Neutralization of the edema-forming, defibrinating and coagulant effects of *Bothrops asper* venom by extracts of plants used by healers in Colombia

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Abstract

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We determined the neutralizing activity of 12 ethanolic extracts of plants against the edema-forming, defibrinating and coagulant effects of *Bothrops asper* venom in Swiss Webster mice. The material used consisted of the leaves and branches of *Bixa orellana* (Bixaceae), *Ficus nymphaeifolia* (Moraceae), *Struthanthus orbicularis* (Loranthaceae) and *Gonzalagunia panamensis* (Rubiaceae); the stem barks of *Brownea rosademonte* (Caesalpiniaceae) and *Tabebuia rosea* (Bignoniaceae); the whole plant of *Pleopeltis percussa* (Polypodiaceae) and *Trichomanes elegans* (Hymenophyllaceae); rhizomes of *Renealmia alpinia* (Zingiberaceae), *Heliconia curtispatha* (Heliconiaceae) and *Dracontium croatii* (Araceae), and the ripe fruit of *Citrus limon* (Rutaceae). After preincubation of varying amounts of each extract with either 1.0 μg venom for the edema-forming effect or 2.0 μg venom for the defibrinating effect, the mixture was injected subcutaneously (*sc*) into the right foot pad or intravenously into the tail, respectively, to groups of four mice (18-20 g). All extracts (6.2-200 μg/mouse) partially neutralized the edema-forming activity of venom in a dose-dependent manner (58-76% inhibition), with *B. orellana*, *S. orbicularis*, *G. panamensis*, *B. rosademonte*, and *D. croatii* showing the highest effect. Ten extracts (3.9-2000 μg/mouse) also showed 100% neutralizing ability against the defibrinating effect of venom, and nine prolonged the coagulation time induced by the venom. When the extracts were administered either before or after venom injection, the neutralization of the edema-forming effect was lower than 40% for all extracts, and none of them neutralized the defibrinating effect of venom. When they were administered *in situ* (*sc* at the same site 5 min after venom injection), the neutralization of edema increased for six extracts, reaching levels up to 64% for *C. limon*.

Introduction

More than 90% of the snakebites reported every year in Latin America are inflicted by snakes of the genus *Bothrops* (1,2). Two of the most relevant signs of bothropic envenomation are edema and defibrination, which may contribute to local ischemic damage, hypotension and hemorrhage (3-5).

The antivenoms commonly used for the serotherapy of snakebites show a very limited efficacy in the neutralization of edema, not only due to the variety of components inducing this alteration, but also due to its rapid onset (6-8). Defibrinating (*in vivo*) and coagulant (*in vitro*) effects are produced by thrombin-like toxins present in the venom which induce fibrinogen consumption and incoagulable blood that may favor systemic bleeding (9).

In northwestern Colombia, 60% of snakebite victims are initially treated by traditional healers who use medicinal plants in different forms according to the clinical condition of the patient (6,10,11). Thus, the experimental validation of such traditional knowledge is very important to elucidate the rational basis of its use. In the present study, we tested 12 ethanolic extracts of plants for their neutralizing ability against the edema-forming, defibrinating and coagulant effects of *Bothrops asper* venom. The extracts had previously demonstrated anti-lethal and/or anti-hemorrhagic capacity against *B. atrox asper* venom in mice (12,13).

Material and Methods

Mice, venom and extract preparation

Swiss Webster mice weighing 18-20 g were used to determine the edema-forming and defibrinating effects of venom. *B. asper* venom was obtained by milking more than 40 specimens captured in Antioquia and Chocó, Northwestern Colombia. Venom was centrifuged and the supernatant lyophilized and stored at -20ºC until use.

The plants were collected together with traditional healers in rural communities from the Atrato River Valley (Antioquia and Chocó) and on the Pacific coast (Chocó) (Table 1), identified and deposited in the Herbarium of the Universidad de Antioquia in Medellín. After drying and crushing, individual samples of each part used were extracted with 96% ethanol at room temperature for 2 days. Extracts were concentrated to a semisolid paste using a BÜCHI - 124 rotavapor (Flawil, Switzerland), lyophilized for 4 days and stored at -20ºC until use (14).

