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Inhibition of edema-forming and hemorrhagic activities of *Bothrops asper* snake venom by *Phenax angustifolius* and *Phenax rugosus* (Urticaceae) extracts

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ABSTRACT

The aim of this work was to investigate the capacity of crude extracts of roots, stems and leaves from *Phenax angustifolius* (H.B.K.) Weddell and *Phenax rugosus* (Poir.) Weddell in DC. (Urticaceae) to inhibit the edema-forming and hemorrhagic activities induced by *Bothrops asper* snake venom. Edema-forming activity was studied plethysmographically in the rat hind paw model. Groups of rats were preinjected intraperitoneally (ip) with various doses each extract and, one hour later, venom was injected subcutaneously (sc) in the right hind paw. Edema was assessed at various time intervals. Results showed that the edematogenic activity was inhibited in those animals that received an injection of either root or stem extracts from *P. angustifolius*. In contrast, extracts from leaves of both plants induced a pro-inflammatory effect, increasing edema in some of the doses tested. On the other hand, the anti-hemorrhagic effect was evaluated by the intradermal (id) mouse skin test. Venom and the extracts were incubated and then injected into groups of mice. Our findings evidenced that root and stem extracts of *P. angustifolius* were able to inhibit hemorrhagic activity. In contrast, extracts from leaves of both plants potentiated the hemorrhagic activity of the venom. Furthermore, a partial inhibition of proteolytic activity of venom on biotinylated casein was achieved with some of the extracts.

Key words: Anti-hemorrhagic activity, anti-edematogenic activity, plant extracts, snake venom, *Bothrops asper*, *Phenax*, Urticaceae.

1. Introduction

Hemorrhage and edema are two of the main local effects provoked by the venom of the viperid snake *Bothrops asper* the species responsible for the majority of snakebites in Central America (1,2). Experimental and clinical observations have shown that these effects are poorly neutralized with commercial antivenoms utilized in the treatment of these envenomations (3,4,5,6). Thus, there is a need to find new therapeutic alternatives to confront the problem of local effects in these envenomations.

Pharmacological studies have shown that certain extracts and fractions obtained from some plants used in traditional medicine have anti-inflammatory, anti-venom activities (7). In the case of snakebite, many plants have been suggested to have anti-venom activity in several countries. For instance, Martz (8) described that at least 578 species of higher plants had some anti-venom action. Thus, natural products obtained from plants constitute an extremely rich source of potential venom-inhibitory substances.

Phenax is a herbaceous genus of the family Urticaceae found throughout Central America. These species grow in the Costa Rican tropical forest (9). In this work we investigated the ability of plant extracts of *P.*

angustifolius and *P. rugosus* (Urticaceae) to inhibit hemorrhagic, proteolytic and edema-inducing activities of *B. asper* venom.

2. Experimental

2.1 Venom

It was obtained from adult specimens of *B. asper* collected in the Pacific region of Costa Rica and corresponding to pools from more than 40 individuals. After lyophilization, the venom was maintained at -20 °C.

2.2 Plant extracts

Plants used were picked during dry season (November and December) in Villa Colón, San José, Costa Rica. They were identified at source by L.J Poveda, School of Biology, Universidad Nacional (Heredia, Costa Rica). The roots, stems and leaves were washed, chopped and dried for 3 days at room temperature, and each one processed through a Wiley-type grinder until they formed fine dust particles, and were extracted with methanol/water (8:2) for two days at room temperature, then filtered and evaporated using a rotary evaporator at 40 °C. Finally, all plant extracts were freeze-dried and kept at 5°C until used.

2.3 Experimental animals

The animals used were adult male Sprague-Dawley rats (*Rattus norvegicus*) with a body weight from 180 to 220 g, and adult male Swiss-Webster mice (*Mus musculus*) weighing from 20 to 22 g. These animals were supplied by the Animal Care Unit (Universidad de Costa Rica) and Instituto Clodomiro Picado, respectively.

2.4 Drugs and chemicals

Indomethacin (Merck), biotinylated N,N-dimethylcasein (Sigma, M.O., USA), sodium chloride (Merck), sodium bicarbonate (Merck), avidine-peroxidase conjugate (Sigma, M.O., USA) were used throughout the study.

