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Full Length Research Paper

The effect of the aqueous extract of *Myrcia guianensis* (AubL) DC and its fractions against the hemorrhagic activity of *Bothrops jararaca* venom

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Brazilian folk medicine utilizes the shrub *Myrcia guianensis* against various snake venom effects. This work aimed to assess the therapeutic potential of aqueous, ethyl acetate and hexane leave extracts of *M. guianensis* and its aqueous residue against the hemorrhagic activity of *Bothrops jararaca* venom. *In vivo* assays were carried out by dorsal intradermic injections of the crude venom in Swiss mice. The *in vitro* effect was determined on agarose gel, using egg yolk as phospholipid source and erythrocytes as substrate and by mixing the aqueous residue with crude *B. jararaca* venom or mice plasma. After incubation, the pellets and the supernatants were analyzed by electrophoresis. The aqueous extract and aqueous residue completely inhibited the *in vivo* hemorrhage (1:1 ratio, w:w); ethyl acetate extract inhibited 90.7% at 1:3 and hexane extract showed no effect at 1:10 (w:w). The aqueous residue completely inhibited *in vivo* jararhagin induced hemorrhage at 1:10 (w:w). However, it showed no action when injected 10 min after the venom. It also completely inhibited the *in vitro* venom phospholipase A2 activity at 1:2 (w:w) and jararhagin induced fibrinolytic at 1:100 (w:w). It is suggested that the antihemorrhagic activity of the aqueous residue is mainly due to protein precipitation, as shown by electrophoresis.

Key words: *Bothrops jararaca*, *Myrcia guianensis*, jararhagin, phospholipase A_2, antiophidian plants.

INTRODUCTION

The World Health Organization lists snakebite envenoming as a neglected tropical disease which brings about mortality or morbidity to many victims (WHO, 2009).

The Brazilian Ministério da Saúde (Brasil, 2011) reported twenty nine thousand snakebite accidents in the year 2010 from four different genera: *Bothrops sensu latu* (jararaca, jararacuçu, urutu, caiçaca, combóia), *Crotalus* (rattlesnakes), *Lachesis* (surucucu-pico-de-jaca) and *Micrurus* (true coral). Plant extracts have been used worldwide as alternatives to snakebite therapy (Pereira et al., 1994). Vilar (2004) reported the antihemorrhagic action of the aqueous extract of *Apodanthera villosa*, *Apodanthera glaziovii*, *Jatropha mollisima* and *Jatropha elliptica* against *Bothrops jararaca* venom while Silva et al. (2005) showed that *Pentaclethra macroloba* aqueous extract inhibits the hemorrhagic effects of venoms from *Bothrops* species and *Lachesis muta*.

The Myrtaceae family are well distributed plants over the tropical region (Ribeiro et al., 1999), growing mainly on acidic and sandy soils. *Myrcia*, one of its major genus, has more than 300 species (Cronquist, 1981); in Amazonia it is conspicuous in open areas such as savannas and bushes and less noticeable in dense and/or swampy forests.

The pharmacological activities of some *Myrcia* species have been studied, including the hypoglycemic properties of infusions and decoctions from *Myrcia multiflora* (Brito and Lanetti, 1996). Myrciatricin I and Myrciaphenone B, obtained from *M. multiflora* leaves, are reported to be hypoglycemic by inhibiting the aldose reductase and alpha glycosidase enzymes (Yoshikawa et al., 1998). Leave extracts from *Myrcia acris* have antibiotic activities (Nadal, 1959) while those from *Myrcia fallax* are active against human nasophariynx (KB) cancer cells (Hecht and Wofor, 1984).

Several Brazilian plants have been utilized in folk medicine as active agents against various snake venoms effects. The inhabitants of the Amazon region use, among others, the macerated leaves of the Pedra-ume-caá shrub (*Myrcia guianensis*) to neutralize these effects. Since there is no study on this shrub, this work aimed to study the therapeutic potential of *M. guianensis* leave extracts against the hemorrhagic activity of *B. jararaca* venom.

It is part of a research project aiming to evaluate the pharmacological activities of Amazonian plants, particularly those of the Myrtaceae family and their antiophidic activities. It focuses on the antihemorrhagic effects of leave extracts from *M. guianensis* (Aubl.) DC. against *B. jararaca* crude venom and its isolated metalloproteases.

MATERIALS AND METHODS

Animals

Swiss mice of both sexes (34 to 41 g body weight) from the animal house of the Universidade Federal do Oeste do Pará, Santarém, Pará (UFOPA) were kept under standard conditions (22 ± 1°C, 12 h dark/light cycle, water and food *ad libitum*). The research followed the Brazilian Federal Law 11,794 and was approved by the Ethics Committee of the Universidade Estadual do Pará (UEPA), protocol number 11/11.