Edema-forming effect of venom and extracts

Groups of four mice were injected subcutaneously (*sc*) into the right foot pad with several quantities of either venom (0.25-4.0 µg) or extract (3.12-400 µg) dissolved in 50 µl phosphate-buffered saline (PBS), pH 7.2. The left foot pad was similarly injected with 50 µl PBS alone (control) according to the method of Yamakawa et al. (15), modified by Gutiérrez et al. (16). Edema was calculated as percentage of increase in the weight of the right foot injected with venom or extract compared to the left foot (PBS alone = control). The minimum edema-forming dose was the venom dose that induced 30% edema within 6 h.

The minimum edema-forming dose of *B. asper* venom was 1.5 ± 0.3 µg/mouse, the dose that also induced intense hemorrhage. For this reason, a lower dose of venom (1.0 µg) was chosen for the experiments of edema neutralization.

Except for *Citrus limon*, *Tabebuia rosea*, *Ficus nymphaeifolia*, *Gonzalagunia panamensis* and *Dracontium croatii*, all of the extracts dissolved in PBS, pH 7.2, and injected *sc* into the foot pad induced edema at doses of 200 to 400 µg/mouse, but none did so at lower doses.

Defibrinating effect of venom and extracts

Groups of 4 mice were injected intravenously (*iv*) into the tail or intramuscularly (*im*) into the gastrocnemius muscle with several doses of either venom or extract dissolved in 0.2 or 0.1 ml PBS, pH 7.2, respectively. A control group of 4 mice received PBS alone under identical conditions. One or 2 h later, mice were anesthetized with halothane and bled to test coagulation status by the method of Theakston and Reid (17), modified by Gutiérrez et al. (18). The minimum defibrinating dose was the venom dose that produced 100% incoagulability in all mice either 1 or 3 h after *iv* or *im* venom injection, respectively. The minimum defibrinating dose of *B. asper* venom was 1.1 ± 0.3 μg/mouse by the *iv* route and 40 μg/mouse by the *im* route. None of the extracts had defibrinating activity.

Coagulant effect of venom

The method described by Theakston and Reid (17) was followed, with small modifications. Briefly, 1.0 μg *B. asper* venom dissolved in 50 μl PBS, pH 7.2, was added to human plasma (0.3 ml) from healthy donors, preincubated at 37ºC and the time required for plasma coagulation was determined.

Neutralization of *Bothrops asper* venom effects

In vitro experiments. Variable doses of each lyophilized extract (3.1-400 μg/mouse for edema, and 1.9-2000 μg for defibrination), were preincubated at 37ºC for 30 min with fixed doses of venom (1.0 μg dissolved in 50 μl PBS for edema, and 2.0 μg dissolved in 200 μl PBS for defibrination). Groups of 4 mice were then injected *sc* or *iv* with the mixture as described above. The control group received venom alone under identical conditions. The results are reported as percentage of edema neutralization within 6 h,

with the edema of the control group being considered 100%. Neutralization of the defibrinating effect is reported as effective dose 100% (ED_{100}), i.e., the extract dose that neutralized 100% of the defibrinating effect of venom in all mice within 1 h.

For the neutralization of the coagulant activity of venom, a constant amount (20 μg) of each lyophilized extract mixed with 1.0 μg venom dissolved in 50 μl PBS, pH 7.2, was incubated at 37ºC for 30 min. The mixture was then added to plasma (300 μl) and the clotting time was recorded.

***In vivo* experiments**

Edema neutralization. The extracts that demonstrated *in vitro* neutralizing ability were then independently administered to groups of 4 mice at different times, doses and routes as follows: 1) 0.5 mg of each extract dissolved in PBS was administered orally (in 50 μl) or intraperitoneally (*ip*) (in 0.5 ml) either 60 min before or 5 min after venom injection (1.0 μg *sc* in the right foot pad); 2) 0.5 mg of each extract dissolved in 0.2 ml PBS, pH 7.2, was injected *iv* either 15 min before or 5 min after venom injection; 3) the extract dose that induced the best *in vitro* neutralization of the edema-forming effect was dissolved in 50 μl PBS and injected *sc* (*in situ*) 5 min after venom injection (1.0 μg *sc* into the right foot pad). Six hours later, mice were sacrificed by halothane inhalation and the percentage of edema neutralization was determined taking as 100% the edema of the control group (venom alone).

