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Characterization of a hemorrhage-inducing component present in *Bitis arietans* venom

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Better characterization of individual snake venom toxins is useful for analyzing the association of their toxic domains and relevant antigenic epitopes. Here we analyzed the *Bitis arietans* hemorrhagic-inducing toxin present in a representative venom sample. Among the 1´ to 5´ protein peaks isolated using a Sephacryl S 100 HR chromatography column, hemorrhagic activity was expressed by all according to the following intensity, P´5 > P´3 > P´2 >, P´4. The proteins were recognized and measured with antibodies present in polyvalent horse F(ab)´$_2$ anti-*B. arietans*, *Bitis* spp., *Lachesis muta, B. atrox*, *Bothrops* spp., *Crotalus* spp., and *Naja* spp. or in IgY anti-*B. arietans* and anti-*Bitis* spp. using enzyme-linked immunosorbent assays and western blotting. In addition, in an *in vitro-in vivo* assay these anti-venoms were able to block hemorrhagic-inducing activity. The evident cross-reactivity expressed by different specific anti-venoms indicates that metalloproteinases induce an immunological signature indicating the presence of similar antigenic epitopes for several snake venoms.

Key words: Snake venoms, anti-venoms, *Bitis arietans,* hemorrhage, metalloproteinases.

INTRODUCTION

In sub-Saharan Africa, snake bites are an important public health problem resulting in permanent disabilities and death (Theakston et al., 2003; Kasturiratne et al., 2008). *Bitis* spp., *Naja* spp., and *Dendroaspis* spp. include the greatest number of snake species that cause envenomation in Mozambique (Broadley, 1968; Manaçás, 1981-1982). Their venoms contain multiple and diverse toxins. *B. arietans* is a common snake found in densely populated savannah areas in sub-Saharan Africa and in the Arabian Peninsula (Navy, 1991). Victims of this snake species exhibit severe local and systemic disturbances, such as swelling, hemorrhage and necrosis (Warrell et al., 1975; Warrell, 1996; Currier et al., 2010; Calvete et al., 2006). Anti-venom supply in the African continent, however, is scarce and often unaffordable.

In 1996, we submitted a proposal to the Conselho Nacional de Ciência Tecnologia – PROÁFRICA, Ministério de Ciência e Tecnologia do Brazil Proc. No: 4800048/2006, including among the main objectives the development of procedures for producing specific anti-*Bitis* spp., anti-*Naja* spp., and anti-*Dendroaspis* spp. anti-venoms. The proposal was granted, and the anti-venoms

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were developed using whole venom as the immunogens and traditional methods of immunization (de Almeida et al. 2008; Guidolin et al. 2010).

To address the specific needs of the African region, high specificity, stable, affordable anti-venoms and poly-specific and lyophilized substitutes must be developed. To accomplish this objective, the initial step is the development of mono-specific anti-venoms. We begin this objective by developing an anti-*Bitis* spp. mono-specific anti-venom.

The metalloproteinases (SVMPs) and A-disintegrin proteinases (ADAMs) (Juárez et al., 2006; Moura-da-Silva et al., 2011; Trummal et al., 2005) comprise the M12b subfamily of zinc-dependent reprolysins. Both enzymes, along with the non-enzyme C-type lectin-like proteins, are important snake venom components (Bode et al., 1993; Stokër et al., 1995; Bjarnason and Fox, 1995). Previous studies indicate that SVMPs and ADAMs are involved in hemorrhage, edema, hypotension, inflammation and necrosis induced by snake venoms (Gutiérrez and Rucovaldo, 2000). Although Viperidae snake venoms are rich in SVMPs, these enzymes have also been observed in certain *Elapidae* and *Colubridae* venoms (Junqueira-de-Azevedo and Ho, 2002; Guo et al., 2007; Ching et al. 2006). SVMPs act by cleaving proteins on the vascular basement membrane and surrounding connective tissues as well as by altering platelet structure and function.

The introduction of toxins responsible for hemorrhage (Gutiérrez et al., 1995; Gutiérrez et al., 2005), complement system activating factors (*Tambourgi* et al., *2015*) and hypotension-inducing components (Kodama et al., 2015) present in *Bitis* sp. venom into an immunogenic mixture is considered essential to obtain high quality mono-specific anti-venom.

In this study, we purify and characterize the biological and immunological properties of a representative *B. arietans* 50-kDa protein endowed with hemorrhage-inducing activity.

MATERIALS AND METHODS

Reagents

The reagents used included: Tris buffer (Tris HCl, 25 mM; pH 7.4); Complete MMT80 (2 mL of Marcol Montanide ISA 50 in 5.0 mL of sodium chloride, 0.15 M, plus 1.0 mL of Tween 80 and 1.0 mg of lyophilized BCG); incomplete MMT80 (Marcol Montanide ISA 50 without BCG); SDS buffer (Tris, 6.25 mM, pH 6.8 plus 0.2% SDS); SDS buffer plus 1 mL of β-mercaptoethanol (SDS buffer plus 8.5 mL of glycerol and 2 mL of 1% bromophenol blue); PBS buffer (potassium chloride, 2.6 mM; monobasic potassium phosphate, 1.5 mM; sodium chloride, 76 mM; disodium phosphate, 8.2 mM; pH 7.2-7.4); AP buffer (Tris HCl, 100 mM, pH 9.5 plus sodium chloride, 100 mM, and magnesium chloride, 0.1 M); NBT solution (NBT, 50 mg; dimethylformamide in 700 µL plus 300 µL of H_2O). BCIP solution (BCIP, 50 mg; dimethylformamide in 1.0 mL of diluent); developing solution for western/dot blotting (AP buffer, 5.0 mL plus NBT solution, 33 and 16.5 µL of BCIP solution); citrate buffer, pH 5.0 (citric acid, 0.1 M, plus 0.2 M monobasic sodium phosphate); OPD solution (20 mg of OPD in 1.0 mL of citric acid); substrate buffer for ELISA (5 mL of citrate buffer plus 100 µL of OPD solution and 5 µL,). All reagents were obtained from Sigma-Aldrich (USA), except for NBT and BCIP, which were obtained from Molecular Probes (USA).