Plants and venom

Lyophilized jararhagin, isolated from *B. jararaca*, and the lyophilized *B. jararaca* crude venom were obtained from the Imunology Laboratory, Instituto Butantan, São Paulo State, Brazil. The crude venom was dissolved to 1 mg/ml in 0.9% NaCl solution (saline) and its protein content measured accordingly to Bradford (1976).

Jararhagin, its major hemorrhagic toxin, was isolated by the HLIB through liquid chromatography using hydrophobic interaction and anion exchange liquid chromatography (Moura-da-Silva et al., 2003). After purification, it was lyophilized, stored at -80°C and diluted to 1 mg/ml in 50 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.2 (PBS), just before use. *M. guianensis* leaves were collected in October 2009 during its sterile period at 02° 32´ 08.9" S and 54° 54´ 23.9" W (São Pedro community, Santarém county, Pará State, Brazil) (GPS – GARMIN Map 60 csx). Exsiccates were deposited in the herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA, Manaus – AM, Brazil) under code number 18193.

Myrcia extracts

The leaves of *M. guianensis* were washed and dried inside a Licit LC-E80 drying-oven at 40°C under air flow. Then they were powdered to approximately 6 mm diameter in a domestic liquefier (Arno-MAGD).

Following, 545 g of the powder were extracted with distilled water at 35 to 40°C for two and a half hour in a water-bath (Marconi-MA127) and left to cool. After cooling, the extract was vacuum filtered, yielding 600 ml of crude aqueous solution (MgAE).

The MgAE (300 ml) was extracted twice with 300 ml hexane, yielding a hexane phase (MgHx) and an aqueous residue I. This was then extracted twice with 300 ml of ethyl acetate, resulting in an organic phase (MgAc) and in a final aqueous residue (MgAR). After drying and lyophilizing (Liotop L101), the MgHx, MgAc and MgAR yielded, respectively, 15.9, 16.9 and 1.7 g. The powders were kept at 4°C and diluted in saline just before use.

Phytochemical profile of the residues

Thin layer chromatography (TLC)

The phytochemical profile of the aqueous residue (MgAR) was obtained by TLC on aluminum plates (M&N) covered with silica gel F_{254} (0.5 × 10 cm) in 20 × 10 cm tanks. An automatic dispenser ATS4, a TLC visualizer and the software WinCats 1.4.4, all from Camag, Muttenz, Switzerland, were used. Four solvent systems of different polarities were used: (A) hexane: ethyl acetate: formic acid (42.5:7.5:2.5 v:v; non polar); (B) ethyl acetate:acetic acid:formic acid:water (25:2.75:2.75:6.75 v:v; acid); (C) ethyl acetate:isopropanol:diethyl amine (9:7:4 v:v; basic); and (D) hexane:ethyl acetate:formic acid (5:5:1 v:v; medium polarity).

Ten milligrams of MgAR were sonicated in 5 ml methanol for 1 min (model 251- Branson) and stored in 1.5 ml vials. Aliquots of 10 µl were used in each TLC plate. After chromatography, the plates

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were photographed under 366 nm ultraviolet (UV) light before and after color development. The developers were sulphuric vanillin for terpenoids (yellow-brown color) and fatty acids (blue); diphenylboryloxethylamine/polyethylene glycol for cumarins (blue-green) and flavonoids (orange-yellow); potassium hydroxide for anthraquinones (yellow-red) and cumarins (blue-green). The standard substances for cumarins, flavonoids and terpenoids were esculin, rutin and thymol (all Sigma-Aldrich, 98% pure, 1 mg/ml). Tannin detection followed Matos (1997): 2 ml of MgAR were transferred to test tubes and 3 drops of $FeCl_3$ 0.5 M added for color development (blue: hydrolytic tannins; green: condensed tannins). A $FeCl_3$ blank was also done.

High performance liquid chromatography (HPLC)

In order to better characterize the main *M. guian*ensis compounds, a sample of the MgAR was purified by HPLC. The sample was diluted in methanol and pre-treated in Strata C-18 cartridges (Phenomex, 3 g stationary phase, 1 ml volume) for solid phase extraction. The cartridges were loaded with 1 ml acetonitrile (ACN) and sonicated for 1 min. Samples were then loaded on top of the cartridge and washed with 1 ml ACN. The eluates were collected, evaporated and another 1 ml ACN added to the dry residue. Twenty microliters were then injected in the HPLC device, eluted with 1 ml/min flux, the mobile phase being a gradient of H_2O and ACN, the last one increasing from 5 to 100% in 60 min. A Shimadzu HPLC equipment was used, with a four Chanel LC-20AT pump, diode detector SPD-M20A adjusted to 200 to 400 nm, membrane degasefier DGU-20As, sample injector SIL-20A, PC Pentium V, interface CBM-20A and software LCsolution.