Neutralization of the defibrinating effect

A 2.0-mg aliquot of each extract dissolved in PBS was administered orally (in 50 μl) or *ip* (in 0.5 ml) either 60 min before or 5 min after *iv* injection of 2.0 μg venom. In a second group of experiments, 1.0 mg of each extract dissolved in 0.2 ml PBS was administered *iv* either 15 min before or 5 min after

the 2.0-µg *iv* venom injection. In a third group of experiments, variable doses (0.5-2.0 mg) of either extract were injected *im* (*in situ*) after the venom injection (40 µg *im*).

A control group of 4 mice received venom alone under identical conditions. One hour or 3 h after *iv* or *im* venom injection, respec-

tively, neutralization of the defibrinating effect of venom was tested as described above.

Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 15% poly-

Figure 1. Dose/response curve of edema neutralization. Experiments were performed by preincubating 3.1-400 µg of extract and fixed doses (1.0 µg) of venom for 30 min at 37ºC (see Material and Methods). *A*, *Brownea rosademonte* extract; *B*, *Bixa orellana* extract; *C*, *Trichomanes elegans* extract; *D*, *Citrus limon* extract. Data are reported as the mean ± SD for each experiment.
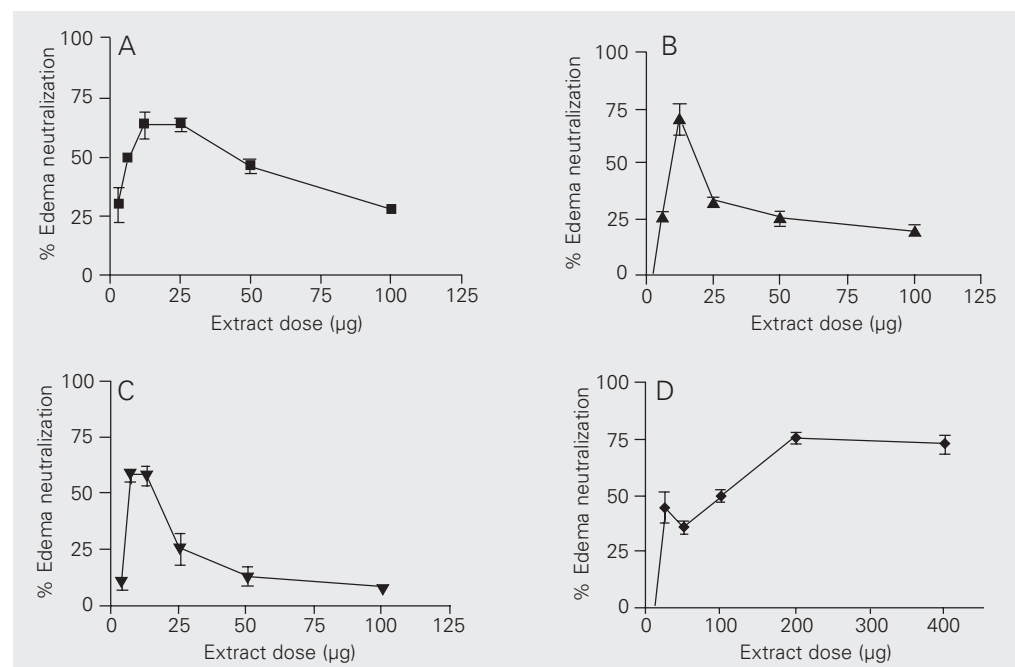

Table 1. *In vitro* neutralizing capacity of plant extracts against the edema-forming and defibrinating effects of *Bothrops asper* venom.

