View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Journal of Ethnopharmacology 145 (2013) 50–58

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Antiophidic activity of the extract of the Amazon plant *Humirianthera ampla* and constituents

Marcelo Abrahão Strauch [a,1], Marcelo Amorim Tomaz [a,1], Marcos Monteiro-Machado [a], Hilmar Dias Ricardo [a], Bruno Lemos Cons [a], Fabrício F.A. Fernandes [a], Camila Z. El-Kik [a], Mariângela Soares Azevedo [b], Paulo A. Melo [a,*]

[a] *Programa de Pós-Graduação em Farmacologia e Química Medicinal, Instituto de Ciências Biomédicas, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Avenida Brigadeiro Trompowski, Rio de Janeiro, RJ 21941-590, Brazil*
[b] *Departamento de Química, Universidade Federal de Rondônia, Porto Velho, RO 78900-500, Brazil*

A R T I C L E I N F O

Article history:
Received 4 August 2012
Received in revised form
18 October 2012
Accepted 19 October 2012
Available online 1 November 2012

Keywords:
Bothrops snake venoms
Plant extract
Humirianthera ampla
Lupeol
Sitosterol

A B S T R A C T

Ethnopharmacological relevance: Although serotherapy against snakebite has been discovered more than one hundred years ago, antivenom is not available all over Brazil. The use of plants from folk medicine is common mainly in the Brazilian Amazon area. One of these plants is named *Humirianthera ampla* (HA).
Materials and Methods: We have investigated HA extract and constituents' antiophidic activity in different experimental protocols against some *Bothrops* snake venoms (*Bothrops jararacussu*, *Bothrops atrox* and *Bothrops jararaca*). The protocols investigated include phospholipase, proteolytic, pro-coagulant, hemorrhagic, edematogenic and myotoxic activities induced by these venoms in Swiss mice.
Results: All the venoms caused an increase in the rate of creatine kinase (CK) release from isolated muscles, indicating damage to the sarcolemma. The crude extract of HA decreased the myotoxic activity in a concentration-dependent fashion. The presence of HA 300 μg/mL decreased up to 96% of *Bothrops jararacussu* and 94% of *Bothrops atrox* myotoxicity after 90 min of exposure. *In vivo* myotoxicity of *Bothrops atrox* venom was decreased in 75% when the venom was preincubated with HA 500 mg/kg. Similar results were observed with lupeol against *Bothrops jararacussu* and *Bothrops atrox* venoms. The hemorrhagic activity was evaluated by intradermal injection of *Bothrops atrox* venom. Preincubation and oral pre- and posttreatment with HA decreased hemorrhage by 100%, 45% and 45%, respectively. *Bothrops atrox* venom also induced formation of edema, which was significantly inhibited by pre- and posttreatment with HA. All the venoms showed extensive pro-coagulating properties, and these activities were inhibited by up to 90% with HA, which presented concentration-dependent inhibition. Finally, proteolytic and phospholipase activities of the venoms were all inhibited by increasing concentrations of HA, lupeol and sitosterol. The inhibition of these activities might help explain the actions against *in vivo* myotoxicity and the *in vivo* effects observed, *i.e.*, edema, myotoxicity, pro-coagulation and hemorrhage.
Conclusions: Altogether, our results give support for the popular use of HA extracts in cases of accidents with snakes, suggesting that it can be used as an adjunct in the management of venomous snakebites.
© 2012 Elsevier Ireland Ltd. Open access under the Elsevier OA license.

1. Introduction

Venomous snakebites represent a significant health problem, especially in tropical countries, where they frequently affect young and economically active men working in the countryside. In Brazil, most accidents are caused by snakes belonging to *Bothrops* genus, which induce extensive local damage, such as myonecrosis and edema (Bochner and Struchiner, 2003; Da Silva et al., 2003).

The treatment available for these accidents is antibothropic antivenom, which has low and limited effectiveness against the local effects of venoms (Da Silva et al., 2003). The antivenom therapy is often administered late after the accident, when tissue destruction is already in process, causing irreversible and disabling damage (Gutiérrez et al., 1980; Homma and Tu, 1971; Nishioka et al., 1992; Rosenfeld, 1971). The use of plants to halt the effect of snake venoms has been proposed by previous studies (Alam and Gomes, 2003; Borges et al., 2005; Cavalcante et al., 2007; Oliveira et al., 2005; Tan et al., 2009). Therefore, our group

* Corresponding author. Tel./fax: +55 21 2610 3726.
E-mail address: melo.pa@gmail.com (P. A. Melo).
[1] These authors contributed equally to this paper.

0378-8741 © 2012 Elsevier Ireland Ltd. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.jep.2012.10.033

has been particularly concerned with the search for new and effective pharmacologically active principles from plants used in folk medicine to treat or prevent damage caused by accidents with venomous snakes. For example, we have proposed the use of *Eclipta prostrata* (Asteraceae) and constituents as an alternative way to prevent some actions of crotalid venoms (Melo et al., 1994; Mors et al., 1989).

In the valley of Purus, Amazon region, rural communities that have no immediate medical treatment make use of the ethanolic extract obtained from the roots of the plant *Humirianthera ampla* Miers, aiming to slow the effects of toxins in snakebites, mainly *Bothrops atrox*, and to achieve some relief for the victims, although up to date there are no scientific data supporting the antiophidic effect (Graebner et al., 2000). *Humirianthera ampla Miers* is a member of the Icacinaceae family that is popularly known as "surucucaína" or "surucuína". Studies of chemical constituents of this plant revealed the presence of the di- and triterpenoids: annonalide, humirianthol, acrenol and lupeol (Dessoy and Scher, 2002; Luiz et al., 2007). In addition, substances as β-sitosterol and glycosylated sitosterol were also isolated from the ethanolic extract (EE) of *Humirianthera ampla* roots (Graebner et al., 2000, 2002; Luiz et al., 2007). The presence of these compounds might explain the exceptional effect of the *Humirianthera ampla* against inflammation and pain caused by snake bite and consequently validates the large use of this plant by the Amazonia native people in this condition. In the present study, the *Humirianthera ampla* extract and the isolated compounds were investigated under different experimental protocols *in vivo* using *Bothrops atrox* venom and *in vitro* using *Bothrops jararaca, Bothrops jararacussu* and *Bothrops atrox* venoms.

2. Material and methods

Bothrops jararaca and *Bothrops jararacussu* venoms were obtained from Instituto Vital Brasil, Rio de Janeiro; *Bothrops atrox* venom was obtained from Embrapa, Goiás; creatine kinase (CK) activity was determined using a CK NAC® kit from BIOCLIN; lupeol and sitosterol were purchased from Sigma-Aldrich Co.; acrenol, annonalide and humirianthol were provided by Dr. Ademir Farias Morel from the Federal University of Santa Maria, Rio Grande do Sul. Male Swiss mice were provided by the Rodent Vivarium of the Institute of Microbiology Paulo de Góes—Federal University of Rio de Janeiro. Mice weighing 25.0 ± 1.0 g used for the study received water and food *ad libitum* and were kept under a natural light cycle. We adhered to protocols approved by the Ethics Committee for the Use of Animals of the Federal University of Rio de Janeiro (CEUA-UFRJ), where all the procedures that could cause pain were performed under anesthesia.

2.1. Plant material

Humirianthera ampla was collected in November 2006 in Porto Velho, State of Rondônia, Brazil, where it is quite abundant (S 08° 41′ 09.9153″; W 063° 52′ 10.02″). The material was collected by Dr. Mariângela Soares de Azevedo's staff group and was identified by a member of the Department of Botany, INPA, Manaus, Brazil, and a voucher specimen was deposited in the Herbarium Dr. Ary Tupinambá Penna Pinheiro (Porto Velho-RO, Brazil) under the number 214579.