Snake venoms

African snake venoms from *B. arietans*, *B. nasicornis*, *B. rhinoceros*, *N. melanoleuca*, and *N. mossambica* were purchased from Venom Supplies Pty Ltd (59 Murray Street, Tanunda, Australia). Although African snake venoms were the focus, Brazilian snake anti-venoms from *Bothrops atrox*, *Bothrops jararaca, Lachesis muta*, and *Crotalus* spp. were also obtained to identify cross-reactions. Brazilian snake venoms were supplied by the Laboratório de Herpetologia, Instituto Butantan, São Paulo, Brazil. All venoms were collected from healthy adult snakes following standard methods, filtered through 0.45-µm membranes, assayed for protein content, and stored at -20°C.

Mice

Swiss and BALB/c isogenic mice (male and female; 18-20 g) were provided by the *Universidade Estadual de Campinas,* sp., Brazil animal facilities. The mice were used to determine the lethality (LD_{50}) of the venoms and the neutralizing potency (DE_{50}) of the anti-venoms. The animals used in this work were maintained and treated under strict ethical conditions according to International Animal Welfare Recommendations (World Health Organization, 981; Remfry, 1987). This study was approved by the *Comissão de Ética de Animais de Laboratório, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense – Darcy Ribeiro*.

Snake anti-venoms

Horse polyclonal $F(ab´)_2$ anti-*B. arietans*, anti-*B. nasicornis* plus *B. rhinoceros*, and anti-*N. melanoleuca* plus *N. mossambica*, anti-*Lachesis muta*, anti-*Bothrops jararaca* and anti-*Crotalus durissus terrificus* were supplied by the Seção de Processamento de Plasmas Hiperimunes-Divisão de Desenvolvimento Tecnológico e Produção, Instituto Butantan, São Paulo, Brazil. Chicken polyclonal IgY anti-*B. arietans*, anti-*B. nasicornis plus B. rhinoceros*, and anti-*N. melanoleuca* plus *N. mossambica*, were prepared at the Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense - Darcy *Ribeiro, Campos do Goytacazes,* RJ (de Almeida et al.,2008). Horse $F(ab´)_2$ anti-venoms were prepared at the Seção de Processamento de Plasmas Hiperimunes-Divisão de Desenvolvimento Tecnológico e Produção, Instituto Butantan, São Paulo, Brazil.

Quantification of proteins

The protein concentrations of the venoms, their purified fractions and the anti-venoms were assessed using the bicinchoninic acid method with a Pierce BCA Protein Assay Kit (Rockford, IL).

Determination of venom lethality (LD_{50})

The median lethal dose (LD_{50}) of each venom was determined in mice. Five serial venom dilutions were prepared in PBS. Groups of four mice were i.p. injected with each venom dilution. The control

group was injected with PBS. The death/survival ratios were determined after 48 h of observation. The LD_{50} was calculated using probit analysis (Finney, 1947; World Health Organization, 1981).

Evaluation of venom hemorrhagic activity (HD_{50})

The median hemorrhagic dose was evaluated by injecting (i.c.) 50-µL aliquots of PBS-saline, pH 7.0, containing different concentrations of crude or purified venom fractions into dorsally depilated mice anesthetized under a CO_2 atmosphere. After 48 h, the mice were again anesthetized under CO_2 atmosphere and bled.

***B. arietans* venom hemorrhagic activity**

Groups of mice were subcutaneously injected with different amounts of crude venom, with chromatograph isolated protein peaks P´1 P´2, P´3, P´5 or with 01 M NaCl (NCs) as negative control. In some experiments, *B. jararaca* venom was included as positive control. 2 h later the mice were anesthetized under CO_2 atmosphere, submitted to total bleeding, the skin dissected, and the hemorrhagic areas were exposed in the internal skin surface. The hemorrhagic areas (Figure 4A1 and 4A2, and 4 negative control NCs), were evaluated with a "SCANNER" program in Image J (Dias, 2007; Schneider et al., 2012), the images were plotted on a square millimeter chart, the plots corresponding to each hemorrhagic area were measured, the hemorrhagic square area was calculated in function of the square millimeter chart and referred as square percentages (Figure 4C). The tissue areas of each hemorrhagic area was removed, the hemoglobin was extracted and spectrophotometrically evaluated at 491 nm.

Neutralization of venom hemorrhagic activities

Samples of *B. arietans* crude venom, its chromatographically isolated protein peaks containing 4 HD_{50}, and various dilutions of anti-venoms were incubated for 30 min at 37°C. The venom samples incubated only with PBS were run in parallel. After incubation, 50 µL aliquots of the mixtures were subcutaneously injected into the mice. Each mixture was tested on a group of five mice. Two hours later, the mice were anesthetized under a CO_2 atmosphere, and the hemorrhagic areas were evaluated as described above. Neutralization activity was expressed as the minimal amount of antivenom reducing 50% of hemorrhagic areas (ED_{50}) produced by untreated antivenoms (HD_{50}).

Isolation of hemorrhagic-inducing metalloproteinases from *B. arietans* venom

Fifty milligrams of freeze-dried venom was dissolved in 5 mL of pH 5.5 acetate buffer (50 mM sodium acetate plus 150 mM NaCl), filtered through a 0.45-µm pore size membrane, and loaded on a Sephacryl S 100 HR (2.5 x 67 cm) molecular exclusion chromatography column previously equilibrated with pH 5.5 acetate buffer in a climate-controlled room. The subsequent elution was also performed with pH 5.5 acetate buffer. The samples were collected at a 60 mL/h flow rate, and their protein content was monitored by recording the absorbance at 280 nm in a spectrophotometer. The eluted fractions corresponding to peaks, P´1, P´2, P´3 and P´5 were pooled, and their protein contents were also determined as described above. The protein concentration of each fraction was adjusted to a common value before being used as antigen in western blotting and ELISA assays or as a hemorrhagic-inducing factor in the mouse skin test.

SDS-PAGE

The protein samples were separated by electrophoresis (Laemmli, 1970). Crude venoms (10 µg) or purified hemorrhagins (2 µg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved on a 12.5% polyacrylamide gel and stained with silver sulfate; molecular mass was determined using molecular markers running in parallel.

