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Rosmarinic acid, a new snake venom phospholipase A_2 inhibitor from *Cordia verbenacea* (Boraginaceae): antiserum action potentiation and molecular interaction

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Abstract

Many plants are used in traditional medicine as active agents against various effects induced by snakebite. The methanolic extract from *Cordia verbenacea* (Cv) significantly inhibited paw edema induced by *Bothrops jararacussu* snake venom and by its main basic phospholipase A_2 homologs, namely bothropstoxins I and II (BthTXs). The active component was isolated by chromatography on Sephadex LH-20 and by RP-HPLC on a C18 column and identified as rosmarinic acid (Cv-RA). Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid [2-*O*-cafeoil-3-(3,4-di-hydroxy-phenyl)-*R*-lactic acid]. This is the first report of RA in the species *C. verbenacea* ('baleeira', 'whaler') and of its anti-inflammatory and antimyotoxic properties against snake venoms and isolated toxins. RA inhibited the edema and myotoxic activity induced by the basic PLA_2s BthTX-I and BthTX-II. It was, however, less efficient to inhibit the PLA_2 activity of BthTX-II and, still less, the PLA_2 and edema-inducing activities of the acidic isoform BthA-I-PLA_2 from the same venom, showing therefore a higher inhibitory activity upon basic PLA_2s. RA also inhibited most of the myotoxic and partially the edema-inducing effects of both basic PLA_2s, thus reinforcing the idea of dissociation between the catalytic and pharmacological domains. The pure compound potentiated the ability of the commercial equine polyvalent antivenom in neutralizing lethal and myotoxic effects of the crude venom and of isolated PLA_2s in

Abbreviations Cv-ME, *Cordia verbenacea* methanolic extract; Cv-RA, rosmarinic acid from *Cordia verbenacea*; PLA_2, phospholipase A_2; PLIs, phospholipase A_2 inhibitors; BthTX-I, *B. jararacussu* bothropstoxin-I; BthTX-II, *B. jararacussu* bothropstoxin-II; BthA-I-PLA_2, *B. jararacussu* acidic phospholipase A_2; COSY, COrrelation SpectroscopY; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; CD, circular dichroism.

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experimental models. CD data presented here suggest that, after binding, no significant conformation changes occur either in the Cv-RA or in the target PLA_2. A possible model for the interaction of rosmarinic acid with Lys49-PLA_2 BthTX-I is proposed.

Keywords: *Cordia verbenacea*; Rosmarinic acid; Anti-inflammatory; Antimyotoxic; Antiophidian; Phospholipase A_2 inhibitor; *Bothrops jararacussu*; Snake venom

1. Introduction

Plants have often been used by humans, sometimes successfully, against numerous diseases caused by different pathological agents. Pharmacological studies have demonstrated that the extracts and fractions from some of these plants used in traditional medicine possess anti-inflammatory, antiviral and antiophidian properties (Phillipson and Anderson, 1989; Martz, 1992; Mors et al., 2000). The antiophidian activity of several plant species in general use in some Brazilian communities has been investigated scientifically (Mors et al., 2000; Batina et al., 2000; Borges et al., 2000, 2001; Biondo et al., 2003, 2004; Januário et al., 2004; Veronese et al., 2005; Esmeraldino and Sampaio, in press; da Silva et al., in press; Oliveira et al., 2005).

Snake venoms are complex mixtures of proteins including phospholipases A_2, myotoxins, hemorrhagic metalloproteases and other proteolytic enzymes, cytotoxins, cardiotoxins and others. The pathophysiology of snake envenomation involves a complex series of events that depend on the combined action of these venom components (Gutiérrez, 2002). Phospholipases A_2 (PLA_2; EC 3.1.1.4) are abundant in snake venoms. Besides playing a digestive role in phospholipid hydrolysis, they may also exert a wide variety of pharmacological activities such as neurotoxicity, myotoxicity, edema-inducing activity and others (Gutiérrez and Lomonte, 1995; Soares et al., 2004b). Local edema, a typical manifestation of *Bothrops* envenomation, usually in addition to pain, is due to the action of the venom upon mastocytes, kininogens and phospholipids, culminating with release of endogenous mediators (Teixeira et al., 2003).

The hydroalcoholic extract from *Cordia verbenacea* ('baleeira', 'whaler') has been used by Brazilian folk as cicatrizant and anti-inflammatory (Sértie et al., 1988). We report now, for the first time, the anti-inflammatory and antimyotoxic activity of the extract from *C. verbenacea* and its active principle, rosmarinic acid, against these effects induced by *Bothrops jararacussu* snake venom and by its main isolated phospholipases A_2. A possible model for the interaction of rosmarinic acid with Lys49-PLA_2 BthTX-I is proposed.

2. Material and methods

2.1. Materials

The leaves from *C. verbenacea* were collected during the blooming period in the Campus of the University of Ribeirão Preto (UNAERP). A voucher specimen (No. 259) identified by specialist Prof. Dr Lin Chau Ming (Departamento de Botânica, UNESP, Botucatu, SP, Brazil) has been preserved in the Unidade de Biotecnologia Herbarium, UNAERP. *B. jararacussu* venom was purchased from Sandrin Bioagents serpentarium, Batatais, SP. *B. jararacussu* PLA_2s were isolated on Sephadex G-75 followed by cation-exchange chromatography as previously described (Andrião-Escarso et al., 2000, 2002). PLA_2 homogeneity was assessed by native and SDS-PAGE and reverse-phase HPLC.

2.2. Preparation of plant extract

After identification, the leaves were dried in a stove with circulating air at 40 °C. They were then grounded (375 g) and macerated with chloroform three times during three days, then with methanol, followed by filtration and evaporation of the methanol in a rotary evaporator wherefrom the dried methanolic extract (Cv-ME) was obtained.

2.3. Purification and identification of rosmarinic acid

A preliminary Sephadex LH-20 column was used for the first fractionation of Cv-ME, using 300 mL of methanol as mobile phase for elution. The resulting fractions, after drying, were analyzed by thin-layer chromatography and revealed with vanillin sulfuric acid reagent. Fraction 3 was then applied on a HPLC semipreparative Supelcosil C18 column, using a concentration gradient of methanol:water at a flow rate of 2 mL/min. Seven new fractions were so obtained, which were assayed for edema inhibition, from which rosmarinic acid (Cv-RA) was fraction 6, as identified by NMR analysis. NMR spectra were recorded with a Brucker DPX-300 spectrophotometer, operating at 300 mHz for ^{1}H and 75 mHz for ^{13}C. For that, 15 mg samples were used, dissolved in dimethyl-d_6-sulfoxide (Aldrich).

2.4. Edema-inducing activity

Edema was induced by i.d. injection, in the right foot pad of male Swiss mice (18–22 g), of *B. jararacussu* venom (25 μg) and its purified PLA_2s (50 μg). Inhibition studies were performed by incubating venom or PLA_2 with Cv-ME/Cv-RA. Control groups were injected with 50 μL of phosphate-buffered saline (PBS, pH 7.2) alone, or Cv-ME/Cv-RA alone. The progression of edema was evaluated with a low pressure pachymeter (Mitutoyo,