The ethanolic extract was prepared from the root (1.562 kg), which was triturated and dried at room temperature. The dried plant material was placed in contact with ethanol and stirred for a period of 8 h. The material was filtered and the solvent evaporated under reduced pressure, yielding 83.21 g of extract.

2.2. Extraction and isolation

Air dried, powdered roots of *Humirianthera ampla* were extracted exhaustively with ethanol at room temperature. Dry ethanol extract was dissolved in H_2O and the solution was successively partitioned between hexane, CH_2Cl_2, EtOAc and *n*-BuOH. The CH_2Cl_2 fraction was subjected to silica gel CC with $CHCl_3$–MeOH mixtures to yield annonalide, humirianthol and acrenol (Graebner et al., 2000).

2.3. Myotoxicity in vitro

In order to assess the loss of creatine kinase from muscle cells, which indicates damage to the sarcolemma, *in vitro* assays were performed as previously described (Melo and Suarez-Kurtz, 1988a; Melo et al., 1993; 1994). Briefly, mouse *extensor digitorum longus* (EDL) muscle was removed, weighed and superfused continuously with physiological saline solution (PSS), which was composed of (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $NaHPO_4$, 1; $NaHCO_3$, 15; and dextrose, 11. The pH of this solution was equilibrated to 7.3 with 5% CO_2/ 95% O_2. During the superfusion, the muscles were exposed to the venoms of *Bothrops jararaca*, *Bothrops jararacussu* and *Bothrops atrox* (25 μg/mL), *Humirianthera ampla* ethanolic crude extract (HA, 100–300 μg/mL) and lupeol (100 μM) that were added to the PSS. Perfusion samples were collected at 30 min intervals and replaced with fresh solution. The collected samples were stored at 4 °C and their CK activity was determined according to previously described procedures (Melo and Suarez-Kurtz, 1988a; 1988b).

2.4. Myotoxicity in vivo

We evaluated the myotoxicity of *Bothrops atrox* venom by measuring the increase of plasma CK activity induced by intramuscular (i.m.) injection of venom alone or associated with *Humirianthera ampla* ethanolic crude extract or lupeol. The venom was dissolved in PSS to a final volume of 0.1 mL (1.0 mg/kg) and they were injected into the rear thigh of the mice as described previously (Melo and Suarez-Kurtz, 1988b; Melo et al., 1993; 1994). Negative controls consisted of mice injected with the same volume of PSS. To evaluate the antimyotoxic activity of the *Humirianthera ampla* ethanolic crude extract and lupeol, three different protocols were used: preincubation: Venom dissolved in PSS was first incubated with the extract (300 mg/kg) or lupeol (10 mg/kg) for 15 min at room temperature prior to injection; pretreatment: 0.2 mL of the extract (1000 mg/kg) or lupeol (10 mg/kg) was administered orally and intraperitoneously (i.p.), respectively, 15 min before the i.m. venom injection; post-treatment: 0.2 mL of the extract (1000 mg/mL) or lupeol (10 mg/kg) was administered orally and intraperitoneously (i.p.) 15 min after the i.m. venom injection. The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones.

2.5. Hemorrhagic activity

The hemorrhagic effect was induced by an intradermic (i.d.) injection of 0.1 mL of *Bothrops atrox* venom (1 mg/kg) in the abdomen of mice and quantified as previously described (Melo et al., 1994). The negative control received 0.1 mL of PSS i.d. injection. To evaluate the antihemorrhagic activity of the *Humirianthera ampla* ethanolic crude extract, three different protocols were used: preincubation: *Bothrops atrox* dissolved in PSS was first incubated with the extract (300 mg/kg) for 15 min at room temperature prior to injection; pretreatment: 0.2 mL of the extract (1000 mg/kg) was administered orally 15 min before the

intradermic (i.d.) venom injection; post-treatment: 0.2 mL of the extract (1000 mg/mL) was administered orally 15 min after the intradermic (i.d.) venom injection. The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones.

Two hours after the venom injection, animals were killed by ether anesthesia, and the skin covering the abdomen was removed, stretched, and dried at room temperature for 72 h. The skin was fixed to a lucite base plate, and the entire area at the injection site and the surrounding area were transilluminated using an incandescent light. Light transmitted over an area of 109 mm^2 was read, and light transmission or the absorbance was normalized by taking the mean values of the readings over skin injected with either PSS (calibrated to zero) or *Bothrops atrox* venom as arbitrary units of absorbance (AU). The hemorrhagic damage induced by the venom alone was considered as 100% of hemorrhage induction.

2.6. Proteolytic and phospholipase activities

The proteolytic assay was carried out as previously described (Garcia et al., 1978). *Bothrops jararacussu, Bothrops jararaca* and *Bothrops atrox* venoms (10 μg/mL) were preincubated with *Humirianthera ampla* ethanolic crude extract (1–300 μg/mL) or lupeol, sitosterol, acrenol, anonalida and humirianthol (1–100 μM) for 30 min at 37 °C. We added the venom alone or the mixtures in a solution containing 0.2% azocasein, 20 mM $CaCl_2$, and 0.2 M Tris–HCl (pH 8.8). The reaction continued for 90 min at 37 °C and was stopped by the addition of 0.4 mL of 15% (vv) trichloracetic acid, and then centrifuged at 20,000 rpm. We then removed 1.0 mL of the supernatant and mixed it with 0.5 mL NaOH (2.0 M). This final solution was analyzed by spectrophotometry at an absorbance of 420 nm.

Phospholipase A_2 activity was assessed by adapting the turbidimetric assay described previously (Marinetti, 1965). We prepared the substrate by shaking one chicken egg yolk in a solution of 150 mM NaCl to a final volume of 100 mL and stored it at 4 °C prior to the reaction. In each assay, we prepared several tubes by taking a final volume (0.25 mL) of a 10% dilution of the egg suspension and adding it to a solution containing 150 mM NaCl, 10 mM $CaCl_2$, 0.01% taurocholic acid, and 5.0 mM Tris–HCl (pH 7.4). The tubes were kept at 37 °C under mild and constant stirring during the procedure. The reactions were started by adding 10 μg/mL of *Bothrops jararacussu, Bothrops jararaca* or *Bothrops atrox* venom alone or preincubated for 30 min at 37 °C with 10–500 μg/mL of the *Humirianthera ampla* extract or with isolated substances (1–100 μM). Thirty minutes after starting the reactions, the absorbance of the solutions was read at 925 nm, and data was expressed as turbidity decrease.

2.7. Clotting time

The clotting time was assessed by the modified Lee–White method (Raphael, 1983). The animals were grouped ($n=4$ for each group) and about 50 μL of blood were collected from the orbital plexus using heparinized microhematocrit capillary tubes. Before collecting blood, the tubes were filled with 20 μL of PSS, *Bothrops jararacussu, Bothrops jararaca* or *Bothrops atrox* crude venoms (1 μg/mL); or venom preincubated with *Humirianthera ampla* ethanolic crude extract (1–30 μg/mL). The extract alone was also tested, to evaluate whether the *Humirianthera ampla* extract could change the clotting time compared to control time (capillaries with PSS). The clotting time was evaluated and compared among the different groups.