Western blotting

Samples of crude venoms (10 µg) or purified fractions (2 µg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved on a 12.5% polyacrylamide gel as described (Laemmili, 1970). Some SDS-PAGE preparations were stained with silver sulfate to be used as protein band profiles. Other SDS-PAGE preparations were electro-blotted to 0.45-µm nitrocellulose membranes (Towbin et al., 1979) to identify the protein bands recognized by anti-venoms. Membranes were incubated overnight in blocking buffer (5% non-fat milk in PBS) and then washed in PBS buffer containing 5% BSA and incubated for 2 hours with primary antibodies (horse or hen IgY anti-*B. arietans* venom antibodies) diluted to 1:5,000 in PBS containing 0.1% BSA for 1 h at room temperature on a horizontal shaker. After washing three times with PBS containing 0.05% Tween-20, the membranes were incubated with rabbit anti-horse IgG or anti-IgY conjugated to alkaline phosphatase (whole molecule) diluted 1:7,500 in PBS containing 0.1% BSA and 0.05% Tween-20. The membranes were then incubated for 1 h at room temperature on a horizontal shaker. The results were imaged after addition of the substrate.

ELISA

ELISA plates (96 wells) were coated with 1.0 µg of crude Brazilian or African snake venom in 100 µL of PBS and stored overnight at 4°C. In some assays, crotoxin or PLA_2 purified from *C. d. terrificus* or purified *B. arietans* metalloproteinase were used as the antigens. The wells were blocked for 2 hours at 37°C with 200 µL of PBS containing 5% BSA. The wells were then washed with 200 µL of PBS. For the initial antibodies, serial dilutions of horse IgG or $F(ab')_2$ preparations (1:4,000 to 2,048,000) in PBS containing 0.1% BSA were prepared, and 100 µL of each dilution was added to the individual wells. The plates were then incubated at 37°C for 1 h. The wells were then washed three times with washing buffer. As the second antibody, rabbit peroxidase-conjugated anti-horse IgG (whole molecule) (Sigma Aldrich, St. Louis, MO) diluted (1:20,000) in PBS containing 0.1% BSA and 0.05% Tween 20 (100 µL/well) was added to the plates. The plates were then incubated for 1 h at 37°C. After three washes with washing buffer, 50 µL of substrate buffer was added to each well, and the plates were incubated at room temperature for 15 min. The reaction was terminated with 50 µL of 4 N sulfuric acid per well. The absorbance was recorded at 492 nm using an ELISA plate reader (Labsystems Multiskan Ex, Thermo Fisher Scientific Inc., Walthan, MA). Horse IgG isolated from serum collected before immunization was used as a negative control. The IgG dilution giving an optical density of 0.2 was used to calculate the U-ELISA per milliliter of undiluted IgG solution. One U-ELISA is defined as the smallest dilution of antibody giving an O.D. of 0.2 under the conditions of the ELISA assay. The calculated value was then used to determine the amounts in 1.0 mL of undiluted anti-venom preparations.

Statistical analysis

The data were subjected to one-way ANOVA followed by Dunn´s

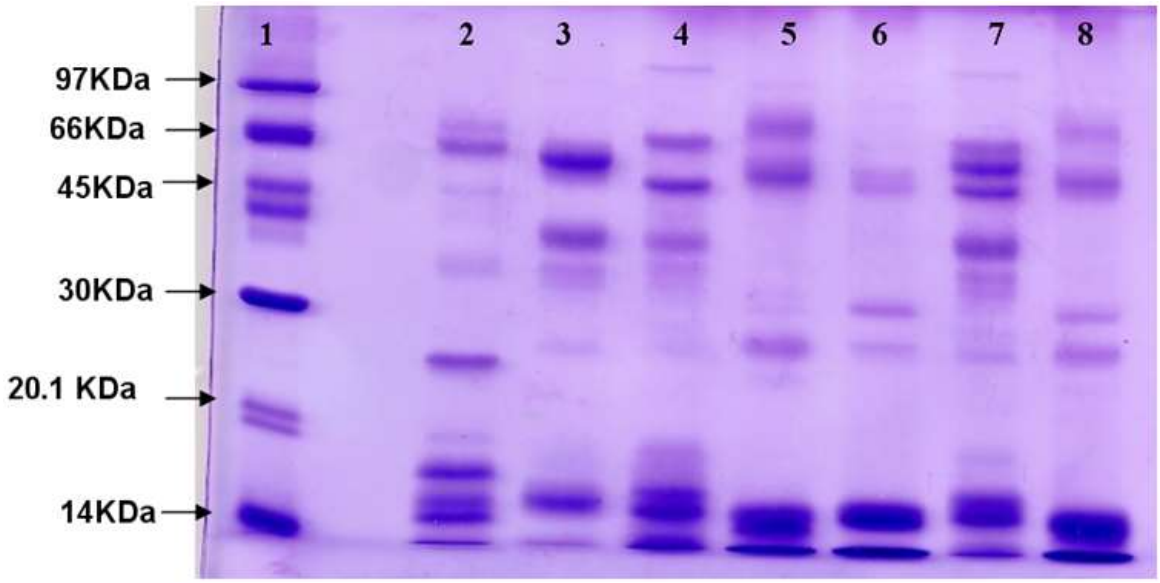

Figure 1. Polyacryalmide gel electrophoresis in SDS of snake venos.(1) Molecular mass ranging from 14 kDa to 97kDa as indicated; (2) *B. arietans* (3), *B. nasicornis* (4), *B. rhinocerus;* (5) *Bitis* spp.; *Naja melanoleuca;* (7) *N. mossambica;* (8) *Naja* spp. Samples of 15 µL containing crude venoms (30 µg) were treated with SDS-PAGE sample buffer under reducing conditions and resolved in a 15% polyacrylamide gel. The protein bands were stained with Coomassie Blue (The SDS-PAGE was performed by David Gitirana da Rocha, Post-graduating student, LBR/UENF, Campos dos Goytacazes, RJ).

multiple comparisons test. The Kruskal-Wallis test was used to compare the hemorrhagic lesions induced by the crude venoms or the chromatographically isolated protein peaks. The Mann-Whitney method was used to compare the hemorrhagic lesions induced by the untreated or antibody-pretreated chromatographically isolated protein peaks. For all analyses, differences were considered to be significant at $P < 0.05$.