Colorimetric assay

The total phenolic content of the lyophilized MgAE, MgAR and MgAc extracts was measured by the Hagerman and Butler colorimetric assay (Mole and Waterman, 1987) at 510 nm in a Quimis Q-108U2VL spectrophotometer. Tanic acid (Sigma) was used as standard at concentrations ranging from 0.05 to 0.8 mg/ml and $FeCl_3$ (Vetec) as the chromogenic agent. The absorbance was read at 510 nm and the correlation coefficient (r) was 0.9999. Total tannins were measured after precipitation of bovine serum albumin in sodium acetate buffer pH 4.9 followed by centrifugation at 3,000 rpm for 15 min (Sigma Laborzentrifugen) (Mole and Waterman, 1987). The pellet was resuspended in a solution of sodium dodecyl sulphate (SDS) (Vetec)/triethanolamine (Sigma) and $FeCl_3$ added for color development. The absorbance was read at 510 nm with tanic acid as standard (concentrations ranging from 0.2 to 0.8 mg/ml, r equal to 0.9994). The condensed tannins were determined following Sun et al. (1998) using hydrated catechin (Sigma) as standard (concentrations from 0.1 to 1.0 mg/ml, r equal to 0.9993, absorbance read at 500 nm). Total flavonoids were determined following Chabariberi et al. (2009) with rutin as standard (from 5 to 50 µg/ml, absorbance read at 425 nm, r equal to 0.9998). All colorimetric assays were done in triplicates.

Electrophoresis

In order to evaluate the effect of *Myrcia* extracts on proteins, crude *B. jararaca* venom or mouse plasma proteins were mixed with MgAR (1:2 and 1:10 w:w for the venom; w:v for the plasma). The mixtures were incubated for 30 min at 37°C and centrifuged at 6,000 g for 5 min (Sigma 3-18K centrifuge). The resulting pellets and supernatants were analyzed by a sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The following controls were also added: mouse plasma protein in saline, and MgAR in saline.

Hemorrhagic activity of B. jararaca venom

The hemorrhagic lesion caused by the *B. jararaca* venom was measured according to Gutierrez et al. (1985). Groups of six Swiss mice were injected intradermically in the dorsum with 50 µl PBS containing increasing amounts of the venom or purified jararhagin; the animals were sacrificed after 60 min, the dorsal tissues removed, photographed and the images digitalized (HP Deskjet Scanner F4280), saved as RGB archives and processed according to Dougherty (2002) using a Matlab Script (Gonzalez et al., 2009); the image components were selected by a threshold device (Guerra et al., 2011) and a morphological gradient processor was used for obtaining better halo limits. The area (in mm^2) and the biggest diameter were measured according to Dougherty and Lotufo (2003). The venom minimal hemorrhagic dose (MHD) was the lowest dose able to cause a hemorrhagic halo of 10 mm diameter after 60 min.

In vivo experiments with M. guianensis extracts

A) Two MHD (10 µg) of crude snake venom were mixed with the plant extracts MgAE, MgHx, MgAc and MgAR in saline at venom/extract ratios of 1:1, 1:2, 1:3, 1:6 and 1:10 (w:w) to a final volume of 50 µl, incubated at 37°C for 30 min, then injected in the mice dorsal skin (groups of six mice). After 60 min, the mice were euthanized and the hemorrhagic halos measured as already described. The controls were: 2 MHD of crude venom incubated in 50 µl saline and 50 µl *Myrcia* extracts in saline solution.

B) Groups of six mice were injected with crude *B. jararaca* venom as already described. After 10 min, the MgAR (20 and 100 µg) was injected at 0.8 cm far from the venom injection. After 60 min, the mice were euthanized and the halos measured. The following controls were added: 2 MHD of crude venom in 50 µl saline; MgAR in saline and 2 MHD of crude venom incubated in 50 µl saline containing antibothropic serum (1:15, w:w);

C) MgAR was incubated with 10 µg jararhagin at 1:10 and 1:100 (w:w, jararhagin/MgAR) for 30 min at 37°C, injected in the mice and the hemorrhagic activity measured after 60 min as already described. The controls were jararhagin plus saline and MgAR plus saline.