2.5 Biological assay

2.5.1 Anti-inflammatory activity

The anti-edematogenic properties of the extracts were quantified in the rat paw edema model by using a plethysmometer (Ugo Basile, model 7140, Italy) (10). Groups of six male rats were injected ip with several doses of each extract (62.5-125-250 mg/kg). One hour later, animals were injected sc in the right hind paw with 50 µg/50 µl of *B. asper* venom, whereas the left hind paw was injected with 50 µl of 0.15 M NaCl. The paw volume was measured plethysmographically at different time intervals (1, 2, 4, 6 and 24 hr) after *B. asper* venom injection. The control group was injected only with *B. asper* venom (50 µg/50 µl) in the right hind paw and with saline solution in the left paw. A group of six rats were treated with indomethacin (50 mg/kg ip) used as standard drug for the anti-inflammatory activity. Edema was expressed as percentage of the difference between the left paw and the right paw volumes and compared with venom control.

2.5.2 Anti-hemorrhagic activity

Hemorrhage was determined in groups of seven mice according to the method of Kondo *et al.* (11), with the modifications of Gutiérrez *et al.* (12). Different plant extract doses (40-10-5.0-1.25 and 0.31 mg) were incubated previously with venom (20 µg/ml) for 30 min at 37 °C. Then 100 µl of the supernatant was injected id in the skin of mice, which corresponded to 10 minimum hemorrhagic doses (MHD) of venom (1 MHD = 2 µg). Mice were sacrificed by CO₂ inhalation after 2 hr and the diameter of hemorrhagic spot in the inner side of the skin was measured. Controls of venom (10 MHD) and extract were carried out. Neutralizing ability was expressed as the effective dose 50 % (ED₅₀), defined as the ratio of mg extract/20 µg venom in which the diameter of hemorrhagic spot was reduced by 50% when compared to the lesions induced by venom alone.

2.6. Proteolytic assay

This activity was followed using a micromethod assay in flat microplates, according to the method Koritsas and

Atkinson (13) with biotinylated N,N-dimethylcasein (Sigma) as substrate, as modified by Franceschi *et al.* (14). Plates were coated with 0.2 µg/100 µl of biotinylated casein, dissolved in coated buffer (0.1 M Tris, 0.15 M NaCl, pH 9.0). After 24 h incubation at room temperature, plates were washed with PBS-Tween 0.05% (v/v) and the free sites in the plates were blocked with 100 µl of a 2 % bovine serum albumin solution diluted in PBS. Then, mixtures of several plant extracts (2.5-5-10 and 40 mg) were incubated with 20 µg of venom for 30 min at 25 °C. Tubes were centrifuged and 100 µl of the supernatant were applied into each well and incubated at 37 °C for 24 h. Plates were washed with PBS-Tween and 100 µl of avidine-peroxidase conjugate, diluted 1:4000 with PBS were added to each well and the plates were incubated for 30 min at 25 °C. After five washing, 100 µl of substrate solution (2 mg/mL O-phenyldiamine, 0.012% H₂O₂ in 0.1 M sodium citrate, pH 5.0) were added and the plates incubated for 3 min at room temperature. The reaction was stopped by adding 50 µl of 2 M HCl. All samples were run in triplicate. Furthermore, controls from venom and extracts were carried out in the same way. Also, a calibration curve was prepared with various amounts of biotinylated casein (from 0 to 200 ng) in each plate. Then, absorbances were recorded at 490 nm in a Dynatech MR500 microplate reader. Results were expressed as the percentage of proteolysis of venom/extract mixture, taking as 100% the absorbance of wells in which biotinylated casein was incubated without venom.

2.7 Statistical analysis

Results are presented as mean ± S.E.M. and the Student's *t* test was used to determine the significance of the differences between experimental groups. *P* values < 0.05 were considered significant.

