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Counteraction of *Bothrops* snake venoms by *Combretum leprosum* root extract and arjunolic acid

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ABSTRACT

Ethnopharmacological relevance: Serotherapy against snakebite is often unavailable in some regions over Brazil, where people make use of plants from folk medicine to deal with ophidic accidents. About 10% of *Combretum* species have some ethnopharmacological use, including treatment of snakebites.
Materials and methods: We evaluated the ability of the extract of *Combretum leprosum* and its component arjunolic acid to reduce some *in vivo* and *in vitro* effects of *Bothrops jararacussu* and *Bothrops jararaca* venoms. The protocols investigated include phospholipase, proteolytic, collagenase, hyaluronidase, procoagulant, hemorrhagic, edematogenic, myotoxic and lethal activities induced by these venoms in Swiss mice.
Results: Oral pre-treatment with arjunolic acid reduced the *Bothrops jararacussu* lethality in up to 75%, while preincubation prevented the death of all the animals. Hemoconcentration effect of *Bothrops jararacussu* venom was confirmed two hours after i.p. injection, while preincubation with arjunolic acid preserved the hematocrit levels. Both *Combretum leprosum* extract and arjunolic acid abolished the myotoxic action of *Bothrops jararacussu* venom. Preincubation of *Bothrops jararacussu* venom with the extract or arjunolic acid prevented the increase of plasma creatine kinase activity in mice. The hemorrhagic activity of *Bothrops jararaca* crude venom was reduced down to about 90% and completely inhibited by preincubation with 10 mg/kg or 100 mg/kg *Combretum leprosum* extract, respectively, while the preincubation and the pretreatment with 30 mg/kg of arjunolic acid reduced the venom hemorrhagic activity down to about 12% and 58%, respectively. The preincubation of the venom with both extract and 30 mg/kg arjunolic acid significantly reduced the bleeding amount induced by *Bothrops jararacussu* venom. The extract of *Combretum leprosum* decreased the edema formation induced by *Bothrops jararacussu* venom both in preincubation and pretreatment, but not in posttreatment. Similarly, arjunolic acid preincubated with the venom abolished edema formation, while pre- and posttreatment have been partially effective. Some enzymatic activities of *Bothrops jararacussu* and *Bothrops jararaca* venoms, *i.e.* phospholipase A_2, collagenase, proteolytic and hyaluronidase activities, were to some extent inhibited by the extract and arjunolic acid in a concentration-dependent manner.
Conclusions: Altogether, our results show that *Combretum leprosum* extract can inhibit different activities of two important Brazilian snake venoms, giving support for its popular use in folk medicine in the management of venomous snakebites.

1. Introduction

Medicinal plants have long been used in the treatment of snakebite, mainly in locations where it is difficult to obtain the specific antivenom. In some countries, knowledge and access to medicinal plant therapy is traditional, and such therapy can be important for many reasons. One is the lack of specific antivenom,

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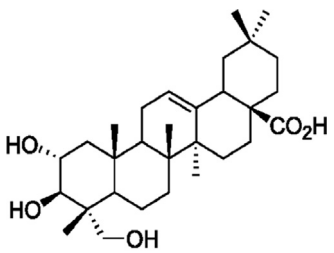
or because of the time needed to travel to a treatment location. Sometimes, therapy with specific antivenom may be partly or wholly ineffective (Mors et al., 1989, 2000a; Chippaux et al., 1991; Da Silva et al., 2007). These natural sources in folk medicine are important in many countries where snakebite treatment is a public-health problem, and its treatment is a challenge for these communities (Watt, 1989; Chippaux and Goyffon, 1997; Mors et al., 2000b; Gutiérrez et al., 2013a).

Although many species of plants are popularly known to be antiophidic agents, only a few have been investigated and had their active components isolated or characterized (Melo et al., 1994; Mors et al., 2000a; Coe and Anderson, 2005; Veronese et al., 2005; Strauch et al., 2013). Plants are important sources of bioactive components such as flavonoids, coumestans and triterpenes, which can help in the treatment of accidents with venomous animals (Mors et al., 1989, 2000a). Generally, substances of medical interest are found in very small quantities in the plants and are affected by many factors such as season of the year, time of collection, growth period, climate, soil composition, part of the plant from which the active component is extracted, or the batch collected (Havsteen, 1983; Mors et al., 2000a, 2000b).