Family/species (voucher specimen)	Part used	Extract dose (µg/mouse)* edema neutralization (%)	Neutralization of defibrination (ED$_{100}$)** (µg)
Bixaceae/*Bixa orellana* L. (RF 6485)	L, BR	12.5 (72 ± 5)[a]	3.9
Caesalpiniaceae/*Brownea rosademonte* Berg. (RF 6455)	SB	12.5 (64 ± 6)[a]	31.2
Rutaceae/*Citrus limon* (L.) Burm.f (RF 6736)	RF	200 (76 ± 6)[b]	NNA
Araceae/*Dracontium croatii* Zhu. (RF 6245)	RH	6.2 (62 ± 10)[a]	2000
Moraceae/*Ficus nymphaeifolia* Miller (RF 6448)	L,BR,ST	200 (64 ± 2)[c]	500
Rubiaceae/*Gonzalagunia panamensis* (Cav.) Schumm. (RF 6405)	L,BR,ST	12.5 (63 ± 8)[a]	500
Heliconiaceae/*Heliconia curtispatha* Petersen (RF 6486)	RH	25 (73 ± 6)[a]	250
Polypodiaceae/*Pleopeltis percussa* (Cav.) Hook & Grev. (RF 6410)	WP	25 (68 ± 8)[a]	31.2
Zingiberaceae/*Renealmia alpinia* (Rottb.) Maas (RF 6456)	RH	100 (66 ± 3)[c]	250
Loranthaceae/*Struthanthus orbicularis* (H.B.K.) Blume (RF 6422)	L,BR	12.5 (65 ± 2)[a]	>2000
Bignoniaceae/*Tabebuia rosea* (Bertold.) DC. (RF 6458)	SB	200 (58 ± 5)[c]	1000
Hymenophyllaceae/*Trichomanes elegans* L.C. Rich (RF 6744)	WP	6.2 (58 ± 2)[a]	31.2

Voucher specimen Herbarium of the Universidad de Antioquia: RF = Ramiro Fonnegra and others (collectors). Part used: BR = branches; L = leaves; RF = ripe fruits; RH = rhizomes; SB = stem bark; ST = stem; WP = whole plant. NNA = no neutralizing ability.

Data are reported as the mean ± SD of three experiments. Values with different superscripts are significantly different (P < 0.05) in each group; first group = extract doses ≤25 µg; second group = extract doses >25 µg.

*Extract dose with the maximal neutralizing effect. **Effective dose 100% (ED$_{100}$) is the extract dose that neutralized 100% the defibrinating effect of venom in all mice.

acrylamide gels (19). The mixture of *B. asper* venom and each extract (1:10 w/w) was preincubated at 37ºC for 30 min. Then, 20 µl of each mixture was run at 150 V for 1 h. The proteins were stained with Coomassie blue.

Statistical analysis

Results are reported as means ± SD. Mean values of venom neutralization by the extracts were compared by analysis of variance (two-way ANOVA) using the STATISTICA 98 software (StatSoft, Tulsa, OK, USA). Differences were analyzed by the Newman-Keuls test. The Dunnett test was used to compare the mean values obtained for the neutralization of the coagulant effect to a control. Differences were considered significant when P < 0.05.

Results

In vitro **neutralization**

All extracts were partially effective in a dose-dependent manner in neutralizing the edema-forming effect of *B. asper* venom at doses ≤200 µg/mouse (Figure 1 and Table 1). The extracts with higher neutralizing potency at low doses (6.2-25 µg/mouse) were those of *Trichomanes elegans* (58 ± 2%), *D. croatii* (62 ± 10%), *Bixa orellana* (72 ± 5%), *Brownea rosademonte* (64 ± 6%), *Struthanthus orbicularis* (65 ± 2%), and *G. panamensis* (63 ± 8%). The extracts of *C. limon*, *Renealmia alpinia*, *T. rosea*, and *F. nymphaeifolia* needed higher doses to produce a similar neutralizing effect (P < 0.05). Nevertheless, the two-phase response observed in the neutralization curve indicated that the preincubated mixture of venom plus extract also induced edema in mice at variable doses according to the extract (>12.5 µg/mouse; Figure 1).

Additionally, 10 of the 12 extracts had 100% neutralizing ability against the defibrinating effect of venom. The *C. limon* extract

was devoid of neutralizing capacity against this effect, and *S. orbicularis* extract was only partially effective (25%) at doses up to 2000 µg/mouse (Table 1). The extract with highest neutralizing potency was that of *B. orellana*.

One microgram of venom induced coagulation of human plasma in 17 ± 3 s. Nine extracts significantly prolonged the coagulation time when preincubated with the venom (P < 0.001). Six of them (*B. rosademonte*, *Pleopeltis percussa*, *T. elegans*, *S. orbicularis*, *B. orellana* and *Heliconia curtispatha*) prolonged the coagulation time beyond 60 s (Figure 2).