3. Results

3.1 Anti-inflammatory activity

Inhibition experiments of edema-forming activity using plant extracts from *P. angustifolius* and *P. rugosus* are shown in Figures 1 and 2. A partial but significant inhibition was observed with the root extract from *P. angustifolius* at various time intervals. Maximum inhibition was achieved with the highest dose tested (250 mg/kg) (Fig. 1A). Similar results were observed with stem extracts of this plant especially at the highest dose (250 mg/kg) (Fig. 1B). On the other hand, at doses of 62.5 and 125 mg/kg, root extracts of *P. rugosus* inhibited edema-forming activity at 4hr after envenomation, whereas a higher inhibitory effect was observed at 2, 4 and 6 hr with a dose of 250 mg/kg (Fig. 2A). Furthermore, an inhibition of edema was also observed in animals pretreated with stem extracts at doses of 125 and 250 mg/kg, being more pronounced with the latter (Fig. 2B). Leaf extract of *P.*

angustifolius and of *P. rugosus* failed to reduce hind paw edema by this venom. Animals treated with all extracts of *P. angustifolius* and *P. rugosus* evidenced a sedative effect. Furthermore, the dose of 500 mg/kg of *P. angustifolius* leaves extract was lethal, since rats died 4 hr after extract administration. They presented somnolence and respiratory depression before dying. Therefore, this dose was not used with the rest of the extracts.

3.2 Anti-hemorrhagic activity

The hemorrhagic activity was abolished completely with doses of 5, 10 and 40 mg of root extracts from *P. angustifolius*, whereas only a partial neutralization was

obtained using 1.25 mg of this extract ($ED_{50} = 0.43$ mg/20 μ g). Likewise, 40 mg of stem extracts neutralized such activity, whereas a lower inhibition was achieved with 10 to 5 mg of extract ($ED_{50} = 2.35$ mg/20 μ g) (Table 1).

Root extracts of *P. rugosus* neutralized the hemorrhagic activity at the highest dose tested (40 mg) ($ED_{50} = 23.93$ mg/20 μ g). However, extracts of stems showed a complete neutralization of hemorrhage at doses of 1.25, 5, 10 and 40 mg ($ED_{50} = 1.80$ mg/20 μ g). Furthermore, extracts from leaves of both plants failed to inhibit this effect. Instead, they increased the hemorrhage spot on the skin of mice (Table 1).

Table 1. Neutralization of hemorrhage induced by *Bothrops asper* venom by plant extracts from *P. angustifolius* and *P. rugosus*.

Concentration mg extract/20 μ g venom	<i>Phenax angustifolius</i> Hemorrhagic diameter*			<i>Phenax rugosus</i> Hemorrhagic diameter*		
	Root	Stem	Leaves	Root	Stem	Leaves
0	14.42 $\pm$ 0.82	15.30 $\pm$ 0.76	14.36 $\pm$ 0.44	13.80 $\pm$ 1.33	10.93 $\pm$ 1.19	16.75 $\pm$ 1.39
0.31	9.66 $\pm$ 1.05*	11.75 $\pm$ 1.08*	ND	15.04 $\pm$ 1.93	8.76 $\pm$ 1.73*	12.52 $\pm$ 0.33
1.25	2.44 $\pm$ 0.68*	9.04 $\pm$ 1.11*	ND	14.29 $\pm$ 0.43	0*	16.05 $\pm$ 0.69
5.0	0*	6.22 $\pm$ 1.21*	14.94 $\pm$ 1.27	12.06 $\pm$ 1.03	0*	16.32 $\pm$ 0.56
10.0	0*	3.46 $\pm$ 1.12*	16.95 $\pm$ 0.53	0*	0*	ND
40.	0*	0*	16.97 $\pm$ 0.96	0*	0*	16.33 $\pm$ 0.97

Results are presented as mean $\pm$ S.E.M. (n=7). * $p < 0.05$; ND: No data

Table 2. Inhibition (percentage) of proteolytic activity by plant extracts from *P. angustifolius* and *P. rugosus*.

mg extract/ 20 μ g venom	<i>Phenax angustifolius</i>			<i>Phenax rugosus</i>		
	Root	Stem	Leaves	Root	Stem	Leaves
2.5	66.14 $\pm$ 0.02	19.25 $\pm$ 0.01	12.41 $\pm$ 0.01	53.44 $\pm$ 0.02	58.63 $\pm$ 0.01	15.44 $\pm$ 0.01
5.0	92.56 $\pm$ 0.01	24.91 $\pm$ 0.01	11.39 $\pm$ 0.01	65.34 $\pm$ 0.02	53.94 $\pm$ 0.01	26.56 $\pm$ 0.01
10.0	62.66 $\pm$ 0.01	54.45 $\pm$ 0.01	10.76 $\pm$ 0.01	63.69 $\pm$ 0.01	49.89 $\pm$ 0.02	55.72 $\pm$ 0.01
40.0	14.56 $\pm$ 0.01	35.84 $\pm$ 0.03	13.42 $\pm$ 0.01	14.81 $\pm$ 0.01	12.28 $\pm$ 0.01	74.71 $\pm$ 0.01

Results are presented as mean $\pm$ S.E.M..