2.8. Mouse tail bleeding time evaluation

Tail vein bleeding tests were performed on mice by a modified method, as previously described (Broze et al., 2001). *Bothrops atrox* venom was administered (0.1 mg/kg, i.v.) in anesthetized mice (intramuscular injection of 0.05 mL anesthetic mixture; pentobarbital 40 mg/kg + xylazine 2.5 mg/kg). Several minutes later, the mice were immobilized in a restraint device and had their tails cut 4 mm from the tip and immersed in 5.0 mL of distilled water at room temperature. Blood loss was evaluated one hour later as a function of absorbance at 540 nm due to the hemoglobin content in the water solution. The absorbance detected for a group that received saline rather than venom was taken as a control of blood loss, and the bleeding time of each procedure was expressed as units of absorbance in 540 nm. For evaluation of the antiophidic activity, three treatment protocols were used: the venom was preincubated at 37 °C with the *Humirianthera ampla* ethanolic crude extract (300 mg/kg) 15 min before the i.v. injection; the extract (1000 mg/kg) was administered orally 15 min before the venom injection; and the oral post-treatment, where the animals received the HA extract (1000 mg/kg) 15 min after the venom injection. The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones.

2.9. Thigh edema

The induction of edema was evaluated by an intramuscular injection of 50 μL *Bothrops atrox* venom (1 mg/kg). Three treatment protocols were used: the venom was preincubated at 37 °C with the *Humirianthera ampla* ethanolic crude extract (300 mg/kg) 15 min before the i.m. injection; the extract (1000 mg/kg) was administered orally 15 min before the venom injection; and the post-treatment, where the animals received the extract injection (1000 mg/kg, oral) 5 min after the venom injection. The doses used here were based on the dose-response study (not shown), from where we chose the most effective ones. The thigh area was measured using a caliper rule and the edema induced by the venom was considered as 100% of edema induction.

2.10. Statistical analysis

Data were expressed as mean ± SEM, and Student's *t*-test was used for statistical analysis. The p value <0.05 was used to indicate a significant difference between means.

3. Results

3.1. Myotoxic activity

Mice isolated *extensor digitorum longus* (EDL) muscles were exposed for 90 min to the venoms of *Bothrops jararacussu, Bothrops atrox* and *Bothrops jararaca* (25 μg/mL). All the venoms caused an increase in the rate of creatine kinase (CK) release from muscles into the bathing media, although *Bothrops jararaca* venom presented a milder *in vitro* myotoxic activity. The rate of CK activity ranged from 0.24 ± 0.15 when exposed only to physiological saline solution (PSS) up to 14.72 ± 2.77; 15.47 ± 1.86; 8.05 ± 4.02 ($U\,g^{-1}\,h^{-1}$, $n=4$) in the presence of the venoms of *Bothrops jararacussu, Bothrops atrox* and *Bothrops jararaca*, respectively. The addition of *Humirianthera ampla* ethanolic crude extract in increasing concentrations (100–300 μg/mL, $n=4$) to the bathing media reduced the rate of CK release (Fig. 1A–C). When we added lupeol, it was able to partially inhibit *Bothrops jararacussu in vitro* myotoxic activity. The rate of CK release from isolated muscles ranged from $0.63 \pm 0.17\ U\,g^{-1}\,h^{-1}$

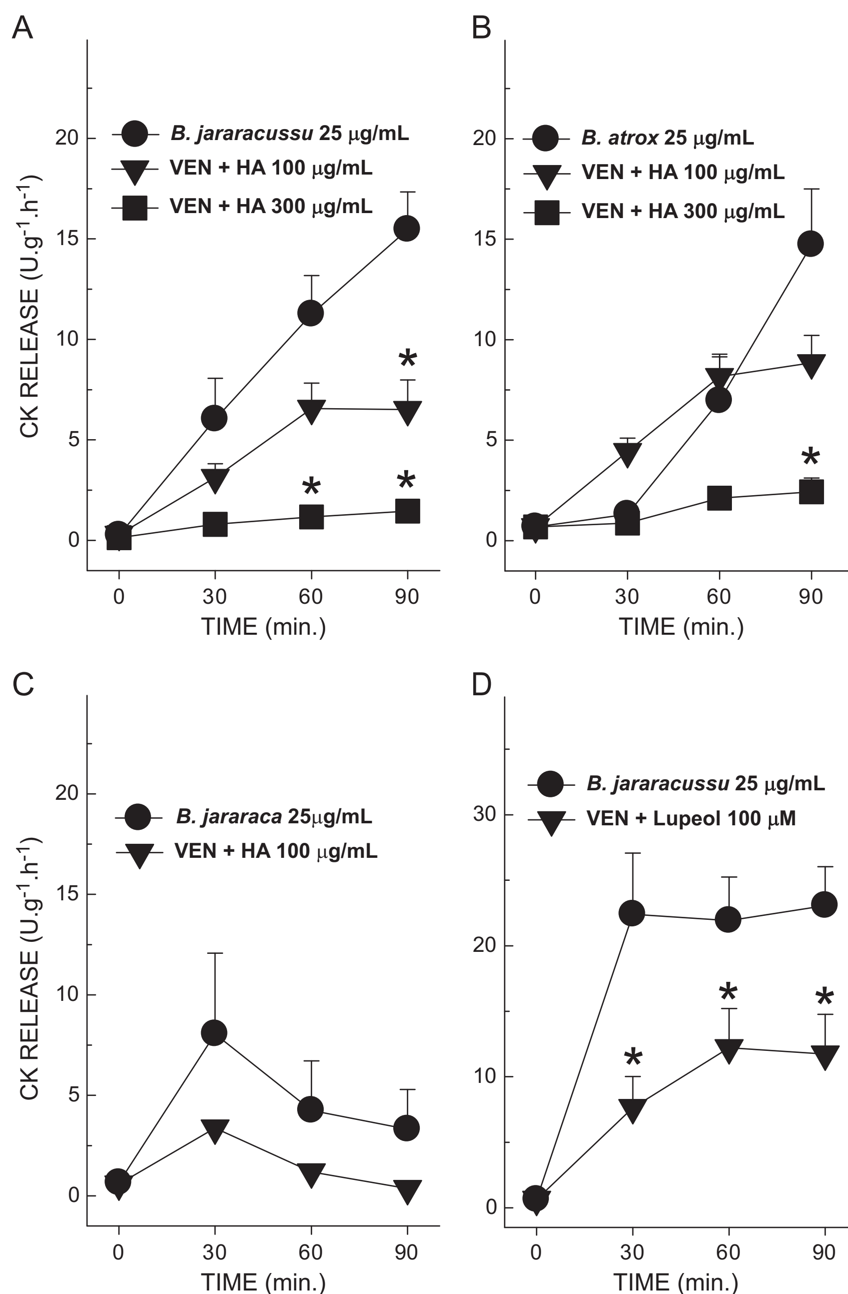

Fig. 1. Effect of *Humirianthera ampla* extract and lupeol on *Bothrops jararacussu, Bothrops atrox* and *Bothrops jararaca* venoms' myotoxic activities *in vitro*.

(basal release) up to 23.07 ± 2.97 U g^{-1} h^{-1} when exposed to *Bothrops jararacussu* venom alone (25 μg/mL) and up to only 11.73 ± 3.05 U g^{-1} h^{-1} when exposed to venom plus lupeol (100 μM) for 90 min (Fig. 1D). The reason for the variability seen in the results with *Bothrops jararacussu* venom alone is that the composition of different lots of venom may exhibit variations associated with the geographical origin, habitat, seasonal variation, diet, age and gender (Queiroz et al., 2008). That was why each treatment was performed using the same venom lot.