RESULTS

Venom LD_{50} values

The venom LD_{50} values were determined by i.p. injecting mice with various amounts of crude venom and calculating the death/survival ratio after 48 h. The following values were obtained: *B. arietans*, 0.96 µg/mouse; *B. nasicornis*, 123.67 µg/mouse; *B. rhinoceros*, 95.28 µg/mouse; *N. melanoleuca*, 13.41 µg/mouse; *N. mossambica*, 22.40 µg/mouse; *Bothrops atrox,* 76 µg/mouse, *Lachesis muta*, 123.4 µg/mouse, *Crotalus durissus terrificus*, 4.32 µg/mouse, and *Bothrops jararaca*, 32.6 µg/mouse. The LD_{50} values were used to determine the ED_{50}, as indicated below. The latter four venoms were included for comparison.

Visualization of the venom protein bands

Different molecular mass components ranging from 14 to 97 kDa were resolved using 15% SDS-PAGE analysis under non-reducing conditions (Figure 1): ten protein bands in *B. arietans*; (well # 2); six bands in *B. nasicornis*; (well # 3); six bands in *B. rhinoceros* (well # 4); six bands in *N. melanoleuca* (well # 6); nine bands in *N. mossambica* (well # 7); and six bands in *Naja* spp.(well # 8). The *B. arietans* venom focused on in this work exhibited five strong protein bands, one 65 kDa, one 25 kDa, one 15 kDa and two 14 kDa.

Fractionation of the *B. arietans* venom

Crude *B. arietans* venom was analyzed chromatographically using a sephacryl S 100 HR (2.5 cm X 67 cm) column equilibrated with 50 mM sodium acetate buffer, 150 mM NaCl at pH 5.5. The chromatography was performed at a 1 mL/min flow rate. Elution was monitored by recording the absorbance at 280 nm in a spectrophotometer. Eluted samples were successively collected, from left to right, and labeled 1 to 48. Five protein peaks (P´1, fractions 1-8; P`2, fractions 9-17; P´3, fractions 18-27; P´4, fractions 33-37; and P´5, fractions 45-48) were identified (Figure 2). After being lyophilized, the selected fractions were dissolved in 0.15 M NaCl, and their protein contents were determined. The recognition of *B. arietans* snake venom components by antibodies present in snake anti-venoms was assayed *in vitro* or by ELISA or western blotting methods. The presence of hemorrhagic-inducing factor was assayed, *in vivo*, by injecting representative aliquots into mouse skin.

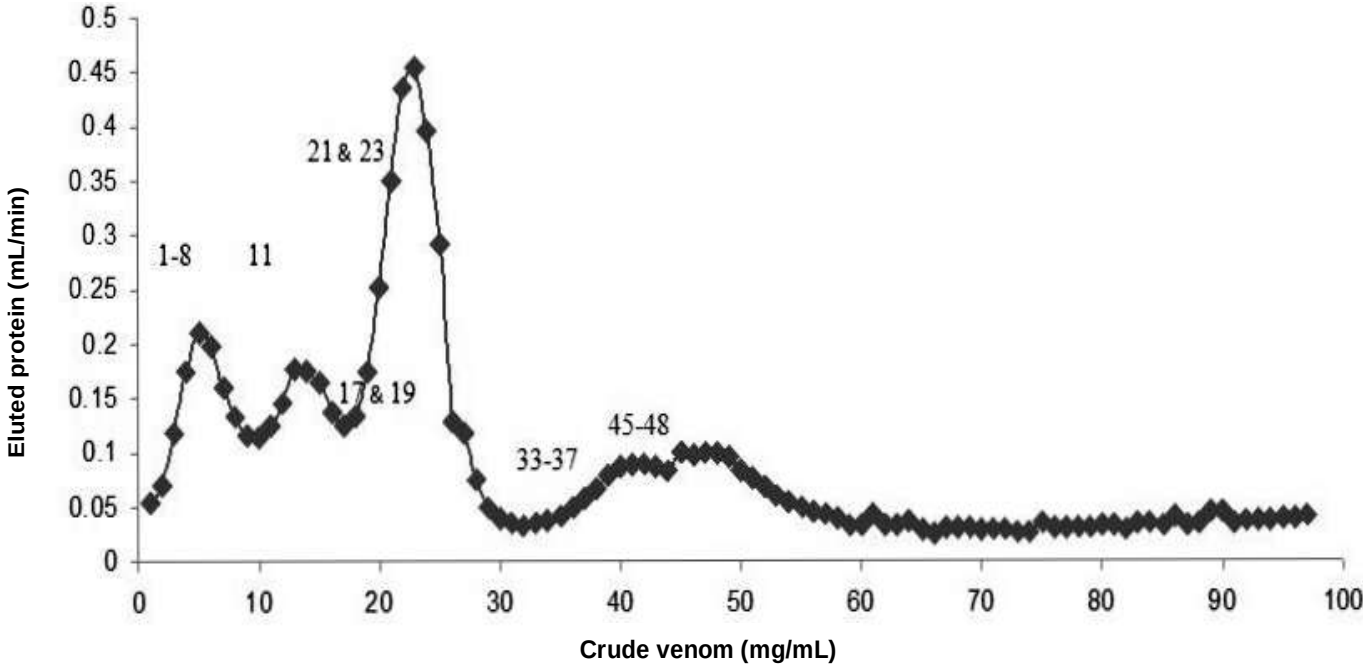

Figure 2. Isolation of hemorrhagic-inducing metalloproteinases from the *B. arietans* venom. Molecular chromatography exclusion of *B. arietans* venom. Five milliliter samples of crude venom (10 mg/mL) in 50 mM acetate-buffer, 150 mM NaCl, pH 5.5 were applied on Sephacryl S 100 HR (2.5 cm X 67 cm) column previously equilibrated with the some buffer. Chromatography was processed at 0.4 mL/min flow-rate. Elution was monitored by recording the absorbance at 280 nm in a UPC-900 Amersham Pharmacia Biotech. Elution samples were successively collected and labeled 1 to 48 from the left to right. Five eluted protein peaks (P´s) were obtained: P1´, fractions 1-8; P`2, fractions 9-17; P´3, fractions 18-27; P´4, fractions 33-37; P´5, fractions 45-48. The protein peaks were lyophilized. Before assays, the peaks were dissolved in the required buffer or solution and the protein contents were determined. The protein peaks were then submitted to, *in vitro*, ELISA and Western blotting assays by using different snake anti-venoms as the first antibodies or, *in vivo*, by injecting into mice skin to evaluated their ability to promote hemorrhage.