In vivo **neutralization**

When the extracts were independently administered by the oral, *ip* or *iv* route, either 15 or 60 min before venom injection, the neutralization of the edema-forming effect
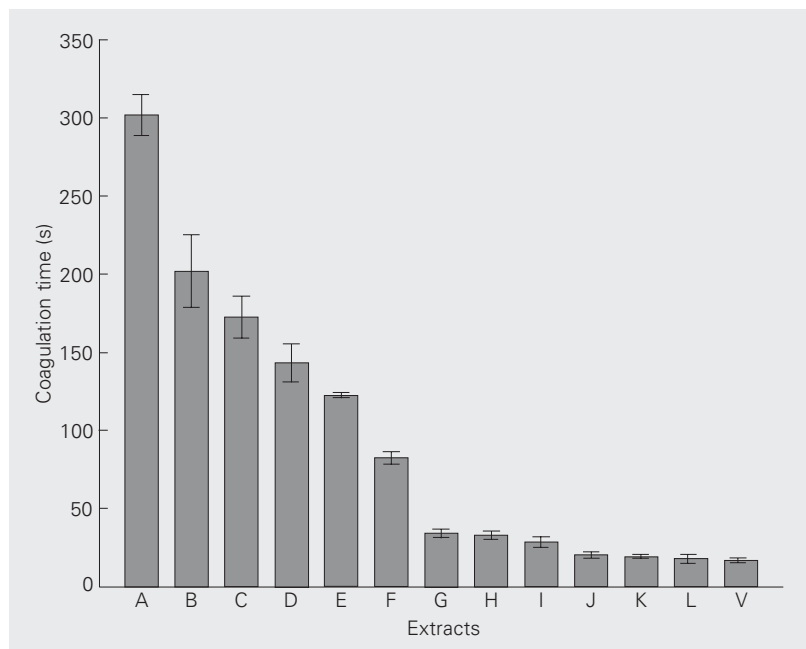

Figure 2. Coagulation time. Fixed doses of each extract (20 µg) and *Bothrops asper* venom (1.0 µg) were preincubated at 37ºC for 30 min. Then, 50 µl of the mixture was added to 300 µl of citrated human plasma and the coagulation time was determined. *A, Heliconia curtispatha*; *B, Pleopeltis percussa*; *C, Brownea rosademonte*; *D, Bixa orellana*; E, *Trichomanes elegans*; F, *Struthanthus orbicularis*; *G, Gonzalagunia panamensis*; *H, Ficus nymphaeifolia*; *I, Tabebuia rosea*; *J, Renealmia alpinia*; *K, Dracontium croatii*; *L, Citrus limon*; V = venom alone. Data are reported as the mean ± SD of three determinations.

of venom varied according to the extract, dose and route of administration (data not shown). Similarly, when the extracts were administered by these routes 5 min after venom injection, they also showed variable neutralizing ability. Overall, neutralization was lower than 40% for all extracts (Table 2). Nevertheless, when the extracts were administered *in situ* immediately after venom injection, all of them showed increased edema neutralization (Table 2). The *C. limon* ex-

tract showed the highest neutralization (64%) by this route (P < 0.001). None of the extracts neutralized the defibrinating effect of *B. asper* venom when they were individually administered by the oral, *ip*, *iv* or *in situ* route, either before or after venom injection (data not shown).

Electrophoretic analysis

After preincubation of extract and venom,

Table 2. Neutralization of the edema-forming effect of *Bothrops asper* venom in experiments with independent administration of extract and venom.