Mice injected intramuscularly with the venom of *Bothrops atrox* (1 mg/kg) presented, 2 h after venom injection, an increased activity of CK in plasma, which ranged from 163.45 ± 19.63 U/L (n=5) in the group receiving the PSS solution, up to 1597.00 ± 154.97 U/L (n=5) in the group receiving the venom. Oral pre- and post-treatment with *Humirianthera ampla* ethanolic crude extract (1000 mg/kg) did not significantly inhibit the myotoxic activity of the venom 2 h after venom injection. On the other hand, in the protocol of pre-incubation, the extract of *Humirianthera ampla* (300 mg/kg) partially inhibited the *in vivo* myotoxic activity of the venom of *Bothrops atrox* (790.46 ± 93.71) (Fig. 2A). In another experiment, mice also received i.m. injection of *Bothrops atrox* venom (1 mg/kg), and we observed, two hours after the injections, an increase in the plasma CK activity that reached up to 2443.91 ± 268.80 U/L, compared to 137.25 ± 32.64 U/L from the group receiving only PSS injection. When we preincubated the venom with lupeol (10 mg/kg) for 15 min before i.m. injection, the plasma CK activity was in the range of 1368.61 ± 250.44 U/L (Fig. 2B).

3.2. Hemorrhagic activity

The intradermic injection of *Bothrops atrox* venom (1 mg/kg) induced a severe hemorrhagic damage surrounding the region injected, which was expressed in arbitrary units of absorbance in

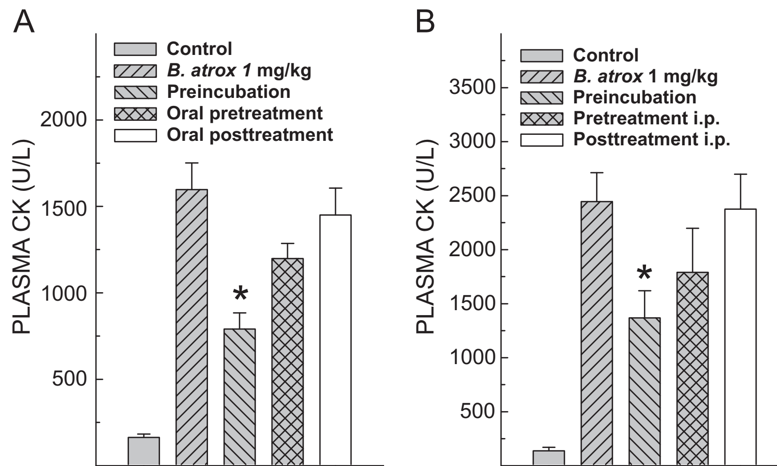

Fig. 2. Effect of *Humirianthera ampla* extract and lupeol on *Bothrops atrox* venom's myotoxicity *in vivo*.

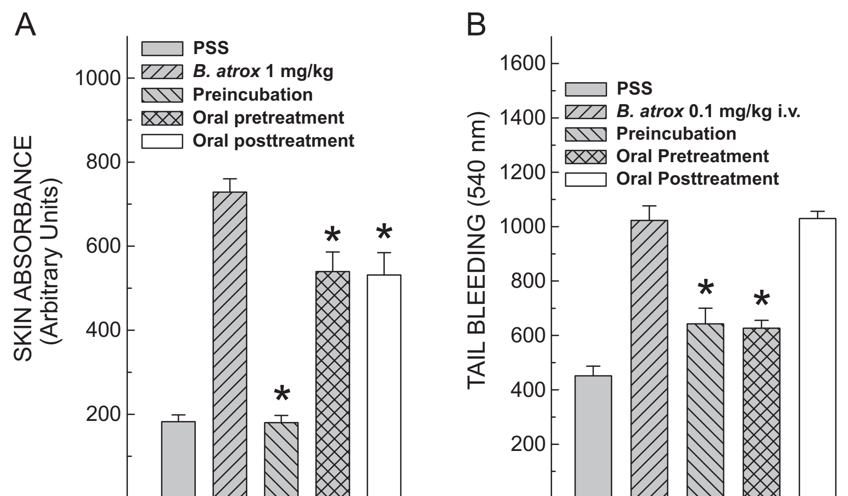

Fig. 3. Effect of *Humirianthera ampla* extract on *Bothrops atrox* venom's hemorrhagic and bleeding activities.

the skin of animals (728.50 ± 31.54 AU), compared to PSS (182.25 ± 16.14 AU). The venom activity was inhibited (180.00 ± 17.16 AU) when pre-incubated with 300 mg/kg of *Humirianthera ampla* ethanolic crude extract, while the oral pre- and post-treatment (1000 mg/kg) reduced the venom activity (539.17 ± 47.08 and 531.25 ± 53.07 AU, respectively) (Fig. 3A). *Bothrops atrox* venom (0.1 mg/kg), when injected i.v. into anesthetized mice that subsequently had their tails cut, induced an important increase in the tail bleeding. Preincubation and oral pretreatment with *Humirianthera ampla* extract reduced the bleeding activity of *Bothrops atrox* venom, while oral posttreatment did not (Fig. 3B).

3.3. Blood clotting activity

The venoms of *Bothrops atrox*, *Bothrops jararaca* and *Bothrops jararacussu* (1 μg/mL) induced an intense decrease in the clotting time of 50 μL of blood collected from the conjunctival sac of anesthetized mice, from control values of 146.50 ± 6.05 s down to 10.30 ± 0.61 s; 17 28 ± 1.10 s and 14.62 ± 1.34 s, respectively (Fig. 4A). The ethanolic crude extract of *Humirianthera ampla* inhibited in a concentration-dependent manner the procoagulant activity induced by the tested venoms, *i.e.*, in the absence of HA extract (zero) all venoms present 100% of procoagulant activity, but as HA extract concentration increases, all three venoms lose their ability to accelerate blood clot (Fig. 4B). The extract alone did not change the clotting time compared to control time (data not shown).

3.4. Edematogenic activity

Following i.m. injection of *Bothrops atrox* venom (1 mg/kg) into the hind limb, the animals presented a significant edema, compared to animals injected only with PSS (41.56 ± 1.43, against 25.50 ± 0.88 mm^2, respectively, 30 min after venom injection). The limb edema induced by the venom was partially inhibited in the protocols of oral pre- and posttreatment and preincubation with the ethanolic crude extract of *Humirianthera ampla* (Fig. 5A and B).

3.5. Phospholipase activity

The turbidimetric assay for phospholipase A_2 activity showed that the venoms of *Bothrops atrox*, *Bothrops jararacussu* and *Bothrops jararaca* reduced the turbidity of egg yolk solutions (0.360 ± 0.004; 0.312 ± 0.006 and 0.404 ± 0.008 absorbance units at 925 nm, for the concentration of 10 μg/mL), respectively, compared to the negative control (0.641 ± 0.028 absorbance units) (Fig. 6A). The ethanolic crude extract of *Humirianthera ampla* (1–500 μg/mL) decreased the enzymatic activity of 10 μg/mL of the three venoms (inhibitions of up to 48%, 42% and 92% for the