Characterization of the *B. arietans* protein peaks separated by sephacryl S 100 HR (2.5 cm X 67 cm) column chromatography

As indicated by polyacrylamide-gel electrophoresis (15%), the main *B. arietans* venom protein P3´ obtained from sephacryl S 100 HR chromatography was a 50-kDa protein similar to the protein present in whole venom (Figure 3A). Densitometric analysis indicated (Figure 3B) that P3´ was also prominent but with smaller-molecular weight protein contaminations. Unexpectedly, the P3´ protein was recognized by antibodies present in the different polyvalent anti-venoms.

The chromatographi-cally isolated samples were also plated in ELISA wells. The horse F(ab)´$_2$ polyvalent anti-venoms anti-*B. arietans*, anti-*Bitis* spp., anti-*Lachesis muta*, anti-*B. atrox*, anti-*Bothrops* spp., anti-*Crotalus* spp., and anti-*Naja* ssp. and the IgY anti-*B. arietans*, anti-*Bitis* spp. and anti-*Naja* spp. anti-venoms were used as primary antibodies (Figure 3C). P3´ was submitted to western blot analysis and subsequently recognized by polyvalent horse anti-venoms and stained (1 - control; 2 - anti-*Bitis arietans*; 3 - anti-*Bitis* spp.; 4 - anti-*Bothrops* spp.; 5 - anti-*Lachesis muta*; 6 - anti-*Naja* spp., 7 - anti-*Crotalus* spp.) (Figure 3D).

Hemorrhagic activity induced by crude venoms or by their isolated protein peaks

The hemorrhagic-inducing properties of *B. arietans* crude venom or of its isolated hemorrhagic-inducing protein fractions were assayed by injecting aliquots of the test samples into mice skin. After 2 h, the mice were anesthetized under a CO_2 atmosphere and bled; the skin was dissected, and the hemorrhagic areas were exposed in the internal skin surface. Figure 4A1 indicates that the hemorrhagic area developed with 5 µg (Figure 4A1a) of *B. arietans* crude venom was larger than that produced with 2 µg (Figure 4A1b) of the same venom.The amounts of hemoglobin extracted from these areas were equally proportional to injected *B. arietans* crude venom doses. Equivalent results were obtained when *B. jararaca* crude venom was used as positive control (Figure 4B). These results were also compatible when the corresponding areas were indirectly measured and expressed in percentage terms of square percentages of hemorrhagic areas: A1a = 12.8%; A1b =24.0%; $A2_1$ =11.7%; $A2_2$

