Evaluation of anti-*Bothrops asper* venom activity of ethanolic extract of *Brownea rosademonte* leaves

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Significant inhibition of the coagulant and hemorrhagic effects of *Bothrops asper* venom was demonstrated by ethanolic extract prepared from the leaves of *Brownea rosademonte*. *In vitro* experiments preincubating 5.5 mg of extract kg^{-1} b.m. for 30 min with a minimum hemorrhagic dose of venom (273.8 ± 16.1 µg of venom kg^{-1} b.m.) lowered the hemorrhagic activity of the venom alone in CD-1 mice by 51.5 ± 2.6 %. Additionally, 1.7 mg extract L^{-1} plasma prolonged 5.1 times the plasma coagulation time. Fractionation of the extract led to the isolation of two compounds: ononitol (**1**) and quercetrin (**2**). The structure of compounds **1** and **2** was established by spectroscopic analyses, including APCI-HR-MS and NMR (^{1}H, ^{13}C, HSQC, HMBC and COSY). A quercetrin concentration of 0.11 µmol L^{-1} prolonged the plasma coagulation time 2.6 times demonstrating that this compound was one of the active constituents of the *Brownea rosademonte* extract.

Keywords: Bothrops asper (Viperidae) *venom,* Brownea rosademonte (Caesalpinaceae)*, quercetrin, anticoagulant, antihemorrhagic, antiproteolytic, antihemolytic*

Snakebite is a neglected public health problem in many tropical and subtropical countries worldwide. Close to 5 million snakebites occur every year, resulting in up to 2.5 million snakebite poisonings. Deaths of at least 100,000, many amputations and other permanent disabilities occur; most cases in Africa, Asia and Latin America. This health problem is often encountered among young people, children and farmers from poor rural settings with scarce medical resources (1–2).

Bothrops asper is mainly responsible (90 %) for snakebites in Panama (3), where about 2500 of such accidents occur every year, and for the incidence of 76 new cases per 100,000 inhabitants per year (4). Its venom induces conspicuous local effects (edema, hemorrhage, blisters, dermonecrosis, myonecrosis) and life-threatening systemic effects such as bleeding, defibrination, thrombocytopenia, cardiovascular shock and nephrotoxicity (5, 6). More

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than 115 years after the beginning of serotherapy (7), antivenoms remain the only specific treatment for snakebites (8). Nevertheless, the lack of appropriate antivenom distribution and availability in distant rural places of tropical countries in Africa and Latin America has caused some snakebite victims to seek initial attention from traditional healers in their rural settings before their arrival to the hospital. Thus, they receive extracts of plants in many ways such as drinks, baths, steams and poultices, as reported by Otero *et al.* (9, 10) in Colombia. Plants could be a potential source for drug discovery in the treatment of neglected tropical diseases like snakebites. A proof is that over 700 plants have been reported in folk medicine for treating snakebites (11). These plants are an invaluable source for obtaining compounds that could be useful to inhibit some of the toxic effects caused by snakebites. Unfortunately, only a few plants have been evaluated in controlled bioassays and information regarding the isolation of active principles is very limited (11). Furthermore, the kinetics of the absorption, distribution, metabolism and excretion of plant extracts as well as their concentration in the tissue, are still unknown (12). Finally, controlled clinical trials in humans, using those plants or their isolated compounds, are not run due to many ethical bans.

In this work, an extract prepared from the leaves of *Brownea rosademonte* was tested for its neutralizing ability against the hemorrhagic, coagulant, hemolytic and proteolytic effects of *B. asper* venom from Panama.

EXPERIMENTAL

Animals and venom

CD-1 mice (50–70 days) weighing 18 to 19 g were used in the experiments. The animals were provided by the Biotery of the University of Panama (Panama City). The animals were kept in plastic cages under a 12 h light/12 h dark cycle at room temperature (23 ± 2 °C). The animals had free access to food and water. Experiments used the smallest possible number of animals and their suffering was avoided by using sevoflurane as general anesthetic. Animal care and the experimental protocol followed the international guiding principles for biomedical research involving animals.

Mice were divided into four groups of four mice (in duplicate) for use in hemorrhagic experiments as follows: group 1 (control mice treated only with venom), group 2 (mice treated only with plant extract), group 3 (control only with PBS), group 4 (mice treated with venom and plant extract).

The experiments involved the hemorrhagic effect of venom and venom neutralization by the extract. *B. asper* venom was obtained by milking more than 90 adult specimens of *B. asper* from four different geographic regions of Panama.