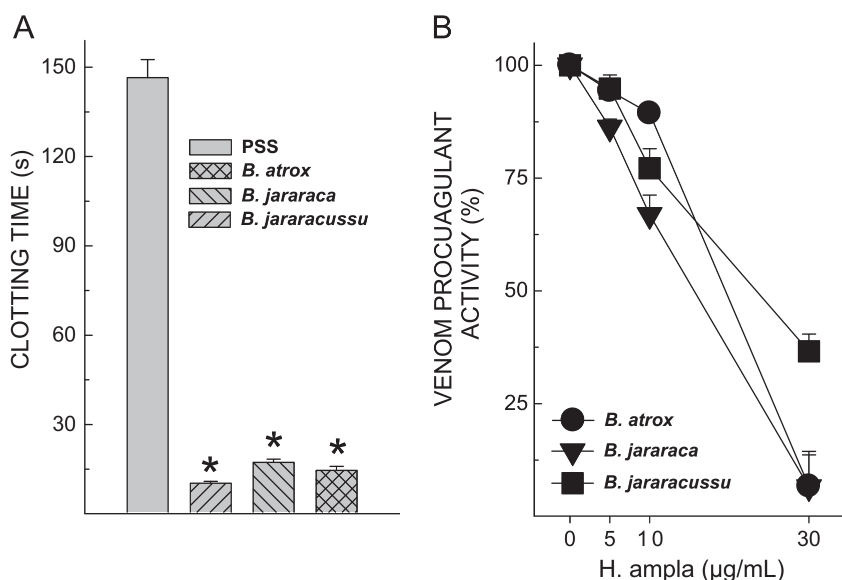

Fig. 4. Effect of *Humirianthera ampla* extract on *Bothrops jararacussu, Bothrops atrox* and *Bothrops jararaca* venoms' clotting times and procoagulant activities.

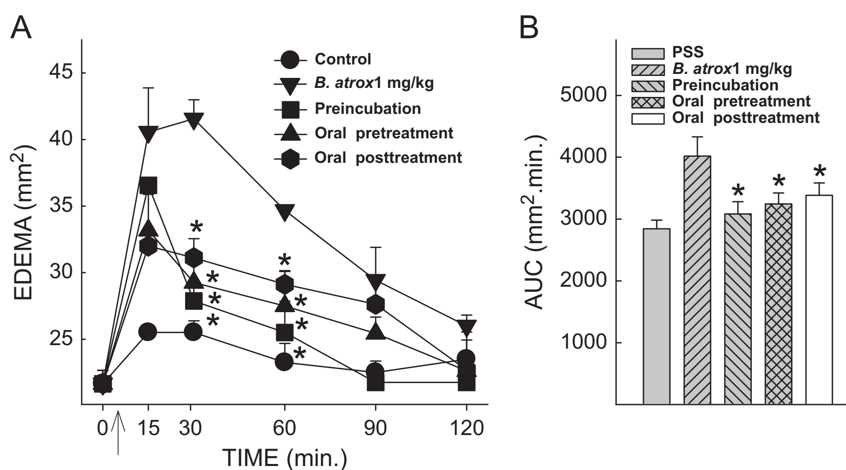

Fig. 5. Effect of *Humirianthera ampla* extract on *Bothrops atrox* venom's edematogenic activity.

concentration of 500 μg/mL, respectively (Fig. 6B). Sitosterol and lupeol presented a concentration-dependent inhibition over the phospholipase activities of 10 μg/mL of *Bothrops jararacussu* and *Bothrops atrox* venoms, respectively (Fig. 6C), while acrenol, humirianthol and annonalide did not present any activity (data not shown).

3.6. Proteolytic activity

The venoms of *Bothrops atrox, Bothrops jararaca* and *Bothrops jararacussu* induced the hydrolysis of azocasein in a concentration-dependent manner, leading to an increase in absorbance units at 420 nm (0.074 ± 0.004, 0.153 ± 0.009, $0.228 + 0.015$, for the concentration of 10 μg/mL), when compared with the respective negative controls (0.008 ± 0.001, 0.014 ± 0.003, 0.012 ± 0.001) (Fig. 7A). The proteolytic activities of the venoms were inhibited in a concentration-dependent manner by the ethanolic crude extract of *Humirianthera ampla* (1–300 μg/mL) (Fig. 7B). Sitosterol and lupeol presented a concentration-dependent inhibition over the proteolytic activities of 10 μg/mL of *Bothrops jararacussu* and *Bothrops atrox* venoms, respectively (Fig. 7C), while acrenol, humirianthol and annonalide were devoid of any activity (data not shown).

4. Discussion

Our results provide for the first time an experimental and scientific support for the use of *Humirianthera ampla* in cases of accidents with *Bothrops atrox, Bothrops jararacussu* and *Bothrops jararaca,* which had some of their venom activities inhibited in experimental conditions by the crude extract and some of its isolated compounds, *i.e*., sitosterol and lupeol.

As previously shown, i.m. injection of the *Bothrops* venoms induces extensive myonecrosis in mice (Homsi-Brandeburgo et al., 1988; Lomonte et al., 2003; Melo and Suarez-Kurtz, 1988a; 1988b; Melo et al., 1993; Queiroz et al., 1984). Our study showed the important *in vivo* myotoxicity of *Bothrops atrox* venom, which was inhibited by both *Humirianthera ampla* ethanolic crude extract and lupeol following preincubation with the venom, and partially inhibited in the protocols of oral pre- and posttreatment. This is of great importance considering the high myotoxicity of *Bothrops* venoms and the lack of protection from this activity by the available treatment, the antibothropic antivenom, leading to functional disabilities due to poor muscle regeneration (Mello et al., 2000). Antimyotoxic properties of *Humirianthera ampla* crude extract and lupeol were also shown

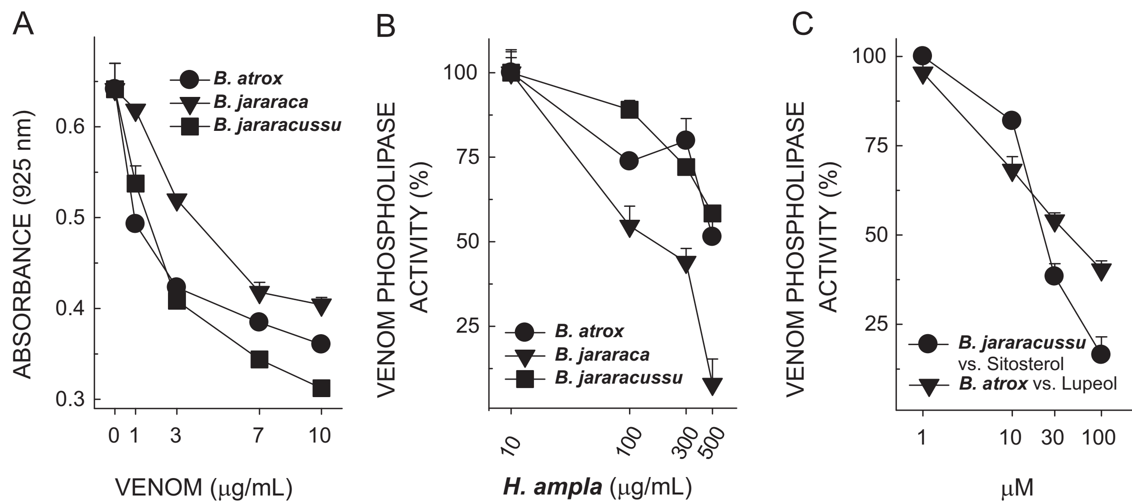

Fig. 6. Effect of *Humirianthera ampla* extract and constituents on *Bothrops jararacussu, Bothrops atrox* and *Bothrops jararaca* venoms' phospholipase A_2 activities.