In vitro experiments

Phospholipase activity: Phospholipase activity was measured by the *in vitro* hemolytic activity on agarose gel, with egg yolk as phospholipid source and human erythrocytes as the substrate. The erythrocyte lysis carried out by the resulting lysophospholipids was assayed in triplicate. The indirect minimum hemolytic dose (iMHD) was determined as the minimum amount of the venom needed to yield a halo of 10 mm diameter in human erythrocyte embedded agarose gels (Gutierrez et al., 1988). For assessing MgAR inhibitory action on venon PLA_2 activity, MgAR was pre-incubated with 2 iMHD (1.24 µg) crude venom for 30 min at 37°C (1:2 and 1:10, w:w, crude venom/MgAR). Enzymatic activity was expressed as the percentage of inhibition, with a value of 100% corresponding to the complete absence of the hemolytic halo. Each assay was run in triplicate.

Fibrinolysis activity: The fibrinolytic activity of MgAR was carried out according to Jespersen and Astrup (1983) with modifications. Thirty milligrams of human fibrinogen (Sigma) were diluted in 10 ml Tris-HCl 50 mM buffer, pH 7.3, with 200 mM NaCl and 50 mM

$CaCl_2$. To this solu-tion, bovine thrombin (Sigma) was added to a final concentration of 2 U/ml. Following 10 ml of 2% agarose low melting (Ambresco) in the same buffer was added. The mixture was rapidly deposited in a plastic Petri dish (100 × 20 mm) on a leveled table until the agarose was polymerized and the fibrin network formed at ambient temperature. Next, 10 µg jararhagin previously incubated with MgAR for 30 min at 37°C (1:1 and 1:100, w:w, jararhagin/MgAR) were applied in standard holes previously perforated in the polymerized fibrin plate. After incubation at 37°C for 18 h, the diameters of the lysis area in the fibrin plate were measured and the areas determined. The fibrinolytic activity of the jararhagin was computed as the area of lysis minus the area of the holes was the samples were applied.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical significance: $p \leq 0.05$, Student's *t* test.

RESULTS AND DISCUSSION

Proteolytic enzymes are important components in *Bothrops* venom toxicity. Together with serineproteases, these enzymes are responsible for the coagulopathy and local and systemic hemorrhage, the most important symptoms in snake poisoning (Moura-da-Silva et al., 2007). To assess the potential of *M. guianensis* to prevent venom-induced hemorrhage, leave extracts were tested for neutralization of venom hemorrhagic activity by *in vivo* injections of a previously incubated mixture of venom and extract, by sequential injections of venom and extracts, by injections of extracts and jararhagin (the major hemorrhagic metalloprotease from *B. jararaca* venom) and by *in vitro* assays of venom-induced hydrolysis of fibrin and PLA_2 activity.

The aqueous fractions MgAE and MgAR completely inhibited 2 DMH (10 µg) of crude *B. jararaca* venom hemorrhagic activity when tested at concentrations of 1:1 (w:w, venom/extract) or higher. MgAc at 1:3 and 1:10 (w:w) inhibited the hemorrhagic halo by 90.7 and 100%, respectively. MgHx had no inhibitory activity even at a 1:10 ratio (w:w) (Table 1). No hemorrhage was produced by the control injections. Such results show a hydrophilic behavior of the inhibitory compound, suggesting that it is a polar molecule.

MgAR completely inhibited jararhagin induced hemorrhage at 1:10 ratio (w:w, jararhagin/MgAR) (Figure 1). Since jararhagin is a metalloprotease, and since all extracts except MgHx were able to inactivate the *in vivo* hemorrhagic action of crude *B. jararaca* venom, it is possible that MgAR, MgAE and, in lesser degree, MgAc have a polar compound that prevents the hemorrhagic activity of such enzymes. However, MgAR inhibition could not be observed if injected 10 min after the crude venom, although the antibothropic serum inhibited the halo by 58% if injected according to a similar protocol. This result could be due to the rapid destruction of the walls of the blood vessels caused by the venom shortly after injected, thus impairing MgAR diffusion through the skin. In accordance with this hypothesis, even the commercial antivenin, which possesses high neutralizing activity, failed to completely inhibit the hemorrhagic lesion.

Table 1. Inhibition of the hemorrhagic activity of *Bothrops jararaca* crude venom by *Myrcia guianensis* leave extracts.