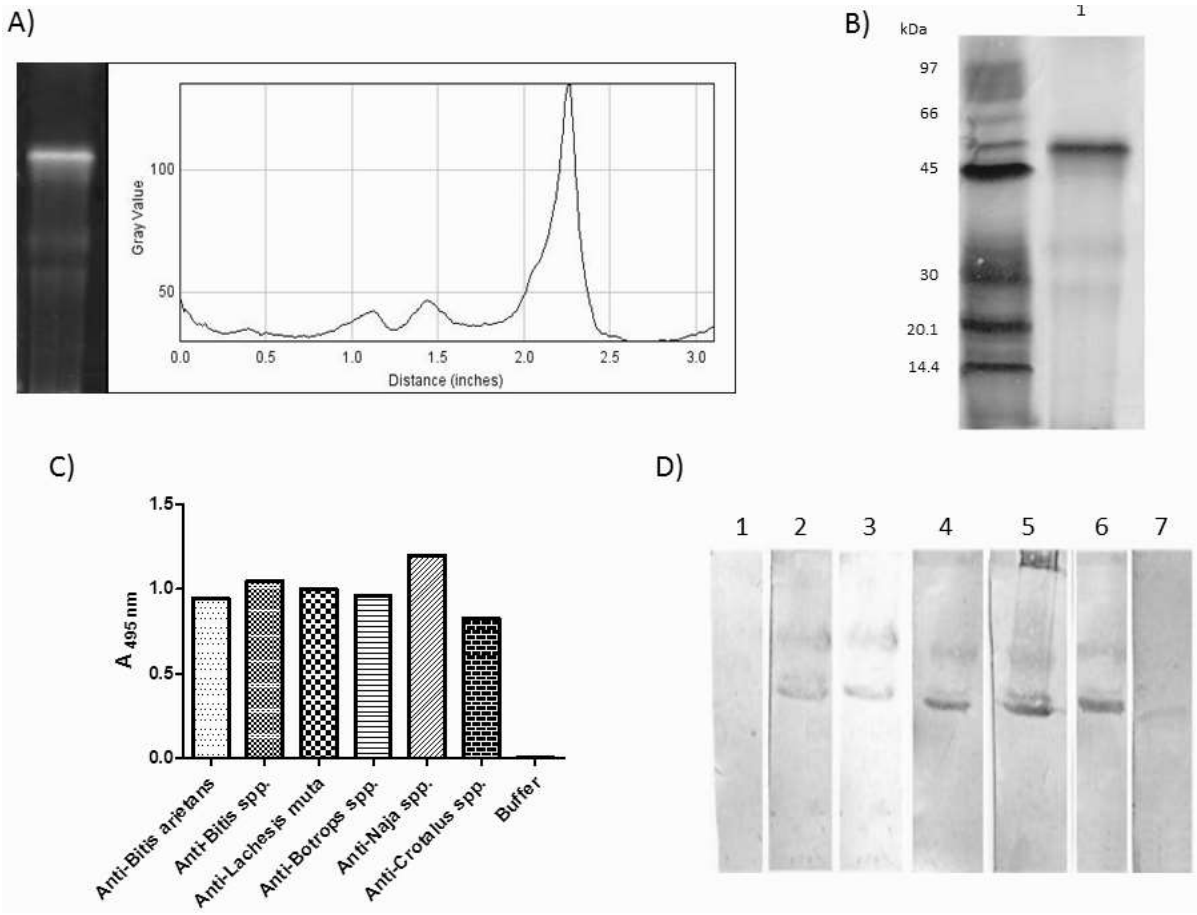

Figure 3. Characterization of the main *B. arietans* venom protein P3´ obtained from Sephacryl S 100 HR (2.5 X 67 cm) chromatography. (**A)** Polyacrilamide-gel electrophoresis (12%) of the *B. arietans* on Superose 12 HR column (10/30, ÄKTA FPLC, Pharmacia, Uppsala, Sweden), eluted protein P'3. A 50kDa band protein present in whole venom is prominent in isolated P'3. (**B)** Upon densitometry analysis the P'3 was also prominent but exhibiting smaller molecular protein contaminations. **(C)** P3´protein was recognized by antibodies present in different polyvalent anti-venoms. Samples of chromatographically isolated was plated on ELISA wells. The horse $F(ab)'_2$ polyvalent anti-venoms anti-*B. arietans*, anti-*Bitis* spp, anti-*Lachesis muta*, anti-*B. atrox*, anti-*Bothrops* spp., anti-*Crotalus* spp., and anti-*Naja* ssp, or IgY anti- *B. arietans*, anti-*Bitis* spp. and anti-*Naja* spp. were used as first antibodies. (**D)** Western blotting analysis of P3. The eluted protein peaks were submitted to polyacrylamide-gel electrophoresis (15%), electrotransferred to nitrocellulose membrane, the protein bands recognized by polyvalent horse anti-venoms and stained. 1, control; 2, anti-*Bitis arietans*; 3, anti-*Bitis* spp.; 4, anti-*Bothrops* spp.; 5, anti-*Lachess muta*; 6, anti-*Naja* spp.; 7, anti-*Crotalus* spp.

=15.6%; $A1_3$ = 18.4%; $A1_5$ = 30.3%; NCs = 0.3% (Figure 4C).

Inhibition of the isolated hemorrhagic activity by polyvalent anti-venoms

Antibodies present in horse polyclonal $F(ab')_2$ or in chicken IgY anti-*Bitis arietans*, anti-*Bitis* spp., anti-*Naja* spp., anti-*Lachesis muta*, anti-*Bothrops* spp., and anti-*Crotalus* spp. and IgY anti-*B. arietans*, anti-*Bitis* spp., and anti-*Naja* spp. were able to inhibit the *B. arietans* inducing-hemorrhagic activity present either in whole venom or in the isolated P3´ fraction (Figure 5).

DISCUSSION

The use of purified relevant snake venom toxins to immunize animals instead of crude venoms avoids anti-venom contamination with non-relevant antibodies directed to non-toxic venom components. Selecting the relevant toxins to be included in the immunogenic mixture is the first step in constructing effective mono-specific anti-venoms.

In this study, we focused on *B. arietans*; its large distribution in densely populated savannah areas in sub-Saharan Africa and in the Arabian Peninsula (Navy, 1991), associated with severity of its bite, justified its selection. Among their toxic components, we examined

