant and Microbial Toxins. Plenum Press, New York, pp. 97–109.

Zamora-Martinez, M.C., Pola, C.N.P., 1992. Medicinal plants used in some rural populations of Oaxaca, Puebla and Veracruz Mexico. J. Ethnopharmacol. 35, 229–257.