3.3 Anti- proteolytic activity

As shown in Table 2, root and stem extracts of *P. angustifolius* and *P. rugosus* partially inhibited proteolytic activity of venom at some doses tested, although at higher doses there was an increment in proteolysis of casein, suggesting that there is proteolytic activity in the extract itself. This was corroborated in experiments where extracts alone were incubated with casein, with evidence of hydrolysis of this substrate (results not shown). However, extracts from leaves of both species lack proteolytic activity. Thus, under these conditions, it is rather difficult to assess the inhibitory effects of these extracts. Extracts from leaves from *P. angustifolius* failed to reduce proteolytic activity of the venom. In contrast, extract from leaves of *P. rugosus* inhibited, in a dose-dependent fashion, proteolytic activity of *B. asper* venom.

4. Discussion

In this work, we demonstrated that extracts of two species of *Phenax* (*Urticaceae*) were able to neutralize two local effects (edema and hemorrhage) induced by *B. asper* venom. Plants from *Urticaceae* family have been previously found to have anti-inflammatory activity (15,16).

Local edema is a complex pharmacological consequence of snakebites, especially in the case of species of the family Viperidae (17,18,19,20). In the case of *B. asper*, the most important snake in Central America (21,22) its venom induces a striking immediate dose-dependent edema in mice (23,20). This effect is responsible for loss of fluid and tissue compression (24), which would contribute to development of cardiovascular disturbances which may lead to shock (25).

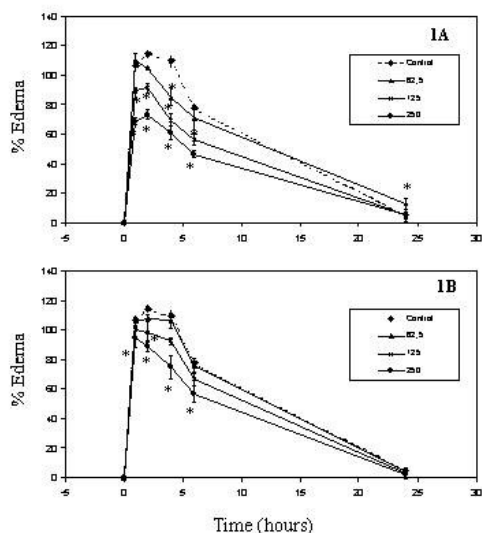


Fig. 1. Inhibition of edema-forming activity by root (A) and stem (B) extracts from *P. angustifolius*. Activity is expressed as percentage of the difference between the left paw and the right paw volumes and compared with venom control. Results are presented as mean $\pm$ S.E.M. ($n=6$). * $p<0.05$.

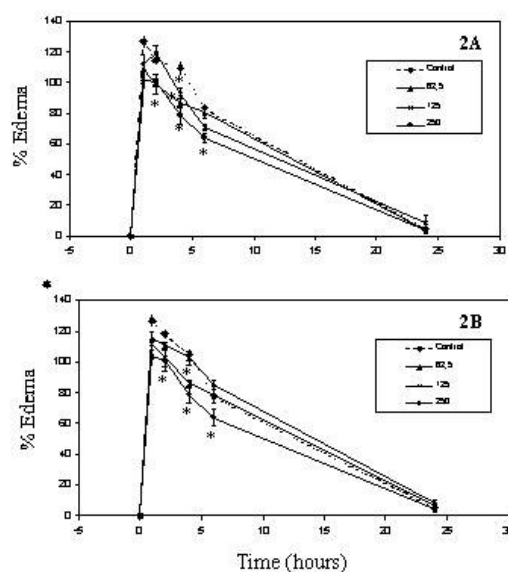


Fig. 2. Inhibition of edema-forming activity by root (A) and stem (B) extracts from *P. rugosus*. Activity is expressed as percentage of the difference between the left paw and the right paw volumes and compared with venom control. Results are presented as mean $\pm$ S.E.M. ($n=6$). * $p<0.05$.

