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Effects of an extract of *Cissampelos pareira* on the hemorrhagic and proteolytic activities from *Bothrops asper* venom

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ABSTRACT

The capacity of an aqueous extract from leaves of *Cissampelos pareira* to neutralize the hemorrhagic and proteolytic activities of the venom of *Bothrops asper* was evaluated. To establish the anti-hemorrhagic activity, the skin of mice was injected with a mixture of extract and venom, and it was found that extract produced a total inhibition of this activity. On the other hand, experiments regarding the anti-proteolytic activity were conducted observing the effect on casein in a test tube or on biotinylated casein in a microplate. None of the two procedures was able to show any inhibitory activity.

KEY WORDS: Plant extracts; Snake venom; Hemorrhage; Proteolysis,

INTRODUCTION

Bothros asper is the most important snake in Central America, as it causes the majority of snakebites (1, 2). This snake is widely distributed in Costa Rica (3), and is commonly named as "terciopelo" or "barba amarilla" (4). Its venom induces remarkable local and systemic effects such as swelling, hemorrhage, myonecrosis, clotting disorders and nephrotoxicity (5).

Arroyo, *et al* (6) report a general incidence of 15.6 snakebites per 100.000 inhabitants with a drastically reduced mortality rate (0.03 per 100.000). These authors estimated that 50 % of these envenomations were caused by *B. asper*. Also, Sasa and Vásquez (7) reviewed snakebite records from the Costa Rican health system during the decade of 1990-2000 showing a relatively constant number of 504 cases of snakebites per year, but the annual incidence per year decreased considerably.

Plants are undoubtedly an enormous source of compounds with high pharmaceutical activity potential (8, 9). They have been used in different countries in traditional medicine to treat snakebite (10, 11).

Extracts of *C. pareira* roots showed antiadiarrhoeal activity on experimentally induced diarrhea, endorsing ethnomedical uses (12), and it was obtained protoberberine alkaloids (13). The aerial part of this plant provided cissampeloflavone, a chalcone-flavone dimer (13) and cissampareine, a cytotoxic alkaloid (14).

In this work, the aqueous extract of *C. pareira L.* (Menispermaceae) leaves, which have been employed ethnomedicaly by costarrican healers (15), (i.e as poultice) on wounds induced by snakes, was evaluated for the neutralization of the hemorrhagic and proteolytic activities of this venom. In order to inhibit these activities we used two reliable assays to evaluate the hemorrhage "*in vivo*" and proteolytic "*in vitro*" effects, which have been described in previous experimental designs.

MATERIALS AND METHODS

Plant material and extract preparation

Plants were collected in the dry season (February) in San Luis de Cutris, San Carlos (Alajuela), Costa Rica, according to botanical information given by the ethnobotanic Luis J. Poveda. Leaves were separated, dried for three days at about 40 ºC and then crushed using a Wiley-type grinder. An aqueous extract (10 %) of the plant material was obtained by infusion with distilled water at 37 ºC, filtered and concentrated in a rotary evaporator. Then, it was lyophilized and stored at 4 ºC until used.

Animals

Swiss Webster mice of 20-22 g were kept in standard environmental conditions and fed with rodent diet and

water *ad libitum*. These animals were supplied by the Animal Care Unit of Instituto Clodomiro Picado (University of Costa Rica).

Hemorrhagic activity and neutralization

Hemorrhage was determined in groups of four mice following the Kondo *et al*. (16) technique as modified by Gutiérrez *et al*. (17). Different amounts of plant extract (60, 40, 30, 20, 10, 5 and 2.5 mg) were pre-incubated for 30 min at room temperature with 200 µg of *B. asper* venom, dissolved in 1mL of PBS, pH 7.2. The supernatant [100 µl, corresponding to 10 minimum hemorrhagic doses (MHD) of venom (1 MHD = 2 µg)] was injected in the abdominal skin of the experimental animals. The diameter of hemorrhage area was measured in the skin. Controls of venom (10 MHD =20 µg) and extract were carried out. Anti-hemorrhagic activity was expressed in percentage, using the diameter of venom control, as 100 percent. Neutralizing ability was expressed as the effective dose 50 % (ED_{50}), defined as the ratio mg extract/200 µg venom at which the diameter of hemorrhagic area was reduced by 50 % when compared to the lesions induced by venom control (10 MHD). Hemorrhagic diameter was calculated with the following formula: d= 2 √area /∏.