Extract	Venom/Extract ratio (w:w)	Inhibition (%)
Controls	-	0
MgAE	1:1	100*
	1:2	100*
	1:3	100*
	1:10	100*
MgAc	1:1	36.71* ± 0.30
	1:2	35.17* ± 0.20
	1:3	90.73* ± 0.46
	1:10	100
MgHx	1:1	0
	1:2	0
	1:3	0
	1:10	0
MgAR	1:1	100*
	1:2	100*
	1:3	100*
	1:10	100*

Extracts were incubated with 2 minimal hemorrhagic dose of *B. jararaca* venom (10 µg) in a final volume of 50 µl and injected in mice dorsal skin. After 1 h the mice were euthanized and the hemorrhagic area measured. 100% inhibition means absence of venom-induced hemorrhage. Controls = 0.9% NaCl solution (saline) and each extract resuspended in saline; MgAE = crude aqueous extract; MgAc= ethyl acetate extract; MgHx= hexane extract; MgAR = final aqueous residue. Data are mean ± SD (n = 6). The signal (*) indicates significant differences ($p < 0.05$) among the control and the test groups. Student`s *t* test.

PLA_2 activity is common to *Bothrops* snakes venoms and may be related to several toxic effects such as myotoxicity, inhibition of blood clotting and platelet aggregation and the inflammatory symptoms which are frequent after snakebites. Table 2 shows the PLA_2 activity with or without MgAR added to the medium. It can be seen that MgAR completely inhibited the *B. jararaca* venom PLA_2 activity when added at 1:2 and 1:10 ratios (m:m). There are several other reports on the neutralizing properties of plant extracts on PLA_2 enzymes present in venom from different snakes (Borges et al., 2005; Maiorano et al., 2005; Biondo et al., 2003). The extract of *Mikania glomerata* leaves, when applied at 1:200 (w:w, venom/extract), inhibited the PLA_2 activity of *Bothrops jararacussu* by only 30% (Maiorano et al., 2005).

Table 2. Inhibition of PLA2 activity of Bothrops jararaca venom by Myrcia guianensis MgAR extract.

Extract	Venom/Extract ratio (w:w)	Inhibition (%)
Control (saline)	-	0
MgAR	1:2	100*
-	1:10	100*

MgAR (aqueous residue of *Myrcia guianensis)* in saline was incubated with 2 indirect minimum hemorrhagic dose of *B. jararaca* venom (1.24 µg) and the PLA_2 activity assayed using egg yolk phospholipids and erythrocyte embedded in agarose gel. 100% inhibition means total absence of venom-induced hemolysis following incubation with MgAR (n = 6). $PLA_{2\ =}$ phospholipase A2; Student's t test, ($p < 0.05$).

Table 3. Phenols, tannins, condensed tannins and flavonoids in *Myrcia guianensis* extracts.

Extract	Total phenols	Total tannins	Condensed tannins	Total flavonoids
MgAE	35.03 ± 0.09	32.52 ± 1.83	86.67 ± 24.34	1.70 ± 0.014
MgAR	33.05 ± 1.40	31.80 ± 3.13	64.24 ± 1.05	0.46 ± 0.006
MgAc	36.81 ± 5.23	10.49 ± 1.50	16.52 ± 3.14	10.58 ± 0.39

Results are expressed as g of the standard/100 g of the dry *M. guianensis* fraction. Data are mean ± SD (n = 3).

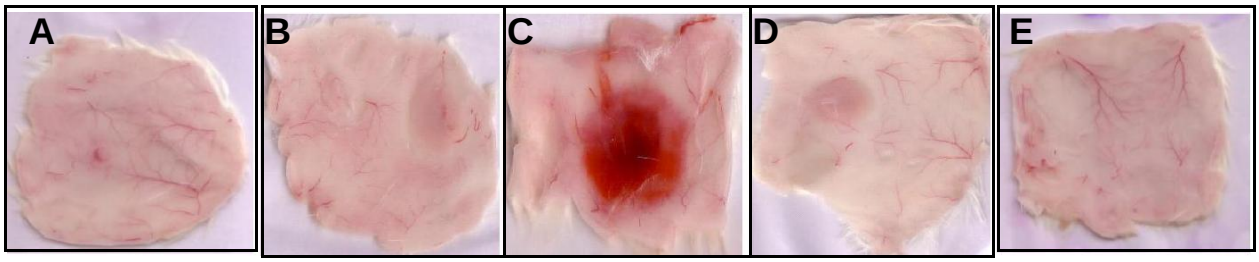

Figure 1. Inhibition of jararhagin hemorrhagic activity by MgAR. Jararhagin (10 µg) was incubated with MgAR (aqueous residue of *Myrcia guianensis*) at 1:10 and 1:100 (w:w, toxin/extract) for 30 min. Then 50 µl of the mixture were injected into mice dorsal skin (n = 6). The induced hemorrhage was measured as described in methods. A = 0.9% NaCl only; B = MgAR only; C =10 µg jararhagin only; D = 10 µg jararhagin + MgAR 1:10; E = 10 µg jararhagin + MgAR 1:100. $p < 0.001$, 100% inhibition for both concentrations, Student`s *t* test.