The venom was centrifuged at 3500 x *g* for 30 min, the supernatant was lyophilized and stored at –40 °C until used. Because of great similarity between *B. asper* and *B. atrox* venoms, the minimum inhibitory doses [minimum hemorrhagic dose (MHD), minimum coagulant dose (MCD), minimum indirect hemolytic dose (MIHD) and minimum proteolytic dose (MPD)] for each evaluated effect were determined based on the data previously reported for *B. atrox* venom (12–15). For the experiments, we used the respective reported minimum inhibitory doses plus two high and two low levels of venom (3, 2, 1, 0.5 and 0.3 MHD). For

example, the previously reported MIHD was 0.13 mg L^{-1}. So, to detect the MIHD of our venom we employed test concentrations of 0.39, 0.26, 0.13, 0.065 and 0.04 mg L^{-1}.

It is important to clarify that experiments carried out to determine the antihemorrhagic effect of *B. rosademonte* on mice are considered to be *in vitro* because the crude plant extract was preincubated together with venom before being injected to test mice.

Plant collection and extract preparation

B. rosademonte (*Caesalpiniaceae*) leaves were collected in the Parque Nacional Alto Chagres Panama; voucher specimens are deposited at the Herbarium of the University of Panama (Panama City, Panama). After sun drying fresh leaves and crushing them in a standard blender to obtain coarse powder, the extract was prepared by maceration with 96 % ethanol for two days. Extract was concentrated to a semisolid paste using a rotary evaporator (Laborota 4010, Germany) to obtain 18.17 g of crude extract (6.2 % of plant yield) and stored at –4 °C until used.

Brine shrimp lethality assay

In vitro lethality assay of *Artemia salina* extract was used to detect cell toxicity. Brine shrimp eggs were placed in seawater [3.8 %, *m/V*, TetraMarine sea salt (United Pet Group, Inc., USA) in distilled water] and incubated at 28 °C. Eggs were hatched within 48 h providing a large number of larvae (nauplii). Ten nauplii were placed in each vial containing 5 mL of seawater and increasing concentrations of *B. rosademonte* extract (10, 50, 100, 500 and 1000 μg mL^{-1}). Each concentration was assessed in triplicate. Percentage of lethality was determined by comparing the mean number of surviving larvae of the test and control vials. LC_{50} values were obtained from the best-fit line of plotted concentration *vs.* percentage of lethality. Potassium dichromate was used as a positive control in the bioassay while the negative control contained only the solvent (methanol) used for the preparation of test samples (16).

Antihemorrhagic activity of the extract

Groups were injected by intradermal route in the abdomen, one group with 5.5 mg of plant extract kg^{-1} b. m. (group 2) and the other with one MHD of venom (venom controls) (273.8 ± 16.1 μg of venom kg^{-1} b. m.) (group 1). In both cases, the solution was prepared in 0.1 mL phosphate buffered saline (PBS), pH 7.2. The control group (group 3) received PBS alone under identical conditions.

The plant extract (5.5 mg of extract kg^{-1} b. m.) was preincubated at 37 °C for 30 min with one MHD of *B. asper* venom (273.8 ± 16.1 μg venom kg^{-1} b. m.) dissolved in 0.1 mL PBS, pH 7.2. Then, the mixture was injected i.d. into four mice (group 4). Two hours later, mice were sacrificed by sevoflurane inhalation, and the diameter of the hemorrhagic area was measured (12).

Neutralization of the proteolytic activity of venom

Proteolytic activity of *B. asper* venom (10–250 μg mL^{-1}) was examined according to the method of Lomonte and Gutierrez with slight modifications (12) using 1 % (*m/V*) casein as a substrate. For neutralization experiments, 800 μg of *B. rosademonte* extract was preincubated for 30 min at 37 °C with one MPD (81 ± 5 U mg^{-1}) of *B. asper* venom. Casein (2 mL) in

PBS pH 7.2 was then added to the mixture and again incubated for 30 min at 37 °C. The reaction was stopped by addition of 5 % trichloroacetic acid. The mixture was centrifuged at 3500x*g* for 10 min and absorbance of the supernatant was measured at 280 nm, in a Lambda 20 UV/VIS spectrometer (Perkin Elmer, USA). The experiment was repeated three times and results were expressed as mean ± SD.

Neutralization of indirect hemolytic activity of venom

The agarose-erythrocyte-egg yolk assay was used (14). Plant extract (25–1600 μg) was tested against one MIHD (195 ± 7 μg mL^{-1}) of venom and the result was expressed as mean ± SD. The experiments were perfomed in duplicate.