Inhibition of proteolytic activity

This activity was determined by following two assays: one is the method on 1% w/v casein -in a test tube- of Friedrich and Tu (18), as modified by Lomonte and Gutiérrez (19), and the other is the technique on microtiter plates of Koritsas and Atkinson (20), as modified by Franceschi *et al*. (21), which uses biotinylated N,N-dimethylcasein (Sigma) as substrate.

In the neutralization experiments using casein as substrate, one minimum proteolytic dose of *B. asper* (1.4 mg/ml) was used as challenge dose. Briefly, samples (1mL) containing either venom, extract (40 mg), or mixtures of extract and venom, were placed in 2 mL of casein 1% for 30 min at 37 ºC. Then, 4 mL of trichloroacetic acid 5% was added to each sample, and were centrifuged after 30 min of incubation at room temperature. Absorbances were recorded at 280 nm (Shimadzu UV-60) and results are expressed in absorbance units per milligram of venom (U/mg).

In the Koritsas and Atkinson assay (20), each well of microtiter plate (Immulon II, Dynatech, USA) was coated with 0.2 µg/100 µl of biotinylated N,N-dimethylcasein-prepared in 0.1 M $NaHCO_3$, pH 8.5 buffer- and dissolved in coating buffer (0.1M Tris, 0,15M NaCl, pH 9.0) according with Franceschi *et al*. (21). Previously, mixtures of extract-venom (40 or 2.5 mg/200 µg venom dissolved in PBS, pH 7.2) were incubated for 30 min at room temperature, with constant stirring, and then a sample of 100 µl of the supernatant of these mixtures was placed in each well and incubated 24 hours at 37 ºC. After incubation, plates were washed five times with PBS-Tween 0.05% (v/v) and 100 µl of avidin-peroxidase conjugate (Sigma), diluted 1:4000 with PBS, was added to each well and the plates were incubated for 30 min at 25ºC. After plates were washed 100 µl of substrate solution (2mg/ml of O-phenylendiamine, 0.012% H_2O_2 in 0,1M sodium citrate, pH 5.0) were added and the plates were incubated for 3 min at room temperature. The reaction was stopped with 50 µl of 2M HCl, and absorbances were recorded at 492 nm in a Spectra SLT microplate reader. A calibration curve was prepared in each plate, containing various amounts of biotinylated casein (0 to 200 ng). Results were expressed as the percentage of inhibition of proteolysis of the extract probed against the measure of 100 % biotinylated casein. In both experiments, all samples were run in triplicate.

Statistical analysis

The data is expressed as mean ±S.E.M. (n= 4) and the Student´s "t" test was used for comparison of the data of the control and standard groups. Probabilities of $p< 0.05$ and $p<0.01$ were considered.

RESULTS

Neutralization of the hemorrhagic effect of venom

C. pareira extract was able to inhibit the hemorrhage completely showing a dose-dependent effect (ED_{50} = 25.1 mg) (Table 1).

Inhibition of proteolytic activity

All the mixtures of extract-venom and venom control gave the same absorbance at 280 nm. The extract amount tested was unable to neutralize one minimum proteolytic dose (1.4 mg) (Table 2).

A microplate assay

None of the extract amounts were able to inhibit the proteolytic activity of the venom (Table 3).

DISCUSSION

A lot of plants have been used in popular medicine as snakebites antidotes (9, 10) however, only few studies of extracts of plants have been published about of the antagonizing properties on any actions produced by snake venoms.