Plant extract*	% Neutralization			
	Oral	*ip*	*iv*	*In situ***
Bixa orellana L.	16 ± 5	25 ± 3	24 ± 3	28 ± 4[c]
Brownea rosademonte Berg.	15 ± 1	21 ± 2	18 ± 2	45 ± 7[b]
Citrus limon (L.) Burm.f	33 ± 9	17 ± 4	28 ± 5	64 ± 6[a]
Dracontium croatii Zhu.	8 ± 3	7 ± 1	28 ± 1	3 ± 2[e]
Ficus nymphaeifolia Miller	8 ± 3	30 ± 0	16 ± 4	27 ± 1[d]
Gonzalagunia panamensis (Cav.) Schumm	23 ± 5	20 ± 7	14 ± 3	26 ± 8[d]
Heliconia curtispatha Petersen	13 ± 1	14 ± 1	19 ± 4	48 ± 4[b]
Pleopeltis percussa (Cav.) Hook & Grev.	0	13 ± 5	11 ± 1	29 ± 5[c]
Renealmia alpinia (Rottb.) Maas	21 ± 6	12 ± 1	28 ± 2	40 ± 8[b]
Struthanthus orbicularis (H.B.K.) Blume	19 ± 4	28 ± 6	0	24 ± 7[d]
Tabebuia rosea (Bertold.) DC.	16 ± 1	35 ± 6	29 ± 6	31 ± 4[c]
Trichomanes elegans L.C. Rich	32 ± 6	24 ± 3	18 ± 4	48 ± 4[b]

The edema in the control group (venom alone) was 14.6 ± 2%. Values with different superscripts are significantly different (P < 0.05).
*A 0.5-mg aliquot of each extract was administered (orally, *ip* or *iv*) 5 min after *sc* venom injection (1.0 µg) into the right foot pad of mice. **The extract dose that induced the best *in vitro* neutralization of the edema-forming effect was injected *sc* (*in situ*) 5 min after the venom.
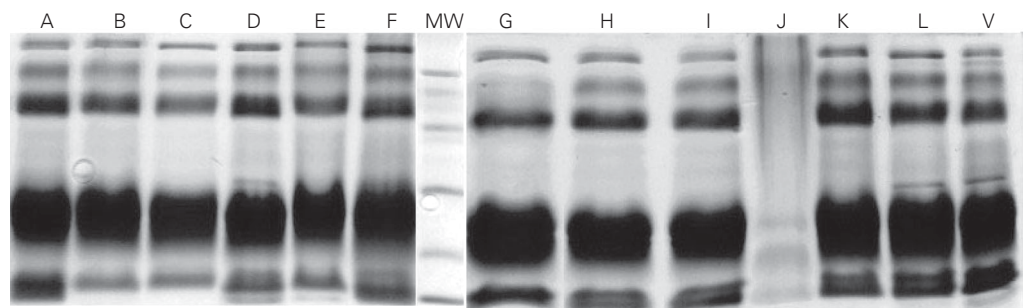

Figure 3. SDS-PAGE of *Bothrops asper* venom after incubation with plant extracts (1:10, w/w, 30 min, 37ºC). *A*, *Gonzalagunia panamensis*; *B*, *Pleopeltis percussa*; *C*, *Struthanthus orbicularis*; *D*, *Dracontium croatii*; *E*, *Brownea rosademonte*; *F*, *Tabebuia rosea*; *G*, *Citrus limon*; *H*, *Trichomanes elegans*; *I*, *Ficus nymphaeifolia*; *J*, *Heliconia curtispatha*; *K*, *Bixa orellana*; *L*, *Renealmia alpinia*; V = venom alone; MW = molecular weight markers (97.4, 66, 45, 31, 21, 14.4 kDa).

only one of the 12 extracts (*H. curtispatha*) induced conspicuous changes in the electrophoretic pattern of venom (Figure 3). All experiments were repeated five times.

Discussion

Some of the factors associated with traditional medicine practices for snakebites in Colombia are social problems, geographical difficulties, and the insufficient production, supply or distribution of antivenoms. Thus, 60% of snakebite victims are initially treated by healers who use medicinal plants administered either by the oral route or by external baths, vapor and poultices applied to the affected extremity (11). Plants constitute rich sources of novel compounds with a variety of pharmacological activities. Therefore, experimental validation of the traditional use of plants is important and can facilitate the development of low-cost phytotherapeutic agents (20).

In viper bites, edema is the result of the synthesis of potent autocoids or eicosanoids triggered by enzymatic components of the venom, as well as by the damage in the microvasculature with plasma extravasation and the release of cytokines. Chaves et al. (21) demonstrated that pretreatment with indomethacin (an inhibitor of the cyclooxygenase pathway), dexamethasone and mepacrine (two drugs that inhibit phospholipase A_2 activity by different mechanisms), reduced the edema induced by *B. asper* venom. Thus, the edema induced by *B. asper* venom in the mouse foot pad model is mediated, at least in part, by phospholipase A_2 activity and by eicosanoid products.

