Journal of Ethnopharmacology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

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

Inhibition of venom serine proteinase and metalloproteinase activities by *Renealmia alpinia* (*Zingiberaceae*) extracts: Comparison of wild and *in vitro* propagated plants

Arley Camilo Patiño*, Dora María Benjumea, Jaime Andrés Pereañez

[a] *Programa de Ofidismo/Escorpionismo, Departamento de Farmacia, Facultad de Química Farmacéutica, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia*

ARTICLE INFO

Article history:
Received 25 May 2013
Received in revised form
23 July 2013
Accepted 28 July 2013

Keywords:
Renealmia alpinia
Snake venoms
Metalloproteinase
Serine proteinase
Anti-venom activity
Micropropagated plants

ABSTRACT

Ethnopharmacological relevance: The plant *Renealmia alpinia* has been used in folk medicine to treat snakebites in the northwest region of Colombia. In addition, it has been shown to neutralize edema-forming, hemorrhagic, lethal, and defibrin(ogen)ating activities of *Bothrops asper* venom. In this work, extracts of *Renealmia alpinia* obtained by micropropagation (*in vitro*) and from specimens collected in the wild were tested and compared in their capacity to inhibit enzymatic and toxic activities of a snake venom metalloproteinase isolated from *Bothrops atrox* (Batx-I) venom and a serine proteinase (Cdc SII) from *Crotalus durissus cumanensis* venom.

Materials and methods: We have investigated the inhibition capacity of *Renealmia alpinia* extracts on enzymatic and toxic actions of isolated toxins, a metalloproteinase and a serine proteinase. The protocols investigated included inhibition of proteolytic activity on azocasein, inhibition of proteolytic activity on fibrinogen, inhibition of pro-coagulant activity, inhibition of hemorrhagic activity and inhibition of edema-forming activity.

Results: Colorimetric assays detected the presence of terpenoids, flavonoids, tannins and coumarins in *Renealmia alpinia* extracts. *Renealmia alpinia* extracts inhibited the enzymatic, hemorrhagic and fibrinogenolytic activities of Batx-I. Extracts also inhibited coagulant, defibrin(ogen)ating and edema-forming activities of Cdc SII. Results highlight that *Renealmia alpinia in vitro* extract displayed comparable inhibitory capacity on venom proteinases that *Renealmia alpinia* wild extract. No alteration was observed in the electrophoretic pattern of venom proteinases after incubation with *Renealmia alpinia* extracts, thus excluding proteolytic degradation or protein denaturation/precipitation as a mechanism of inhibition.

Conclusions: Our results showed that *Renealmia alpinia* wild and *in vitro* extracts contain compounds that neutralize metallo- and serine proteinases present in snake venoms. The mechanism of inhibition is not related to proteolytic degradation of the enzymes nor protein aggregation, but is likely to depend on molecular interactions of secondary metabolites in the plant with these venom proteinases.

1. Introduction

Envenomation by snakebites is a relevant public health issue in many regions of the world, particularly in tropical and subtropical countries of Africa, Asia, Latin America and Oceania (Gutiérrez et al., 2010). In the case of snakes of the family *Viperidae*, which inflict the vast majority of accidents in the Americas, the pathophysiology of envenomation includes both local and systemic manifestations associated with hemorrhage, necrosis, edema, hypovolemia, nephrotoxicity, coagulopathy and cardiovascular shock (Warrell, 2004). This complex clinical picture is the result of the action of various venom components, predominantly proteinases, both metallo- and serine proteinases, phospholipases A_2, C-type lectin-like proteins, and other minor components (Calvete, 2011).