Several investigations with extracts of plants or their components have demonstrated an effective neutralization or inhibition of lethality (23,24,25,26), myotoxicity (22,24,25), hemorrhagic effects (26,27,28,29,30) as well as anticoagulant and partial

Table 1.*Inhibitory effect of Cissampelos pareira leaves aqueous extract in hemorrhagic activity induced by B. asper venom.*

Concentration (mg extract/ 200 µg venom)	Hemorrhagic diameter (mm)	% of inhibition
0.0	18.22 ± 1.20	0.0
2.5	19.69 ± 1.06	0.0
5.0	18.07 ± 1.71	0.82
10.0	15.35 ± 2.37	15.75
20.0	13.96 ± 3.91	23.38
30.0	5.00 ± 2.79*	75.56**
40.0	0.69 ± 1.38 *	96.21**
60.0	0.036 ± 0.001*	99.80**

*Values are mean ±S.E.M.. *p<0.05, ** p<0.01 significantly different from control (Student´s "t"-test)*

Table 2.*Inhibition of proteolytic activity by Cissampelos pareira leaves aqueous extract on casein substrate.*

	Absorbance at 280 nm	U/mg A_{280}/mg venom X 100
Cissampelos pareira	2.50 ± 0.008	0.0
Venom	0.31 ± 0.003	22.14

Values are mean ±S.E.M. Samples were run in triplicate

Table 3. *Inhibition of proteolytic activity by Cissampelos pareira leaves aqueous extract in Microplate assay.*

	Absorbance (mg extract/200 µg venom)		Absorbance (mg extract/ml PBS)	
	2.50	40.00	2.50	40.00
Cissampelos pareira	0.156 ± 0.008	0.207 ± 0.003	0.346 ± 0.040	0.371 ± 0.065

Venom absorbance was 0.265 ±0.03. Values are mean ±S.E.M. Samples were run in triplicate.

reduction of edema-forming activities (23) against snake venoms from Elapidae and Viperidae families.

Despite the availably of antivenom for snakebites, in several countries of Central America many people still use herbs as treatment during an envenomation. So, in our approach, we first want to study if any of these plants used by healers or members of different communities in our country, shows any inhibitory properties to some of the toxic components of venom. We are looking for any plant extract that might solve specifically the local effects, such as myonecrosis, edema-forming, dermonecrosis and hemorrhage (31). Such effects are very important during an envenomation because they develop very fast after a snakebite causing irreversible damages and the use of antivenom only neutralized poorly almost all local effects mentioned above.

C. pareira extract was able of neutralize completely the hemorrhage activity in mice. Although, the mechanism of action of active compounds of these plants has not been elucidated yet, perhaps a chelating action of zinc ion could be responsible of this inhibition. Castro *et al*. (28), evaluated fifty alcoholic extracts of Costa Rican plants, of which only eighteen had anti-hemorrhagic activity and after chemical analysis of these extracts, flavones, catequines, tannins and anthocyanines were identified. Interestingly, all of them had the capacity to inhibit the hemorrhage by chelation of the zinc required for the catalytic activity of venom metalloproteases (28).We did not find any correlation between anti-proteolytic and anti-hemorrhagic activities.

The hemorrhage is one of the most important local effects provoked by the venom of *B asper*. This activity is produced by several enzymes called metalloproteinases (32). These enzymes are zinc dependent (33, 34); and four of them have been isolated and characterized from this venom (5, 21, 35). Besides, several inhibitors of hemorrhagic toxins such as salts of EDTA and hydroxamates, which chelate zinc ions, have been studied (36).

In previous works in plants, different compounds as flavonoids, condensed tannins, cathequines, and anthocyanines have been found which are able to chelate zinc required for the catalytic activity of venom hemorrhagic metalloproteinases and maybe could be responsible for the inhibitory effect observed in these plants studied (37).

Generally, local effects such as hemorrhage, edema, necrosis and dermonecrosis (38) developed very fast. Therefore, finding any plant extract or compound that helps solving the local effects caused by viperid snakes would be of great help as additional therapy in patients with this kind of envenomations.

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