The rapid neutralization of venom in the bloodstream does not guarantee the halting of edema progression within a short time. In several experimental and clinical trials it has been demonstrated that antivenoms are of limited value to stop edema progression within the first 12-24 h of treatment, while they are highly efficient in restoring blood coagulation status within the same time interval (7,22,23).

Several reports have shown that plant-derived compounds with activity on arachidonic acid metabolism, lead to a marked inhibition of edema. Some of them have been characterized as inhibitors of cyclooxygenase and lipoxygenase activities (24). Additionally, some plants active against the lethal effect of snake venoms (25,26, present study) are also active against the edema induced by venoms. The herbal compound (2-OH-4-methoxy benzoic acid) isolated from the root extract of *Hemidesmus indicus* R. BR effectively neutralized the inflammation induced by *Vipera russelli* venom in male albino mice and reduced the cotton pellet-induced granuloma in rats (27). The *Casearia sylvestris* (Flacourtiaceae) extract inhibited by 50% the edema-inducing activity of both whole venom and myotoxin II of *B. moojeni* when assayed in the mouse foot pad model in preincubation experiments (28).

In the present study, 12 extracts which had previously demonstrated *in vitro* an antilethal and/or antihemorrhagic effect against *B. asper* venom (12,13) also showed *in vitro* neutralizing ability against the edema-forming effect. When the extracts were administered individually (*in vivo* experiments) by the oral, *ip* or *iv* route they showed a low, but significant neutralizing ability against edema. This capacity was increased for six extracts when they were injected *in situ*. This may indicate that close contact between the extract and venom is essential for a maximal neutralizing effect.

Venoms of vipers such as *B. asper* have *in vitro* coagulant effect and *in vivo* defibrinating effect on human plasma (3,9,17). Accordingly, ten of the extracts studied here completely neutralized the defibrinating effect of venom and nine neutralized the coagulant effect in experiments with preincubation of venom and extract. Five of them, with the highest neutralizing capacity against the defibrinating effect of venom (*B. orellana*, *B. rosademonte*, *P. percussa*, *T. elegans*, *H.*

curtispatha) also showed high neutralizing potency against the coagulant effect (Table 1, Figure 2).

Nevertheless, the extracts were unable to neutralize the defibrinating effect of venom when they were administered before or after venom injection, even when they were administered almost simultaneously at the same site of venom injection. The lack of this neutralizing ability may be related to the fact that low concentrations of venom are sufficient to induce defibrination, as well as to the rapid action of thrombin-like components (29).

Although the mechanism of action of these extracts is unknown, a change in the electrophoretic pattern of *B. asper* venom induced by the *H. curtispatha* extract indicates that protein hydrolysis is a potential mechanism. When varying the incubation time of venom and extract (5, 15, 30 and 60 min; results not shown) a similar electrophoretic pattern was obtained. Other mechanisms of action (inhibition of the catalytic activity of snake venoms and their enzymes, i.e., phospholipase A_2, metalloproteinase

inhibition by the presence of metal-chelator substances in the extracts) have been proposed for plant extracts in venom neutralization (12,13,30-32). With the exception of *C. limon*, *D. croatii* and *F. nymphaeifolia*, all the extracts studied here showed neutralizing ability against phospholipase A_2 activity (indirect hemolysis) of *B. asper* venom as well as partial neutralization of myonecrosis induced by this venom (12, and Otero R, Núñez V, Barona J, Jiménez S, Fonnegra R and Osorio RG, unpublished observations).

Despite the reduction of the neutralizing capacity of the extracts against the toxic activities of *B. asper* venom when they were administered individually, the extracts could be used in combination with serotherapy in the treatment of viper bites, mainly as palliative treatment of some local signs of envenomation such as edema, hemorrhage and myonecrosis (13, and Otero R, Núñez V, Barona J, Jiménez S, Fonnegra R and Osorio RG, unpublished observations, present study). This might be particularly true if the extract is administered very soon at the same site of venom injection.

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