A number of inflammatory mediators participate in the genesis of edema in a variety of inflammatory conditions (26,27). In the case of snake venoms, edema could be induced by several mediators such as histamine, prostaglandins, kinins and leukotrienes, among others (28,20).

P. angustifolius root and stem extracts, as well as *P. rugosus* root extract, showed a dose-dependent anti-edematogenic activity. Since inhibition was observed from 1 to 6 hr, it is likely that compounds present in

these extracts are acting on the first and second phases of this inflammatory model (28,16,28,19,27). In addition, animals treated with *P. rugosus* stem extract showed anti-inflammatory activity on the second phase of the inflammatory process, and only with the highest dose employed (250 mg/kg).

On the other hand, hemorrhage is one of the main pathophysiological effects observed in *B. asper* envenomations (29,30,31). Hemorrhage is provoked by zinc-dependent metalloproteinases of different molecular weights that degrade basal lamina components and cause microvasculature damage, with the consequent extravasation (32,30,33). Five hemorrhagic factors have been isolated from this venom, named BaH1, BaH2, BH3, BaP1 and BaH4 (34,31,14). These components induce severe local and systemic alterations associated not only with bleeding, but also with other pathological effects (18,30,35,36).

Our results clearly indicate that both extracts of roots and stem of *P. angustifolius* and *P. rugosus* were able to inhibit hemorrhage activity induced by *B. asper* venom. Interestingly, an enhancement of hemorrhagic activity was observed when venom was incubated with extract from leaves of both plants. Since the extracts alone are not hemorrhagic, these results evidence the existence of molecules that potentiate metalloproteinase-induced microvessel damage. Although this study was not designed to investigate the mechanism of inhibition of hemorrhage by plant extracts, it is suggested that such inhibition may be due to a chelating effect on the zinc ion essential for enzymatic activity of metalloproteinases (37,32). However, other possibilities have to be considered, such as the presence of competitive inhibitors (38, 39).

Rastrelli *et al.* have isolated two lignanes and vitexina, isovitexina and quercetine from *P. angustifolius* leaves (9). Moreover, flavonoids, as well as catechins, condensed tannins and anthocyanines, have been found in ethanolic, ethyl acetate and aqueous extracts of tropical plants of Costa Rica, which are likely to be responsible for the inhibition of *B. asper*-induced hemorrhage (39). In this regard, several studies have reported that flavonoids display a wide spectrum of actions or properties, such as antioxidant (40, 41), anxiolytic (42), binding affinity to biological polymers and heavy metal ions (43), anti-allergic (41), and anti-inflammatory (44, 45,46) and anticarcinogenic (47,48) effects. Thus, the isolation and characterization of active constituents from stem and roots of *P. angustifolius* and *P. rugosus* separately needs to be performed.

Horse or sheep-derived antivenins are the mainstay scientific treatment for snakebite envenomations (49). In the case of *B. asper* bites, polyvalent (Crotalinae) antivenom is highly effective in the neutralization of the systemic effects, whereas local injurious effects are

neutralized only to a partial extent (3,12,6). This has been attributed to the fast development of local hemorrhage, edema and myonecrosis after venom injection (3,30,35,20). Thus, it is necessary to develop complementary treatments aimed at the inhibition of toxins responsible of local tissue damage. In this regard, previous studies performed with crude extracts and fractions of various plants have shown that they possess anti-snake venom activity (8). For instance, the aqueous extract and an active component obtained from the ethanolic extract of *Tabernaemontana catharinensis* (Apocynaceae) inhibited the lethal and myotoxic activities of *Crotalus durissus terrificus* (50). Moreover, the neutralization of these activities by extracts and purified compounds derived from *Eclipta prostrata* (Asteraceae) had been reported (51). Borges *et al.* (52) demonstrated the capacity of extract from *Casearia sylvestris* to inhibit phospholipase A₂, myotoxic, anticoagulant, and edema-forming activities of some snake and bee venoms and of various phospholipases A₂ isolated from these venoms.

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