Although *B. jararaca* and *B. jararacussu* PLA_2 may differ, the striking differences between the activities of extracts from *M. guianensis* and from *M. glomerata* makes it possible that that of *M. guianensis* is a more potent inhibitor than that of *M. glomerata*.

MgAR inhibited by 25% jararhagin-induced fibrinolytic at 1:2 ratio (w:w, jararhagin/MgAR) and by 100% at 1:100 (Figure 2). Jararhagin causes hemorrhage by disrupting the blood vessels walls and by cleaving fibrinogen and other compounds that favors blood coagulation (Swenson and Markland, 2005). Our results from the *in vitro* experiments suggest that MgAR antihemorrhagic action against *B. jararaca* venom is at least partially due to jararhagin and PLA_2 inactivation.

The TLC analysis and the chemical reaction with $FeCl_3$ showed that MgAR has neither anthraquinones nor terpenoids but is composed of fatty acids, cumarins, flavonoids and hydrolytic and condensed tannins, which can inactivate proteins (data not shown). Table 3 shows the results of the colorimetric assays. MgAE, MgAR and MgAc fractions have approximately the same amount of total phenols. However, there is at least three times more total and condensed tannins present in MgAE and MgAR than in the MgAc fraction. On the other hand, the total flavonoids present in the MgAc fraction is at least five times bigger than the amounts found in the MgAE and MgAR fractions.

Flavonoids can bind metallic ions (Voet and Voet, 1995), are soluble in ethyl acetate, and metalloproteases need divalent ions such as zinc to be active (Fox and Serrano, 2009). Also, tannins, mainly condensed tannins, can precipitate metalloproteases (Martins et al., 2000). So, although it is possible that flavonoids could be at least in part responsible for the results found in this study,