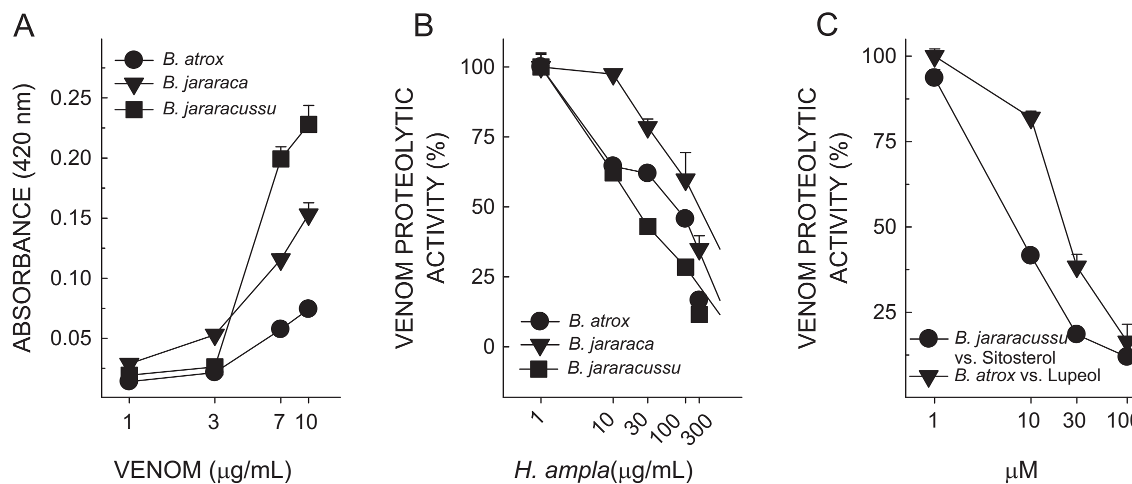

Fig. 7. Effect of *Humirianthera ampla* extract and constituents on *Bothrops jararacussu, Bothrops atrox* and *Bothrops jararaca* venoms' proteolytic activities.

in vitro, where the venoms and treatments are put together before bathing the isolated muscles. These data give support for the treatment carried out by rural communities in the Amazon region, which make use of the plant extract in oral prophylactic and therapeutic preparations (Graebner et al.2002).

Local and systemic skeletal muscle degeneration is a common consequence of envenomations due to snakebites. Phospholipases A_2 (PLA_2) are important myotoxic components in *Bothrops* venoms, inducing a similar pattern of degenerative events in muscle cells. Myotoxic PLA_2s bind to acceptors in the plasma membrane, which might be lipids or proteins and which may differ in their affinity for the PLA_2s. Upon binding, myotoxic PLA_2s disrupt the integrity of the plasma membrane by catalytically dependent or independent mechanisms, provoking a pronounced Ca^{2+} influx which, in turn, initiates a complex series of degenerative events associated with hypercontraction, activation of calpains and cytosolic Ca^{2+}-dependent PLA_2s, and mitochondrial Ca^{2+} overload (Gutiérrez and Ownby, 2003). Our experiments showed concentration-dependent inhibition of *Bothrops atrox*, *Bothrops jararaca* and *Bothrops jararacussu* phospholipase A_2 activities by *Humirianthera ampla* extract, as well as inhibition of *Bothrops jararacussu* and *Bothrops atrox* by sitosterol and lupeol, respectively. These data strongly suggest that the observed decrease in the myotoxicity of *Bothrops* venoms is related to inhibition of venom PLA_2 activity.

Another remarkable result was the effect of *Humirianthera ampla* extract against the procoagulant and hemorrhagic activities of the *Bothrops* venoms. Bleeding is one of the most relevant local and systemic signs of envenomation by snakes of this genus. Several types of bleeding distant from the site of the bite are observed, such as gum bleeding, epistaxis, hemoptysis, hematuria, uterine, intrathoracic or intrabdominal bleeding (Kamiguti and Sano-Martins, 1995; Otero et al., 2002). Severe anemia and shock can occur as complications of these hemorrhages causing deaths and sequelae (Otero et al., 2000). For this reason, one of the mainstays of treatment of these envenomations should be inhibition of bleeding. The extract showed not only an extensive inhibitory response against the procoagulant activities of the venoms, but also reduced the skin hemorrhagic activity of *Bothrops atrox* venom. The hemorrhagic activity of *Bothrops* venoms is due to angiorrexis attributed to metalloproteases, proteins that target the vascular wall. Among the substrates of hemorrhagic toxins are integrins, present in the vascular wall and the basal lamina (Baramova et al., 1990; Bjarnason and Fox, 1994; Fujimura et al., 1995; Kamiguti et al., 1986; 1991; Paine et al., 1992). Since some plant substances have the ability to bind and neutralize toxins from snake venoms and inhibit their catalytic activity in a concentration-dependent manner, an anti-proteolytic mechanism has been proposed to explain the effect of antihemorrhagic plant extracts (Melo and Ownby, 1999; Melo et al., 1994). Studies have shown that *Bothrops* venoms contain

procoagulant toxins that act at various levels of the coagulation cascade, such as batroxobin from *Bothrops atrox*, BjussuSP-I and BJ-48 from *Bothrops jararacussu*, and jararassin-I from *Bothrops jararaca* (Hofmann and Bon, 1987; Nahas et al., 1979; Sant'Ana et al., 2008; Silva-Junior et al., 2007; Stocker and Barlow, 1976; Vieira et al., 2004). The snake venom thrombin-like enzymes comprise a number of serine proteases functionally and structurally related to thrombin. Thus, these enzymes present "thrombin-like" effect, accelerating the production of fibrin (procoagulant effect), causing depletion of the content of fibrinogen, leading to hemorrhagic process (Braud et al., 2000; Sajevic et al., 2011). Inhibition of hemorrhagic activities of *Bothrops* venoms by *Humirianthera ampla* extract can be directly related to the concentration-dependent ability of components of the extract to inhibit the proteolytic activities of these venoms.

Edema is also an important effect of *Bothrops* venoms, which along with tissue necrosis can be severe enough to cause functional loss or even compartment syndrome (Anz et al., 2010). *Humirianthera ampla* extract was able to inhibit the activity of the venom in all protocols, especially when pre-incubated with the venom, but also significantly in the prophylactic and therapeutic protocols. Like paw edema, hind limb edema has been described as an inflammatory process induced by components that involve local mediators derived from arachidonic acid, autacoids such as histamine and serotonin, and is not well blocked by antivenom (Rojas et al., 2005; Soares et al., 1998), constituting a major complication related to accidents caused by *Bothrops* in large animals.

It is known that PLA_2 inhibitors, such as *p*-bromofenacil bromide (*p*-BPB), significantly reduced the inflammatory edema induced by some *Bothrops* venom (Andrião-Escarso et al., 2000; Landucci et al., 2000; Zuliani et al., 2005). In fact, the chemical constituents present in the roots of the *Humirianthera ampla*: diterpenoids, triterpenoids and sterols have been reported as potent anti-inflammatory agents (Fernandez et al., 2001; Hernandez-Perez et al., 1995; Liu and Lin, 2006; Yamashita et al., 2002; Spessoto et al., 2003). In our experiments, *Humirianthera ampla* extract, sitosterol and lupeol inhibited the phospholipase and proteolytic activities of the venoms of *Bothrops atrox*, *Bothrops jararacussu* and *Bothrops jararaca* in a concentration-dependent manner, suggesting that some of the effects observed *in vivo* may depend on these enzymatic activities.

It is important to state that there are no descriptions of adverse effects or intoxication in humans following the use of HA ethanolic crude extracts in folk medicine, which was confirmed by the lack of physiological complications in the animals that received the extract both orally and intraperitoneal (data not shown).