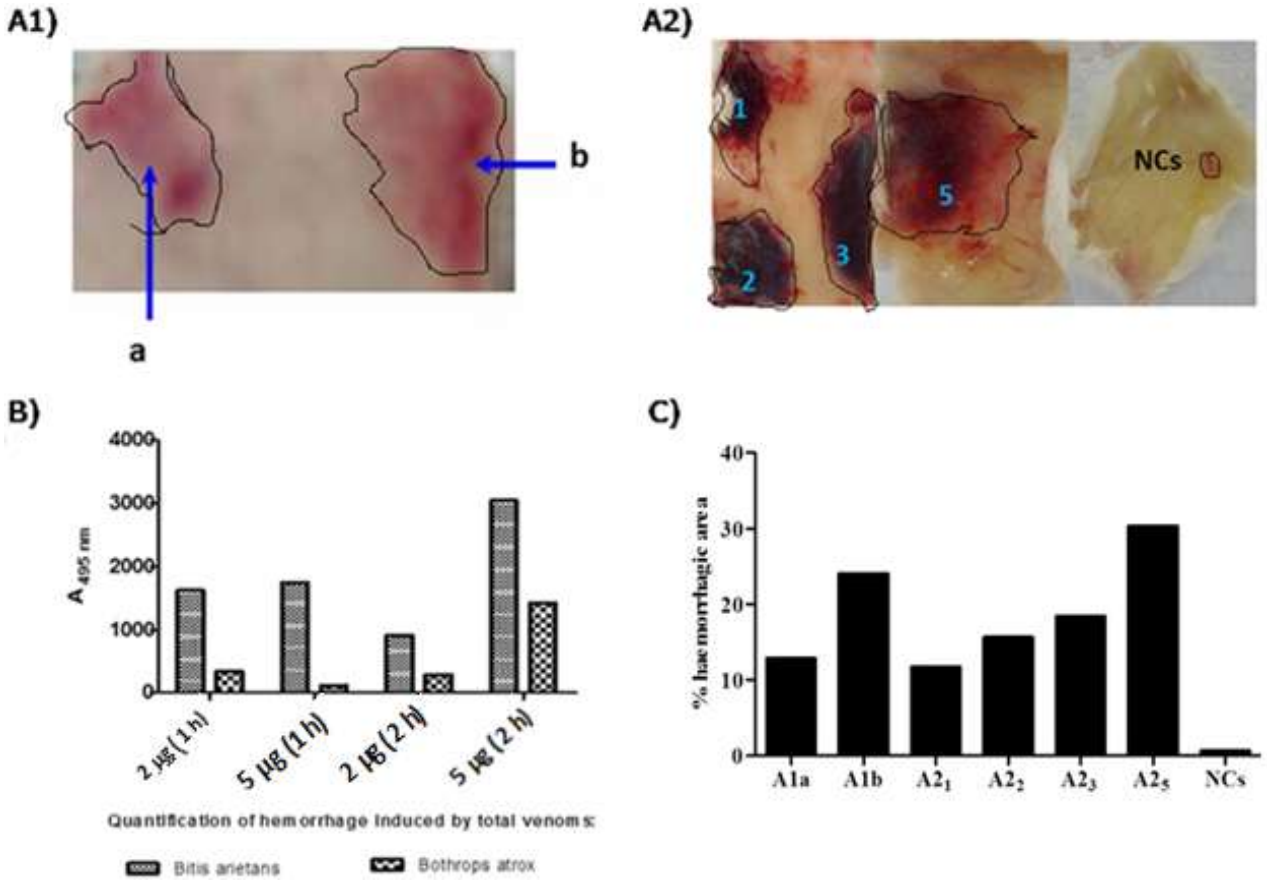

Figure 4. *B. arietans* venom hemorrhagic activity. Groups of mice were subcutaneously injected with different amounts of crude venom, with chromatograph isolated protein peaks P´1 P´2, P´3, P´5 or with 01M NaCl (NCs) as negative control. In some experiments, *B. jararaca* venom was included as positive control. Two hours later the mice were anesthetized under CO_2 atmosphere, submitted to total bleeding, the skin dissected, and the hemorrhagic areas were exposed in the internal skin surface. The hemorrhagic areas (4A1 and 4A2, and negative control NCs), were evaluated with a "SCANNER" program in Image J (Schneider, 2012), the images were plotted on a square millimeter chart, the plots corresponding to each hemorrhagic area were measured, the hemorrhagic square area was calculated in function of the square millimeter chart and referred as square percentages (**C)**. The tissue areas of each hemorrhagic area was removed, the hemoglobin was extracted and spectrophotometrically evaluated at 491 nm. (**A)** Skin fragments of hemorrhagic areas resulting from subcutaneous injections with 2 µg (A1a) or 5 µg (A1b) of *B. arietans* crude venom or with Sephacryl S 100 HR chromatography protein peaks P´1, P´ 2, ´P´3 and P´ 5 ($A2_1$, $A2_2$, $A2_3$, $A2_5$) or with 0.15 M NaCl solution (NCs**)**. (**B)** Local tissue deposited hemoglobin. (**C)** Square percentages of hemorrhagic areas: A1a = 12.8%; A1b =24.0%; $A2_1$ =11.7%; $A2_2$ =15.6%; $A1_3$ = 18.4%; $A1_5$ = 30.3%; NCs = 0.3%.

the hemorrhage-inducing factors contained in *B. arietans* venom. Data from past and recent publications have indicated that the M12b subfamily of zinc-dependent reprolysins is causative agents of local tissue lesions and systemic symptoms in *Bitis* spp. accidents (Bode et al.1993; Stokër et al., 1995; Gutiérrez and Rucovaldo, 2000). SVMPs is remarkable present in *Elapidae* and *Colubridae* venoms (Junqueira-de-Azevedo and Ho, 2002; Guo et al. 2007).

Among the *B. arietans* venom isolated fractions P´1, P´2, P´3, P´4 and P´5, P´3 were identified as having 50-kDa, P´1, P´2 and P´4 and P´5 were admittedly having lesser or higher molecular mass, respectively (Figure 2); as it was expected that SVMPs include 20-30 kDa and over 50 kDa enzymes could all mimicked venom hemorrhagic activities. In fact, the isolated *B. arietans* venom protein fractions, P'1, P'2, P'3 and P'5 exhibited, with different degree, hemorrhagic activity (Figure 4). Considering the relative molecular mass of 50 kDa for P'3, 20-30kDa for P'1 and P'2, over 50kDa for P'5, all described SVMPs were partially purified. P'3 protein fraction may exhibits the typical SVMP disintegrin domain in the presence of an MGD sequence replacing the RGD sequence found in many disintegrins (Nikai et al., 2000). The immunochemical properties of this protein were evaluated *in vitro* by standard ELISA and Western blotting methods, and the ability of this protein to induce hemorrhage was analyzed *in vivo* in mouse skin. As