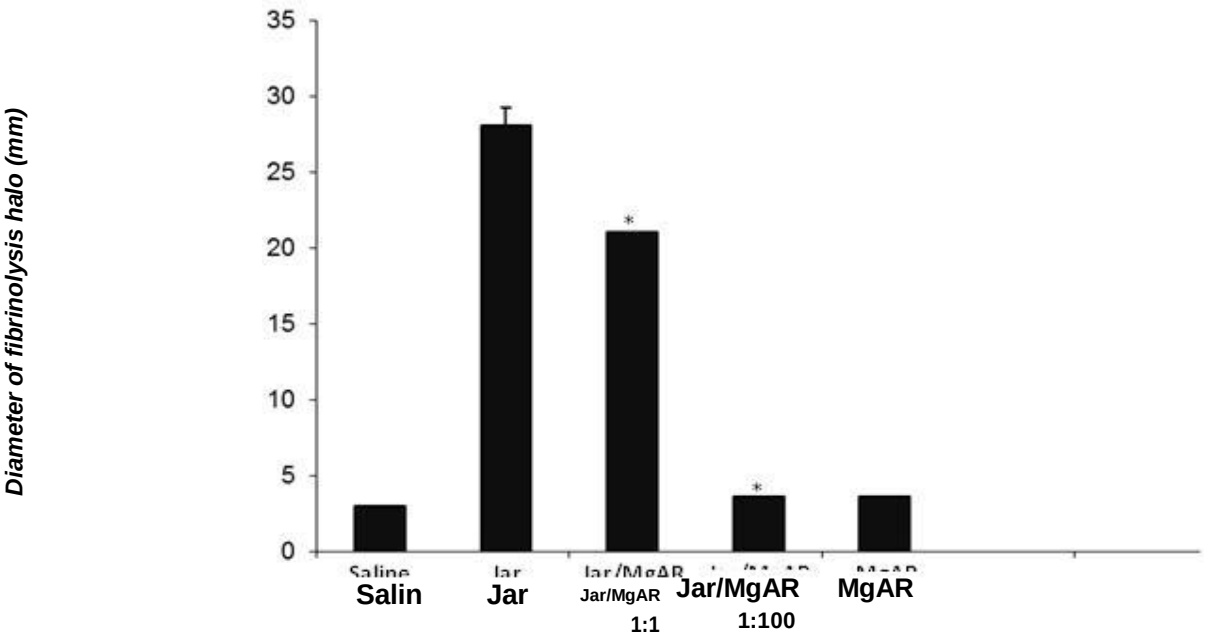

Figure 2. Neutralization of jararhagin fibrinolytic activity by MgAR. Jararhagin (jar 10 µg) was incubated with 0.9% NaCl saline solution (control) or MgAR (aqueous residue of *Myrcia guianensis*) in saline at 1:1 and 1:100 (w:w, toxin/ MgAR). After 30 min, fibrinogenolytic activity was measured as described in methods. % inhibition: 25 % for 1:1 and 100 % for 1:100 jararhagin/MgAR. $p < 0.05$ for both concentrations, n = 6, Student`s *t* test.

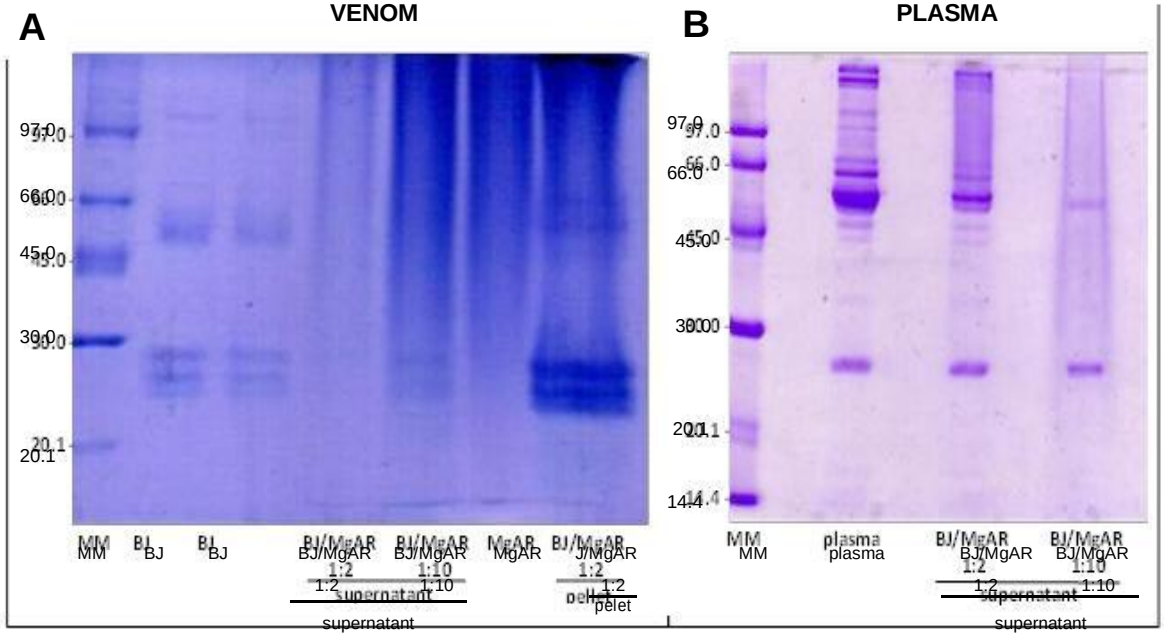

Figure 3. Direct effect of MgAR on venom and plasma proteins. *Bothrops jararaca* venom (BJ) (A) or mice plasma proteins (B) were incubated with MgAR at 1:2 and 1:10 (w:w, protein/ MgAR) for 30 min. The mixture was centrifuged and the pellet and supernatants analyzed by SDS-PAGE.

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tannins are the principal components of the MgAE and MgAR fractions. As such, it is suggested that precipitation is the principal mechanism which explains the results found. Accordingly, the *in vitro* incubation of MgAR or