5. Conclusion

Our study is promising because, as stated by Graebner and colleagues (2002), *Humirianthera ampla* have active components, which according to our results inhibit some properties of the venoms of *Bothrops atrox, Bothrops jararacussu* and *Bothrops jararaca*. Therefore, the set of our results give scientific support for the use of *Humirianthera ampla* ethanolic crude extract in folk medicine, suggesting that it can be useful as an adjuvant in the treatment of snakebite along with the antivenom.

Disclosure statement

The authors declare that there is no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

Acknowledgements

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Programa de Apoio aos Núcleos de Excelência (PRONEX). We are thankfull to Dr. Ademir Farias Morel from the Federal University of Santa Maria, Rio Grande do Sul, Brazil for providing the compounds acrenol, annonalide and humirianthol.

References

Alam, M.I., Gomes, A., 2003. Snake venom neutralization by Indian medicinal plants (*Vitex negundo* and *Emblica officinalis*) root extracts. Journal of Ethnopharmacology 86, 75–80.

Andrião-Escarso, S.H., Soares, A.M., Rodrigues, V.M., Ângulo, Y., Díaz, C., Lomonte, B., Gutiérrez, J.M., Giglio, J.R., 2000. Myotoxic phospholipases A_2 in *Bothrops* snake venoms: effect of chemical modifications on the enzymatic and pharmacological properties of bothropstoxins from *Bothrops jararacussu*. Biochimie 82, 755–763.

Anz, A.W., Schweppe, M., Halvorson, J., Bushnell, B., Sternberg, M., Andrew Koman, L., 2010. Management of venomous snakebite injury to the extremities. The Journal of the American Academy Orthopaedic Surgeons 18, 749–759.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B., Fox, J.W., 1990. Identification of cleavage sites by a hemorrhagic metalloproteinase in type IV collagen. Matrix 10, 91–97.

Bjarnason, J.B., Fox, J.W., 1994. Hemorrhagic metalloproteinases from snake venoms. Pharmacology and Therapeutics 62, 325–372.

Bochner, R., Struchiner, C.J., 2003. Snake bite epidemiology in the last 100 years in Brazil: a review. Cadernos de Saúde Pública 19, 7–16.

Borges, M.H., Alves, D.L.F., Raslan, D.S., Piló-Veloso, D., Rodrigues, V.M., Homsi-Brandeburgo, M.I., de Lima, M.E., 2005. Neutralizing properties of *Musa paradisiaca* L. (Musaceae) juice on phospholipase A_2, myotoxic, hemorrhagic and lethal activities of crotalidae venoms. Journal of Ethnopharmacology 98, 21–29.

Braud, S., Bon, C., Wisner, A., 2000. Snake venom proteins acting on hemostasis. Biochimie 82, 851–859.

Broze Jr., G.J., Yin, Z.F., Lasky, N., 2001. A tail vein bleeding time model and delayed bleeding in hemophiliac mice. Thrombosis and Haemostasis 85, 747–748.

Cavalcante, W.L.G., Campos, T.O., Pai-Silva, M.D., Pereira, P.S., Oliveira, C.Z., Soares, A.M., Gallacci, M., 2007. Neutralization of snake venom phospholipase A_2 toxins by aqueous extract of *Casearia sylvestris* (Flacourtiaceae) in mouse neuromuscular preparation. Journal of Ethnopharmacology 112, 490–497.

da Silva, C.J., Jorge, M.T., Ribeiro, L.A., 2003. Epidemiology of snakebite in a central region of Brazil. Toxicon 41, 251–255.

Dessoy, E.C., Scher, A., 2002. Diterpenos isolados de *Humirianthera ampla* Miers. Revista Brasileira de Farmacognosia 12, 80–81.

Fernandez, M.A., de las Heras, B., Garcia, M.D., Saenz, M.T., Villar, A., 2001. New insights into the mechanism of action of the anti-inflammatory triterpene lupeol. Journal of Pharmacy and Pharmacology 53, 1533–1539.

Fujimura, Y., Ikeda, Y., Miura, S., Suzuki, M., Titani, K., Kawasaki, T., 1995. Isolation and characterizatin of jararaca GPIb-BP, a snake venom antagonist specific to platelet glycoprotein Ib. Thrombosis and Haemostasis 74, 743–750.

Garcia, E.S., Guimaraes, J.A., Prado, J.L., 1978. Purification and characterization of a sulfhydryl-dependent protease from *Rhodnius prolixus* midgut. Archives of Biochemistry and Biophysics 188, 315–322.

Graebner, I.B., Mostardeiro, M.A., Ethur, E.M., Burrow, R.A., Dessoy, E.C., Morel, A.F., 2000. Diterpenoids from *Humirianthera ampla*. Phytochemistry 53, 955–959.

Graebner, I.B., Morel, A.F., Burrow, R.A., Mostardeiro, M.A., Ethur, E.M., Dessoy, E.C., Scher, A., 2002. Diterpenos isolados de *Humirianthera ampla* Miers. Revista Brasileira de Farmacognosia 12, 80–81.

Gutiérrez, J.M., Arroyo, O., Bolaños, R., 1980. Myonecrosis, hemorrhage and edema induced by *Bothrops asper* venom in white mice (author's transl). Toxicon 18, 603–610.

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.

Hernandez-Perez, M., Rabanal, R.M., de la Torre, M.C., Rodriguez, B., 1995. Analgesic, anti-inflammatory, antipyretic and haematological effects of aethiopinone, an *o*-naphthoquinone diterpenoid from *Salvia aethiopis* roots and two hemisynthetic derivatives. Planta Medica 61, 505–509.

Hofmann, H., Bon, C., 1987. Blood coagulation induced by the venom of *Bothrops atrox*. Identification, purification, and properties of a prothrombin activator. Biochemistry 26, 772–780.

Homma, M., Tu, A.T., 1971. Morphology of local tissue damage in experimental snake envenomation. British Journal of Experimental Pathology 52, 538–542.

Homsi-Brandeburgo, M.I., Queiroz, L.S., Santo-Neto, H., Rodrigues-Simioni, L., Giglio, J.R., 1988. Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. Toxicon 26, 615–627.

Kamiguti, A.S., Sano-Martins, I.S., 1995. South American snake venoms affecting homeostasis. Journal of Toxicology 14, 359–374.

Kamiguti, A.S., Cardoso, J.L.C., Theakston, I.S., Sano-Martins, I.S., Hutton, R.A., Rugman, F.P., Warrel, D.A., Hay, C.R.M., 1991. Coagulation and haemorrage victims of *Bothrops jararaca* envenoming in Brazil. Toxicon 29, 961–972.

Kamiguti, A.S., Matsunaga, S., Spir, M., Sano-Martins, I.S., Nahas, L., 1986. Alterations of the blood coagulation system after accidental human inoculation by *Bothrops jararaca* venom. Brazilian Journal of Medical Biological Research 19, 199–204.

Landucci, E.C., de Castro, R.C., Toyama, M., Giglio, J.R., Marangoni, S., De Nucci, G., Antunes, E., 2000. Inflammatory oedema induced by the lys-49 phospholipase A_2 homologue piratoxin-i in the rat and rabbit. Effect of polyanions and *p*-bromophenacyl bromide. Biochemical Pharmacology 59, 1289–1294.

Liu, C.F., Lin, N., 2006. Progress in research on mechanisms of anti-rheumatoid arthritis of triptolide. Zhongguo Zhong Yao Za Zhi 31, 1575–1579.

Lomonte, B., Angulo, Y., Calderon, 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.