In Brazil, the majority of accidents with venomous snakes are due to *Bothrops* sp. envenomation, which causes mainly local tissue damage, hemorrhage, edema and myonecrosis. Administration of the specific antivenom may prevent death but does not prevent local tissue damage and resultant disabilities (Da Silva et al., 2007). Antiophidic plants have been a target of previous studies from our group, such as *Eclipta prostrata*, which showed strong effects against venoms of snakes of the genera *Bothrops*, *Lachesis*, and *Crotalus* or some of their purified toxins, such as bothropstoxin, bothropasin, and crotoxin (Mors et al., 1989; Melo et al., 1994). The crude extract, as well as the isolated components wedelolactone, sitosterol, and stigmasterol, have shown intense antimyotoxic, antihemorrhagic, and antilethal activities against some snake venoms and isolated toxins (Mors et al., 1989; Melo et al., 1994; Melo and Ownby, 1999). Studies with *Casearia sylvestris*, *Harpalyce brasiliana* and *Humiriantherа ampla* have also demonstrated that these plants inhibited the myotoxic, edematogenic, and anticoagulant activities of several *Bothrops* venoms and isolated toxins (Borges et al., 2001; Da Silva et al., 2004; Strauch et al., 2013).

We have noticed that the plants from *Combretum* genus have been used as antivenom in folk medicine in Brazil and other regions of the world, especially in Africa. This plant is a member of the family Combretaceae, constituted by *circa* 600 species in 18 genera, of which *Teminalia* and *Combretum* are the most important. Worldwide, species of *Combretum* are popularly used against several diseases, most of the times used as infusions or decoctions of the leafs, flowers or roots (Facundo et al., 1993, 2005; Agra, 1996; Mors et al., 2000b; McGaw et al., 2001) including snakebites (Hutchings et al., 1996; Van Wyk et al., 1997). *Combretum leprosum* is found in Northeast Brazil, growing mainly along riverbanks, where it is called “mofumbo”, “mufumbo” or “pente de macaco” (Pio Corrêa, 1984). The phytochemical analysis of *Combretum leprosum* extract showed the presence of monosaccharides as the major compounds (80%), followed by triterpenes (10%) such as arjunolic acid (Fig. 1), and yet oligosaccharides (5%) and fatty acids (3%) (Facundo et al., 1993, 2005).



Arjunolic Acid

Fig. 1. The chemical structure of the pentacyclic triterpene arjunolic acid isolated from *Combretum leprosum*.

In the present study, the *Combretum leprosum* extract and the isolated triterpene arjunolic acid were investigated under different experimental protocols *in vivo* and *in vitro* against some important activities of *Bothrops jararacussu* and *Bothrops jararaca* venoms.

2. Material and methods

Bothrops jararaca and *Bothrops jararacussu* venoms and the antibothropic polyvalent antivenom (PAV) were obtained from Instituto Vital Brazil, Niterói, RJ. Bothropstoxin II was obtained from Faculdade de Ciências Farmacêuticas/USP, Ribeirão Preto-SP. Creatine kinase (CK) activity was determined using a CK NAC® kit from BIOCLIN®. Azocasein®, azocoll®, and hyaluronic acid were purchased from Sigma Chemical Co., USA. Male Swiss mice were provided by the Rodent Vivarium of the Institute of Microbiology Paulo de Góes – Federal University of Rio de Janeiro. Mice (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).

2.1. Plant material, extraction and isolation

Botanical material (*Combretum leprosum*) was collected in May 2001 in Viçosa, State of Ceará, Brazil. The material was identified at the Prisco Bezerra herbarium of the Biology Department, Federal University of Ceará, and a voucher specimen was deposited under number 12446.

Ethanolic extract was obtained from the roots (2.7 kg), which were placed in contact with ethanol 98% (5 L) at room temperature for 72 h. The material was filtered and the solvent evaporated under reduced pressure, yielding 58.3 g of extract. The isolation of arjunolic acid was performed as previously described (Facundo et al., 1993). Part of the extract (34.0 g) was submitted to coarse chromatography over silica gel using as eluents hexane, clorofórmium, ethyl acetate, and methanol. In the fraction eluted with ethyl acetate the presence of a white precipitate was observed, which, through recrystallization in methanol, was identified as the triterpene arjunolic acid. Spectral data, mainly mass spectrometry, ^{1}H and ^{13}C NMR were in agreement with literature values (Facundo et al., 1993).

2.2. Myotoxicity in vivo and in vitro

We evaluated the *in vivo* myotoxicity of *Bothrops jararacussu* venom by measuring the increase of plasma CK activity induced by perimuscular injection of venom alone or associated with *Combretum leprosum* extract or arjunolic acid. The venom was dissolved in physiological saline solution (PSS) to a final volume of 0.1 mL (1.0 mg/kg) and injected next to the *extensor digitorum longus* (EDL) muscles, in order not to cause direct mechanical damage to the muscle, as previously described (Calil-Elias et al., 2002b). Negative controls consisted of mice injected with the same volume of PSS. To evaluate the antimyotoxic activity of the *Combretum leprosum* extract (250 mg/kg) and arjunolic acid (70 mg/kg), three different protocols were used, as follows. Pre-incubation: venom dissolved in PSS was first incubated with the extract or arjunolic acid for 30 min at room temperature prior to injection; pretreatment: 0.15 mL of the extract or arjunolic acid was injected intraperitoneously (i.p.) 15 min before the i.m. venom injection; post-treatment: 0.15 mL of the extract or arjunolic acid