Snake venom metalloproteinases (SVMPs) are abundant in viperid snake venoms (Calvete, 2011), and belong to the reprolysin subgroup of metalloproteinases (Fox and Serrano, 2005). SVMPs participate in the hemorrhagic process by proteolytic degradation of extracellular matrix components of the basement membrane of the microvasculature involved in the maintenance of capillary structure and integrity, leading to disruption of capillary networks, edema and hemorrhage (Gutiérrez et al., 2005; Escalante et al., 2006; Fox and Serrano, 2005). SVMPs classification is based on their different domain constitution as follows: P-I (SVMPs comprised by single metalloproteinase domain), P-IIa to P-IIe (containing metalloproteinase and disintegrin domains),

* Corresponding author. Tel.: +57 4 2196536; fax: +57 4 2631914.
E-mail addresses: arleycamilo@gmail.com (A. Camilo Patiño), doraben2000@gmail.com (D. María Benjumea), andrespj20@gmail.com (J. Andrés Pereañez).

0378-8741/$ - see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.jep.2013.07.033

P-IIIa to P-IIIc (containing metalloproteinase, disintegrin-like and cysteine-rich domains), and P-IIId, formerly known as P-IV (containing the P-III structure and two C-type lectin-like domains connected by disulfide bonds to the cysteine-rich domain) (Fox and Serrano, 2008).

On the other hand, many serine proteinases have been isolated and characterized from snake venoms (Serrano and Maroun, 2005). Despite sharing similar structural features, venom serine proteinases display a highly diverse pharmacological profile. This includes actions on proteins of the coagulation cascade, such as thrombin-like activity on fibrinogen, activation of factor *V*, activation of protein C, fibrinogenolysis, activation of plasminogen, and induction of platelet aggregation (Markland, 1998; Serrano and Maroun, 2005). In addition, some viperid venom serine proteinases exert kallikrein-like activity, releasing bradykinin or Lys-bradykinin, and, therefore, promoting an increase in vascular permeability and hypotension (Serrano and Maroun, 2005). Most of these activities are associated with systemic disturbances occurring in viperid snakebite envenomings.

The therapy for snakebite envenomations has been based on the intravenous administration of antivenoms (Bon, 1996; Gutiérrez et al., 2011). However, it has been demonstrated that antivenoms have a limited efficacy against the local tissue damaging activities of venoms (Gutiérrez et al., 1998, 2007). Thus, it is important to search for alternative sources of venom inhibitors, either synthetic or natural, that would complement the action of antivenoms, particularly regarding neutralization of local tissue damage.

Medicinal plants represent a vital source of novel bioactive compounds with several pharmacological activities, and constitute possible alternatives for inhibiting venom components which, eventually, might complement the therapeutic action of conventional antivenom therapy (Soares et al., 2005). *Renealmia alpinia* (Rottb.) Maas (*Zingiberaceae*) is known as guaiporé, pintura negra, jazmín de monte, matandrea o achira de monte (Villalobos, 1994; Acero, 1979; Standley and Steyermark, 1952), and it has been used to treat snakebites in the northwest region of Colombia (Otero et al., 2000a). In addition, extracts of this plant have been effective to neutralize edema-forming, hemorrhagic, lethal, and defibrinating activities of *Bothrops asper* venom (Otero et al., 2000b, 2000c; Núñez et al., 2004), thus constituting a valuable source of inhibitory substances. In order to increase the productivity and homogeneity of *Renealmia alpinia* extract, our group carried out a study involving the micropropagation of this plant, with the aim of obtaining enough plant material, which would not be possible to achieve with traditional methods of collection (Alarcón et al., 2008). Moreover, extracts from roots and leaves of this plant grown *in vitro* inhibited the proteolytic, coagulant, and indirect-hemolytic activities of *Bothrops asper* venom (Fernández et al., 2010).

In this work, extracts of *Renealmia alpinia* obtained by micropropagation (*in vitro*) and wild forms were tested and compared for their capacity to inhibit enzymatic and toxic activities of a P-I SVMP isolated from *Bothrops atrox* venom and a serine proteinase from *Crotalus durissus cumanensis* venom.

2. Materials and methods

2.1. Toxins, chemicals and reagents

The SVMP Batx-I was isolated from *Bothrops atrox* snake venom as previously described (Patiño et al., 2010). The serine proteinase Cdc SII was isolated from *Crotalus durissus cumanensis* snake venom as previously described (Patiño et al., 2013). All chemicals and reagents used in this work were of analytical grade.