Luiz, A.P., Moura, J.D., Meotti, F.C., Guginski, G., Guimarães, C.L., Azevedo, M.S., Rodrigues, A.L., Santos, A.R., 2007. Antinociceptive action of ethanolic extract obtained from roots of *Humirianthera ampla* Miers. Journal of Ethnopharmacology 114, 355–363.

Marinetti, G.V., 1965. The action of phospholipase A on lipoproteins. Biochimica et Biophysica Acta 3, 554–565.

Mello, L.F., Barcelos, M.G., Meohas, W., Pinto, L.W., Melo, P.A., Nogueira Neto, N.C., Smith, J., 2000. Chronic ulceration of the leg following extensive scarring due to a snake bite complicated by squamous cell carcinoma. Skeletal Radiology 29, 298–301.

Melo, P.A., Suarez-Kurtz, G., 1988a. Release of creatine kinase from skeletal muscle by *Bothrops* venoms: heparin potentiation of inhibition by antivenim. Brazilian of Journal Medical and Biological Research 21, 545–548.

Melo, P.A., Suarez-Kurtz, G., 1988b. Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: antagonism by heparin and by the serum of South American marsupials. Toxicon 26, 87–95.

Melo, P.A., Homsi-Brandeburgo, M.I., Giglio, J.R., Suarez-Kurtz, G., 1993. Antagonism of the myotoxic effects of *Bothrops jararacussu* venom and Bothropstoxin by polyanions. Toxicon 31, 285–291.

Melo, P.A., Nascimento, M.C., Mors, W.B., Suarez-Kurtz, G., 1994. Inhibition of the myotoxic and hemorrhagic activities of crotalid venoms by *Eclipta prostrata* (Asteraceae) extracts and constituents. Toxicon 32, 595–603.

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

Mors, W.B., do Nascimento, M.C., Parente, J.P., da Silva, M.H., Melo, P.A., Suarez-Kurtz, G., 1989. Neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of the plant *Eclipta prostrata* (Asteraceae). Toxicon 27, 1003–1009.

Nahas, L., Kamiguti, A.S., Barros, M.A., 1979. Thrombin-like and factor X-activator components of *Bothrops* snake venoms. Thrombosis and Haemostasis 41, 314–328.

Nishioka, S., de, A., Silveira, P.V., 1992. A clinical and epidemiologic study of 292 cases of lance-headed viper bite in a Brazilian teaching hospital. The American Journal of Tropical Medicine and Hygiene 47, 805–810.

Oliveira, C.Z., Maiorano, V.A., Marcussi, S., Sant'ana, C.D., Januário, A.H., Lourenço, M.V., Sampaio, S.V., França, S.C., Pereira, P.S., Soares, A.M., 2005. Anticoagulant and antifibrinogenolytic properties of the aqueous extract from *Bauhinia forficata* against snake venoms. Journal of Ethnopharmacology 98, 213–216.

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., Núnez, V., Díaz, A., 2002. Complications of *Bothrops*, *Porthidium*, and *Bothriechis* snakebites in Colombia. A clinical and epidemiological study of 39 cases attended in a university hospital. Toxicon 40, 1107–1114.

Otero, R., Núñez, V., Barona, J., Fonnegra, R., Jiménez, S.L., Osorio, R.G., Saldarriaga, M., Díaz, A., 2000. Snakebites and ethnobotany in the northwest region of Colombia. Part III: Neutralization of the haemorrhagic effect of Bothrops atrox venom. Journal of Ethnopharmacology 73, 233–241.

Paine, M.J., Desmond, H.P., Theakston, R.D., Crampton, J.M., 1992. Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from *Bothrops jararaca* venom. Insights into the disintegrin gene family. The Journal of Biological Chemistry 267, 22869–22876.

Queiroz, L.S., Santo-Neto, H., Rodrigues-Simioni, L., Prado-Franceschi, J., 1984. Muscle necrosis and regeneration after envenomation by *Bothrops jararacussu* snake venom. Toxicon 22, 339–346.

Queiroz, G.P., Pessoa, L.A., Portaro, F.C., Furtado, M.F., Tambourgi, D.V., 2008. Interspecific variation in venom composition and toxicity of Brazilian snakes from *Bothrops* genus. Toxicon 52, 842–851.

Raphael, L.S., 1983. Medical Laboratory Technology, fourth ed. Saunders International Edition 742.

Rojas, E., Quesada, L., Arce, V., Lomonte, B., Rojas, G., Gutiérrez, J.M., 2005. Neutralization of four Peruvian *Bothrops* sp. snake venoms by polyvalent antivenoms produced in Peru and Costa Rica: preclinical assessment. Acta Tropica 93, 85–95.

Rosenfeld, G., 1971. Symptomatology, pathology and treatment of snake bites. South America. Venomous Animals and their Venoms. Academic Press, New York 345–384.

Sajevic, T., Leonardi, A., Križaj, I., 2011. Haemostatically active proteins in snake venoms. Toxicon 57, 627–645.

Sant'Ana, C.D., Ticli, F.K., Oliveira, L.L., Giglio, J.R., Rechia, C.G., Fuly, A.L., Selistre de Araújo, H.S., Franco, J.J., Stabeli, R.G., Soares, A.M., Sampaio, S.V., 2008. BjussuSP-I: a new thrombin-like enzyme isolated from *Bothrops jararacussu* snake venom. Comparative Biochemistry and Physiology—Part A: Molecular & Integrative Physiology 151, 443–454.

Silva-Junior, F.P., Guedes, H.L., Garvey, L.C., Aguiar, A.S., Bourguignon, S.C., Di Cera, E., Giovanni-De-Simone, S., 2007. BJ-48, a novel thrombinlike enzyme from the *Bothrops jararacussu* venom with high selectivity for Arg over Lys in P1: role of N-glycosylation in thermostability and active site accessibility. Toxicon 50, 18–31.

Spessoto, M.A., Ferreira, D.S., Crotti, A.E., Silva, M.L., Cunha, W.R., 2003. Evaluation of the analgesic activity of extracts of *Miconia rubiginosa* (Melastomataceae). Phytomedicine 10, 606–609.

Soares, D.M., Aguiar, A.S., Melgarejo, A.R., De Carvalho, L.C., 1998. Pharmacological aspects of mouse hind-paw oedema induced by *Lachesis muta rhombeata* venom. Toxicon 36, 771–780.

Stocker, K., Barlow, G.H., 1976. The coagulant enzyme from *Bothrops atrox* venom (batroxobin). Methods in Enzymology 45, 214–223.

Tan, N.H., Fung, S.Y., Sim, S.M., Marinello, E., Guerranti, R., Aguiyi, J.C., 2009. The protective effect of Mucuna pruriens seeds against snake venom poisoning. Journal of Ethnopharmacology 123, 356–358.

Vieira, D.F., Watanabe, L., Sant'ana, C.D., Marcussi, S., Sampaio, S.V., Soares, A.M., Arni, R.K., 2004. Purification and characterization of jararassin-I, a thrombin-like enzyme from *Bothrops jararaca* snake venom. Acta Biochimica et Biophysica Sinica 36, 798–802.

Yamashita, K., Lu, H., Lu, J., Chen, G., Yokoyama, T., Sagara, Y., Manabe, M., Kodama, H., 2002. Effect of three triterpenoids, lupeol, betulin, and betulinic acid on the stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils. Clinica Chimica Acta 325, 91–96.

Zuliani, J.P., Gutiérrez, J.M., Casais e Silva, L.L., Coccuzzo Sampaio, S., Lomonte, B., Pereira Teixeira Cde, F., 2005. Activation of cellular functions in macrophages by venom secretory Asp-49 and Lys-49 phospholipases A(2). Toxicon 46, 523–532.