Neutralization of coagulant effect of venom

Human plasma was used to assess the coagulant effect. Plasma was donated by the National Blood Bank (Panama City, Panama). The plasma came from healthy donors and complied with the Caja del Seguro Social (CSS, Panama National Hospital) requirements for use in biological experimentation and with permission from the donors. The method described by Theakston and Reid (18) was used with small modifications. Briefly, 1.0 μg of *B. asper* venom dissolved in 50 μL PBS, pH 7.2, was added to human plasma (0.3 mL), pre-incubated at 37 °C and the time required for plasma coagulation was determined. To evaluate the neutralizing capacity of the plant extract against the coagulant effect of venom, one MCD (1.7 μg mL^{-1}) of *B. asper* venom was dissolved in 50 μL PBS, pH 7.2, and preincubated at 37 °C for 30 min with the plant extract (100 or 200 μg) and then, the mixture was added to human plasma (0.3 mL) and preincubated at 37 °C. The time required for plasma coagulation was determined (15).

Chemical procedures

NMR spectra were acquired on a Jeol Eclipse 400 MHz spectrometer (Jeol, Japan) and referenced to residual solvent: ^{1}H and ^{13}C signals [δ_H 3.34, δ_C 49.86 for CD$_3$OD and δ_H 2.54, δ_C 40.45 for (CD$_3$)$_2$SO]. APCIHR-MS were acquired on a JEOL LC-mate (Jeol) mass spectrometer.

Purification of the compounds was carried out by gel permeation column chromatography using Sephadex LH-20 (Sigma-Aldrich, USA) and column chromatography on silica gel 60 (0.063–0.210 mm, Merck, Germany). TLC (analytical) was performed on precoated silica gel 60 F254 plates (Merck). All solvents were of analytical reagent grade (ACS) grade and were used without further purification.

Extraction and isolation of ononitol and quercetrin from B. rosademonte *extract*

A portion of crude extract (10 g) was dissolved in a methanol/water mixture (90:10) and partitioned successively with ethyl acetate and hexane. All three fractions were evaporated to dryness using a rotar evaporator at low temperature (30 °C). Water fraction (2.5 g) was fractionated by gel permeation column chromatography using Sephadex LH-20 (50 g) and eluted with a methanol/water mixture (90:10, *V/V*) for three days, giving 36 fractions. A

major and active fraction (F4, 800 mg) was fractionated by column chromatography on silica gel 60 (50 g). The column was eluted with hexane, followed by a gradient of hexane/CHCl$_3$ (1:0→0:1) and finally with a gradient of CHCl$_3$/methanol (1:0→1:1). Altogether, 10 secondary fractions (FI to FX) were obtained. Because of the low yield and high complexity of fractions, we proceeded to work with major fractions only. From major fraction FIII eluted with chloroform/methanol (8:2, *V/V*), 300 mg of ononitol (0.019 % of plant yield, compound **1**) was purified by re-crystallization (17). Fraction FVII (40 mg) was further subjected to silica gel 60 column chromatography and eluted with a gradient of hexane/chloroform (1:0→0:1) and chloroform/methanol (1:0→1:1). This process led to fifteen secondary fractions (F7-1 to F7-15). Fraction F7-9 eluted with 100 % ethyl acetate was filtered and 12.1 mg of quercetrin (0.007 % of plant yield, compound **2**) was obtained in the supernatant (18).

RESULTS AND DISCUSSION

Looking for alternatives to combat snakebites in Panama, we have chosen the leaves of *B. rosademonte* to evaluate their properties against some *B. asper* venom toxic effects. This plant is used by traditional healers and in a previous study its stem bark extract showed *in vitro* neutralizing ability against the lethal, hemorrhagic, edema formating, defibrinating and coagulant effects of *B. atrox asper* venom (15).

As a first step, we evaluated the lethality of crude extract against brine shrimp (*A. salina* larvae) to verify the intrinsic toxicity of *B. rosademonte*. In the brine shrimp test, the plant extract showed an LC_{50} above 1000 µg mL^{-1}, suggesting the absence of cytotoxicity. The next step was to find the MHD, MCD, MIHD and MPD of venom. The lowest dose of venom that induced a hemorrhagic area of 10 mm in diameter in 2 h (mean ± SD of three experiments) was 273.8 ± 16.1 µg venom kg^{-1} b. m. Furthermore, we found that the plant extract did not produce any hemorrhagic effect in mouse tissue, this being another sign of the harmlessness of the plant. The MIHD of *B. asper* venom was 195 ± 7 mg L^{-1}. This dose induced a 20 mm diameter hemolysis halo after 20 h of incubation at 37 °C. MCD and MPD were 1.7 ± 0.2 µg mL^{-1} and 81 ± 5 U mg^{-1}, respectively.