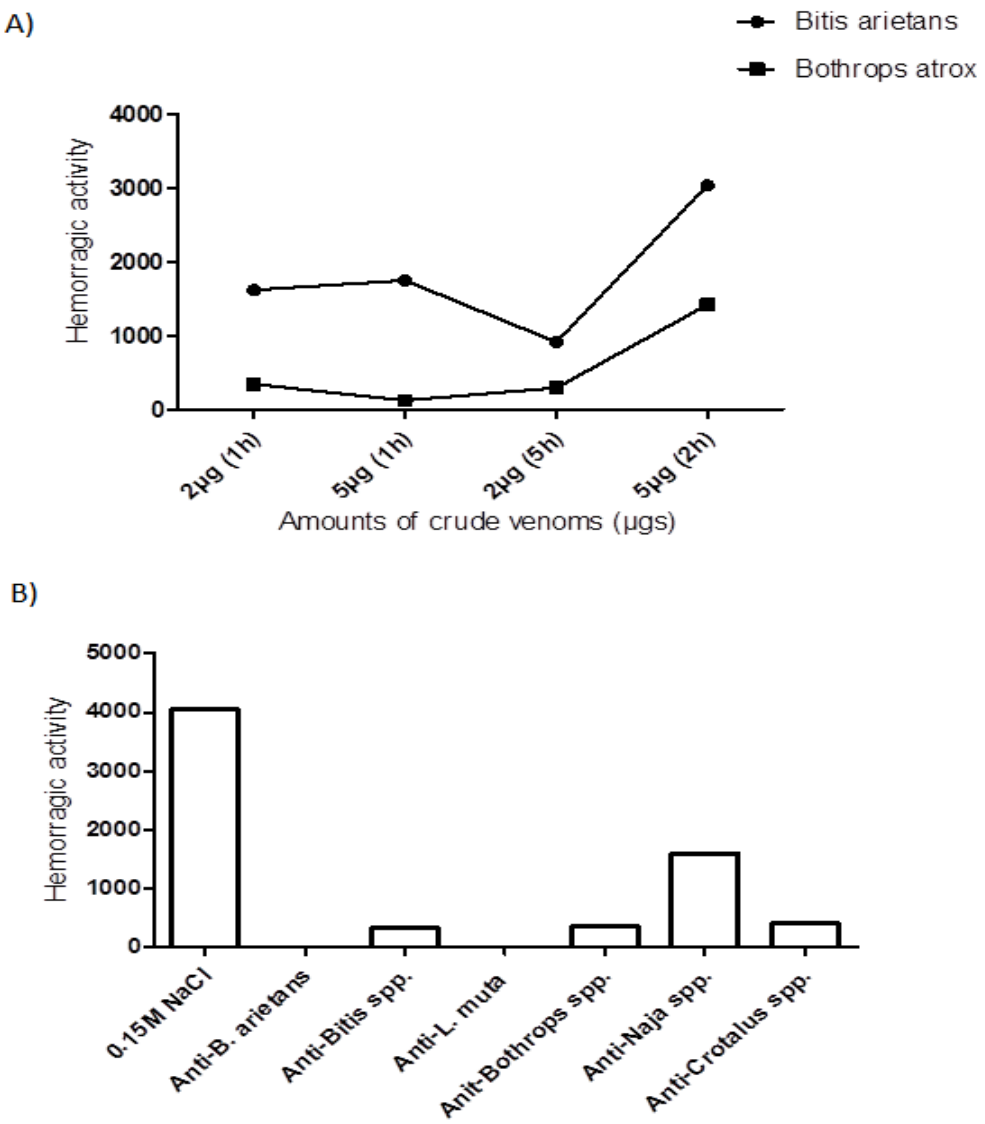

Figure 5. Antibodies developed by different crude snake venoms inhibit the hemorrhagic activities present in *B. arietans* whole venom or in their isolated fraction P3´. Mice (n=20) were subcutaneously injected with 100 µL of 0.15 M NaCl containing 20 µg of P3` eluted from on Sephacryl S 100 HR (2.5 X 67 cm) column pretreated at 37°C for 2 h with Horse $F(ab´)_2$ anti-*B. arietans*, anti-*Bitis*, anti-*Lachesis*, anti-*B.atrox,* anti-*Bothrops* anti-venoms from "Instituto Butantan" or IgY anti-*B. arietans*, anti-*Bitis* spp*., and* anti-*Naja* spp. produced in hens. The hemorrhagic areas were measured, the hemoglobin extracted and spectrophotometrically quantified at 410 nm.

expected from published data, *B. arietans* venom protein contains antigenic epitopes recognized by antibodies generated by non-Viperidae snake venoms (Junqueira-de-Azevedo and Ho, 2002; Guo et al., 2007; Ching et al., 2006). In fact, in this study, we experimentally demonstrated that the isolated 50-kDa protein was recognized by antibodies present in horse polyclonal $F(ab´)_2$ or in chicken IgY anti-*Bitis arietans*, anti-*Bitis* spp., anti-*Naja* spp., anti-*Lachesis muta*, anti-*Bothrops* spp., and anti-*Crotalus* spp. and IgY anti-*B. arietans*, anti-*Bitis* spp., and anti-*Naja* spp. (Figure 3). These antibodies were also able to inhibit the *B. arietans* hemorrhagic activity present in either *B. arietans* venom or the isolated P3´ fraction (Figure 5).

Although not tested, the 50-kDa purified protein may exhibit some biological activities similar to SVMPs, for example, fibrinolytic activity (Retzios and Markland Jr, 1998), non-hemorrhagic fibrinolytic protease activity (Willis and Tu, 1988; Kini, 2005; Kornalik and Blombäk, 1975; Yamada et al., 1996; Siigur et al., 2004; Tans and

Rosing, 2002; Brenes et al., 2010; Trumal et al., 2005; Han et al., 2010), platelet aggregation inhibition (Kamiguti et al., 1996; Laing and Moura-da_Silva, 2005; Wang et al., 2005; Zhou et al., 1996), pro-inflammatory action (Gutiérrez et al., 2005; Moura-da-Siva et al., 2007; Rucavado et al.,1995), and blood serine protease inhibitor inactivation (Kress, 1986; Krees and Catanese, 1980; Kress and Hufnagel, 1984).

The purified 50-kDa protein fraction can therefore be included in immunogenic mixtures intended to induce mono-specific anti-*Bitis* spp. venoms. This mixture has been enriched by the inclusion of purified *B. arietans* blood pressure-hypotensive factor (Kodama et al., 2015).

The immunogenic mixture will be submitted to a pre-clinical test in mice and in laying hens. Specifically, two H-2 distinct mice inbreed strains, C57BL/6 (k) and BALB/c (b), and white leghorn lineage hens (*Gallus gallus domesticus*) will be used as test animals. The immunogenic mixture containing the purified 50-kDa hemorrhagic fraction and blood pressure-hypotensive factor in various proportions will be subcutaneously (in mice) or intramuscularly (in hens) injected four times at 15-day intervals. In the primary immunization, the immunogenic mixture will be adsorbed in mesoporous silica SBA-15 as an adjuvant (Carvalho et al., 2010) prior to injection. In the three subsequent boosters, the immunizations will contain venom components without adjuvants. Blood samples will be collected before each immunization, and the sera will be used to evaluate the antibody titers and their ability to neutralize *B. arietans* venom hemorrhage, blood pressure hypotensive and lethality-inducing activities. The obtained antibodies will be evaluated in parallel assays with horse anti- *B. arietans* or *B. g. rhinoceros* plus *B. nasicornis* venoms (Guidolin et al., 2012). These anti-venoms inhibit *Bitis* spp. toxic activities (Paixão et al., 2015).

The antibody potencies for neutralizing lethality (ED_{50}), skin hemorrhage (EH_{50}), complement-inactivation (ECa_{50}), and blood-pressure hypotensive-inducing activities (EBh_{50}) will be evaluated as recommended by WHO (2010). Antibodies endowed with high specific and affinity qualities may be the first step in producing engineered mini-anti-venom antibodies as anti-snake venoms (Holliger and Hudson, 2005).

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

Tahís Louvain de Souza carried out the experiments; Fábio C. Magnoli purified the metalloproteinases; Wilmar Dias da Silva analyzed the results and wrote the paper.

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