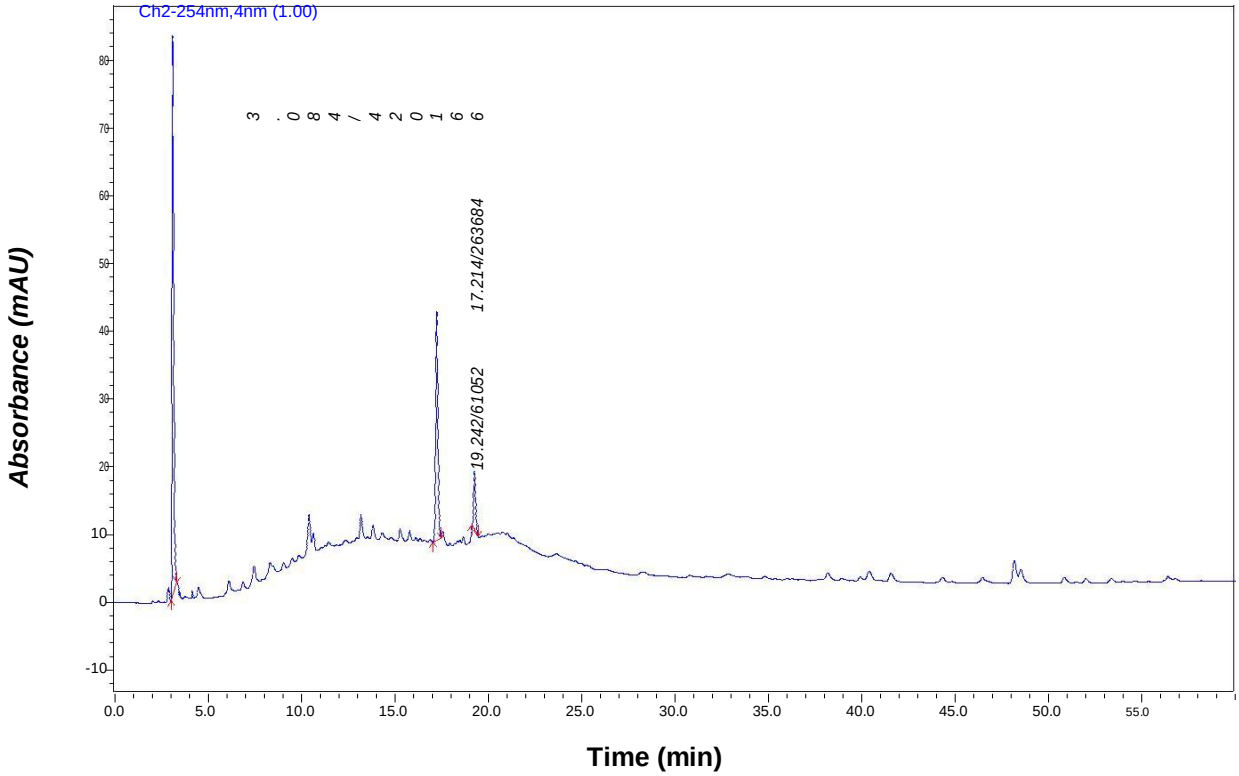

Figure 4. HPLC profile of MgAR (aqueous residue of *Myrcia guianensis*), showing two main compounds at retention times of 17 and 19 min. Twenty millilitres MgAR were injected in the HPLC (Shimadzu) device (C-18 Phenomex), eluted with 1ml/min flux, the mobile phase being a gradient of H_2O and acetonitrile, the last one increasing from 5 to 100% in 60 min. Diode detector SPD-M20A adjusted to 200 to 400 nm.

mouse plasma protein with crude *B. jararaca* venom followed by electrophoresis showed high protein concentration in the pellet when compared with the supernatant (Figure 3). Besides, plasma proteins with the highest molecular weight were not seen in the supernatant, further suggesting that MgAR can cause their precipitation. No conspicuous protein decomposition could be observed, reinforcing the hypothesis that the inhibition of the hemorrhagic halo caused by MgAR is mainly due to protein precipitation.

On HPLC analysis*, M. guianensis* aqueous residue (MgAR) showed two main compounds with retention times of 17 and 19 min at 254 nm (Figure 4). Studies are being done to identify these compounds.

Taken together, the *in vivo* assays suggest that the MgAE, MgAR and MgAc inhibitory activity could be mainly due to the precipitation of metalloproteases present in *B. jararaca* venom.

Conclusions

M. guianensis crude aqueous extract (MgAE), its acetate fraction (MgAc) and its residue after extraction with organic solvents (MgAR) can inhibit the hemorrhagic lesion produced by intradermic injections of both *B. jararaca* crude venom and jararhagin. MgAR can also precipitate proteins, inhibit phospholipase activity and has antifibrinolytic activity.

TLC analysis showed the presence of tannins and flavonoids in all *M. guianensis* fractions with inhibitory activity, and colorimetric measurements showed that tannins are the principal compounds in both MgAE and MgAR fractions. Although flavonoids can inactivate metalloproteases, the relatively small amounts of these compounds as compared to the amounts of total and condensed tannins in the MgAE and MgAR makes it possible that metalloprotease precipitation due to the tannins is the principal mechanism of the antibothropic activity of the extracts. This hypothesis is further reinforced by the electrophoretic results. However, a synergistic action of both tannins and flavonoids cannot be discarded at this stage of the research.

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