Once the minimum doses were determined, we proceeded to evaluate the effect of the plant against those caused by snake venom and it was found that ethanolic extract from the leaves of *B. rosademonte* had significant *in vitro* activity against coagulant and hemorrhagic effects provoked by *B. asper* venom at 5.5 mg kg^{-1} b. m. significantly lowered 51.5 ± 2.6 % the hemorrhagic activity of *B. asper* venom (Fig. 1) and at 1.7 mg extract L^{-1} plasma prolonged the plasma coagulation time induced by the venom until 5.1 times. This may indicate the possible inhibition of venom metalloproteinases responsible for local and systemic hemorrhage, as well as the thrombin-like enzymes of venom responsible for plasma coagulation by this plant extract. Ethanolic extract significantly prolonged the plasma coagulation time when preincubated with the venom (one microgram of venom-induced coagulation of human plasma in 30.1 ± 1.5 s). This effect was dose-dependent because 1.7 mg of extract L^{-1} of plasma showed a coagulation time of 155.3 ± 2.4 s, whereas 3.4 mg of extract L^{-1} plasma delayed the coagulation time to 350.4 ± 3.9 s (*p* < 0.001). Proteolytic and indirect hemolytic activities of *B. asper* venom were not neutralized by the ethanolic extract prepared from the leaves of *B. rosademonte*.
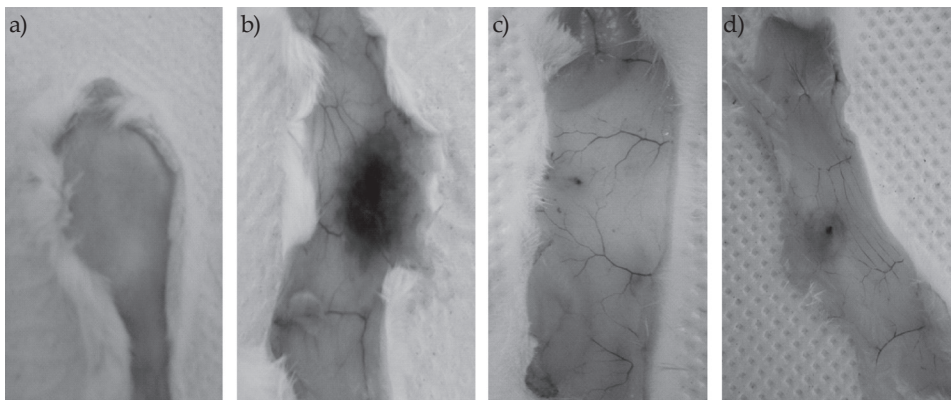
Fig. 1. Determination of the hemorrhagic effect of *B. asper* venom before and after *B. rosademonte* leaf ethanol extract administration: a) control (PBS, pH 7.2, and DMSO); b) 1 MHD of *B. asper* venom (273.8 ± 16.1 µg of venom kg⁻¹ b. m.); c) 5.5 mg of *B. rosademonte* extract kg⁻¹ b. m.; d) 1 MHD of *B. asper* venom (273.8 ± 16.1 µg of venom kg⁻¹ b. m.) and 5.5 mg of plant extract kg⁻¹ b. m.

Chemistry

Steroids, triterpenes, flavonoids and tannins were detected in the plant extract by TLC analysis (21).

Subsequent extract fractionation and compound purification were carried out by several chromatographic methods. These processes led isolation of two major components: ononitol (**1**) and quercetrin (**2**) from *B. rosademonte* (Fig. 2).

Compound **1** was isolated as a colorless crystalline solid. Its molecular formula was determined as $C_7H_{15}O_6$ by APCI-HR-MS. The ^{13}C NMR spectrum for **1** (Table I) revealed 7 resonances, interpreted from the multiplicity-edited HSQC and DEPT (135 and 90) data as six methane and one methyl group. The ^1H NMR spectrum (Table I) showed three triplets (at δ_H 3.59, 3.57 and 3.30 ppm), two doublets of doublets (at δ_H 3.76, and 3.71 ppm), a quartet (at δ_H 4.08 ppm), and a singlet (at δ_H 3.62 ppm). This evidence suggested that compound **1** was a cyclohexitol methyl ether. Based on detailed analysis of the spectroscopic data and
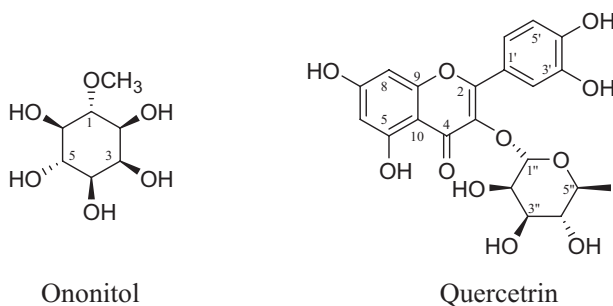