2.2. Animals

Swiss Webster mice (18–20 g body weight) were used for *in vivo* assays. All experiments were conducted in accordance with guidelines of the Universidad de Antioquia Ethics Committee (Protocol number 53, June 16, 2009) (Medellín, Colombia).

2.3. Plant material and preparation of extracts of Renealmia alpinia

The leaves of *Renealmia alpinia* were collected in the wild from plants grown in the campus of the University of Antioquia (1.454 m.a.s.l.; voucher 107316, registration number 6456 RF at the Herbarium of the University of Antioquia (HUA)). On the other hand, samples of *Renealmia alpinia* grown *in vitro* were obtained by the method described by Alarcón et al. (2008). Whole plants were used to prepare the extract. Plant material from *Renealmia alpinia* wild and *in vitro*, respectively, was dried at 37 °C. Then, dried and milled plant material was extracted overnight with 90% ethanol three times. The resultant ethanol extract was concentrated at a temperature below 40 °C to a semisolid paste using a rotary evaporator (BÜCHI–124 Flawil, Switzerland). Finally, extracts were lyophilized and stored at −20 °C until their use. In addition, a preliminary colorimetric phytochemical analysis was carried out on both extracts, according to the methods described by Trease and Evans (2002).

2.4. Inhibition of proteolytic activity

Proteolytic activity was tested on azocasein (Wang et al., 2004). Batx-I (20 μg) dissolved in 10 μL of 25 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.4, was incubated with 100 μL of a 10 mg/mL azocasein solution (Sigma-Aldrich). After incubation at 37 °C for 90 min, the reaction was stopped by the addition of 200 μL of 5% trichloroacetic acid. After centrifugation at 100 × *g*, 100 μL of supernatant was diluted with 100 μL of 0.5 M NaOH, and the absorbance at 450 nm was recorded. The absorbances of samples of azocasein incubated with buffer alone were subtracted from the values of absorbances of samples incubated with Batx-I. For inhibition experiments, several w/w ratios of Batx-I:extract (1:1, 1:2.5, 1:5, 1:10 and 1:20) were incubated with a fixed concentration of Batx-I for 30 min at 37 °C. Then, proteolytic activity was determined as described above. Controls included Batx-I incubated without extracts, extracts incubated without Batx-I, and buffer alone.

2.5. Inhibition of fibrinogenolytic activity

The methodology described by Rodrígues et al. (2000), with some modifications, was used. Samples of 50 μL of bovine fibrinogen (2 mg/mL) were incubated with Batx-I (8 μg) at 37 °C for 2 h. The reaction was stopped by the addition of 25 μL of 0.05 M Tris–HCl buffer, pH 8.8, containing 10% (v/v) glycerol, 10% (v/v) mercaptoethanol, 2% (w/v) SDS, and 0.05% (w/v) bromophenol blue. Samples were then analyzed by 12% (w/v) SDS-PAGE. The effect of *Renealmia alpinia* extracts on the fibrinogenolytic activity was evaluated after preincubation for 30 min at 37 °C of Batx-I (8 μg) with several amounts of the extract, in order to achieve various w/w Batx-I:extract ratios (1:5, 1:10 and 1:20).

2.6. Inhibition of coagulant activity

The method described by Theakston and Reid (1983) was followed. Inhibition assays were carried out by incubating several w/w ratios of Cdc SII (1.0 μg) and the extract (1:1, 1:2.5, 1:5, 1:10 and 1:20) for 30 min at 37 °C. Then, these mixtures were added to 200 μL of human citrated plasma from healthy donors, and the

Please cite this article as: Camilo Patiño, A., et al., Inhibition of venom serine proteinase and metalloproteinase activities by *Renealmia alpinia* (*Zingiberaceae*) extracts: Comparison of.... Journal of Ethnopharmacology (2013), http://dx.doi.org/10.1016/j.jep.2013.07.033