Ononitol

Quercetrin

Fig. 2. Chemical structures of major compounds isolated from *B. rosademonte*.

comparison with the data reported by Vernon *et al.* (19), compound **1** was identified as ononitol.

Compound **2** was obtained as a yellow amorphous powder (m.p. 181–182 °C). The high resolution mass spectrum APCI (positive mode) showed a pseudo-molecular ion at *m/z* 449.1079 [M + H]⁺, corresponding to the molecular formula $C_{21}H_{21}O_{11}$. The ^{13}C NMR spectrum (Table I) showed the presence of resonances characteristic of a flavonol core (δ_C 157.0, 134.8, 178.1, 161.9, 99.2, 164.8, 94.3, 157.5, 104. 7, 121.6, 116.2, 149.0, 145.8, 116.0, 121.2 ppm). The ^1H NMR spectrum of **2** (Table I) showed two doublets at δ_H 6.21 and 6.40 ppm, indicating that the compound contained a 1,3 disubtituted aromatic ring A. Moreover, the resonances at δ_H 7.36, 7.30 and 6.87 ppm suggested the presence of a 1,3,4-trisubstituted aromatic ring B. In adition, two resonances at δ_H 5.25 (d, *J* = 1.5 Hz, H-1″) and 0.86 ppm (d, *J* = 5.6 Hz, H-6″) suggested the presence of a rhamnose unit in the structure of compound **2**. Based on the above evidence, the structure of **2** was defined as quercetrin. The structure of compound

Table I. NMR data for compounds isolated from B. rosademonte[a]

Position	Ononitol (in CD₃OD)		Quercetrin (in DMSO-d_6)	
	^{13}C	^1H		
1	83.6	3.59 (t, 9.4)		
2	71.2	4.08 (q, 3.8)	157.0	
3	70.7	3.71 (dd, 2.8)	134.8	
4	72.4	3.30 (t, 9.4)	178.1	
5	73.0	3.57 (t, 9.4)	161.9	
6	72.1	3.76 (dd, 2.8)	99.2	6.21 (d, 2.2)
7	59.5	3.62 (s)	164.8	
8			94.2	6.40 (d, 1.8)
9			157.5	
10			104.7	
1′			121.6	
2′			116.2	7.30 (d, 1.8)
3′			149.0	
4′			145.8	
5′			116.0	6.87 (d, 8.4)
6′			121.2	7.36 (dd, 8.1, 1.8)
1″			102.4	5.25 (d, 1.5)
2″			70.9	4.20 (m)
3″			71.2	3.73 (dd, 9.2, 3.4)
4″			70.6	3.31 (m)
5″			71.5	3.38 (m)
6″			18.1	0.90 (d, 6.6)

[a] Coupling constants (*J* in Hz) are given in brackets.

2 was confirmed by comparing our experimental data with those reported by Hanamura *et al.* (20).

Compounds **1** and **2** (with approximately 98 % purity level) were tested against the hemorrhagic and coagulant activities of *B. asper* venom. Only compound **2** showed neutralizing capacity against the coagulant effect of venom. A concentration of 0.11 µmol L^{-1} of quercetrin prolonged the plasma coagulation time in the presence of 1 µg of venom 2.6 times. Unfortunately, it was not possible to isolate other pure active compounds with the methodology used. However, we found out that highly polar fractions were those that showed the antihemorrhagic effect. This fact shows that highly hydroxylated compounds may be the main antihemorrhagic components of the plant.

CONCLUSIONS

Biological assessments performed with *B. rosademonte* ethanolic extract allowed us to demonstrate that this plant possesses significant activity *in vitro* against coagulant and hemorrhagic effects induced by *B. asper* venom. In the brine shrimp test, the organic extract of *B. rosademonte* showed no cytotoxicity (*LC*$_{50}$ above 1000 µg mL^{-1}). On the other hand, it was demonstrated that quercetrin is one of the major anti-coagulant compounds of that plant. To our knowledge, this is the first report on the isolation and structural characterization of some compounds produced by *Brownea rosademonte*.

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