ORIGINAL PAPER

Neutralization of pharmacological and toxic activities of *Bothrops jararacussu* snake venom and isolated myotoxins by *Serjania erecta* methanolic extract and its fractions

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Abstract: Most of the snakebites recorded in Brazil are caused by the *Bothrops* genus. Given that the local tissue damage caused by this genus cannot be treated by antivenom therapy, numerous studies are focusing on supplementary alternatives, such as the use of medicinal plants. *Serjania erecta* has already demonstrated anti-inflammatory, antiseptic and healing properties. In the current study, the aerial parts of *S. erecta* were extracted with methanol, then submitted to chromatographic fractionation on a Sephadex LH20 column and eluted with methanol, which resulted in four main fractions. The crude extract and fractions neutralized the toxic activities of *Bothrops jararacussu* snake venom and isolated myotoxins (BthTX-I and II). Results showed that phospholipase A_2, fibrinogenolytic, myotoxic and hemorrhagic activities were inhibited by the extract. Moreover, the myotoxic and edematous activities induced by BthTX-I, and phospholipase A_2 activity induced by BthTX-II, were inhibited by the extract of *S. erecta* and its fraction. The clotting time on bovine plasma was significantly prolonged by the inhibitory action of fractions SF3 and SF4. This extract is a promising source of natural inhibitors, such as flavonoids and tannins, which act by forming complexes with metal ions and proteins, inhibiting the action of serineproteases, metalloproteases and phospholipases A_2.

Key words: medicinal plants, antiophidian properties, *Serjania erecta*, *Bothrops jararacussu* snake venom, myotoxins.

INTRODUCTION

Snake venoms are a complex mixture of toxic enzymes and proteins such as phospholipases A_2, myotoxins, hemorrhagic metalloproteases, clotting serineproteases, neurotoxins, cytotoxins and others. In Brazil, *Bothrops* and *Crotalus* snakes are responsible for most ophidian envenomations, which induce mainly local tissue damage such as hemorrhage, necrosis, edema and alterations in blood coagulation. Snakebite envenomations are frequently treated with parenteral administration of horse- or sheep-derived antivenoms aiming at the neutralization of toxins. But despite the success of serum therapy, it is important to search

for different venom inhibitors, either synthetic or natural, which would complement the action of antivenoms, particularly in relation to the neutralization of local tissue damage (1). Plant extracts constitute an extremely rich source of pharmacologically active compounds, and a number of extracts has been shown to act against snake venom (2). The medicinal value associated with a plant can be confirmed by the successful use of its extract on snakebite wounds (3-6). Application of medicinal plants with anti-snake-venom activities might be useful as first aid treatment for victims of snakebites, which is particularly important in local areas where antivenoms are not readily available (7-10).

In many countries, plant extracts have been used traditionally in the treatment of snakebite envenomations. Thus, vegetal extracts have been found to constitute an excellent alternative with a range of anti-snake-venom properties. However, in most cases, scientific evidence of their antiophidian activity is still needed. Several plants have already shown antiophidian activity and the Brazilian flora has a wide variety of medicinal plants with anti-snake-venom potential (2, 3, 5, 6, 11).

The vegetal kingdom is the main source of pharmacologically active compounds (12). The Sapindaceae family is widely distributed in the tropical regions of the world and some species are found in Brazil, including *Serjania erecta* Radlk, commonly called *retrato de teiú*, *cinco-folhas* or *cipó-cinco-folhas* (13). The popular use of *S. erecta* in Brazil is related to the treatment of inflammatory and ulcerative diseases, but several studies showed that different species of the *Serjania* genus and some of their isolated compounds present analgesic, antibacterial, antifungal, molluscicidal activity and anti-inflammatory effect (14-17). Phytochemical screening tests have demonstrated the presence of alkaloids, saponins, flavonoids, tannins, triterpenoids, steroids, catechins, coumarins, anthranoids and quinines in *S. erecta* extract (16). The present study aimed to investigate the healing properties of *S. erecta* against the toxic effects induced by *Bothrops jararacussu* snake venom.

MATERIALS AND METHODS

Reagents and Animals

Snake venoms were purchased from the Bioactive Proteins Serpentarium, Batatais, São Paulo state, Brazil. BthTX-I and II were isolated from *Bothrops jararacussu* snake venom (Bjussu) as previously described (18). Male Swiss mice (18-25 g) were obtained from the animal house at the School of Pharmaceutical Sciences of Ribeirão Preto, São Paulo state, Brazil. Experiments reported in this study were performed after approval by the Institutional Ethics Committee of the University of São Paulo (protocol n. 07.1.202.53.1).

Plant Material and Chromatographic Fractionation

Plant material of *Serjania erecta* was collected in Altinópolis (from a rural area in the state of São Paulo). A voucher specimen (n. HPMU 835) has been deposited at the Medicinal Plant Herbarium of the Biotechnology Unit of the University of Ribeirão Preto (UNAERP), Ribeirão Preto, SP, Brazil. Aerial parts (stem and leaf) were dried in an oven at 60°C and plant material was pulverized into a dry powder and extracted by applying methanol for three days by maceration. The extract was then concentrated at a reduced temperature (50°C). The crude extract (5 g) was dissolved in methanol and subjected to chromatographic fractionation on a Sephadex LH20 column (110 x 3 cm) and eluted with methanol. This fractionation resulted in four main fractions that were evaluated by phytochemical screening tests. The fractions were evaporated under reduced pressure and the concentrations were expressed in terms of dry weight.

Phospholipase A$_2$ Activity

Phospholipase A$_2$ activity was measured via an indirect hemolytic assay on agarose-erythrocyte-egg yolk gel plates, following methods described by Gutiérrez *et al.* (19). The minimum indirect hemolytic dose (MIHD) of BthTX-II or *B. jararacussu* snake venom (10 μg) induced hemolytic halos after incubation of plates for 18 hours at 37°C. The extracts and venom/toxin (1:30, w/w) were preincubated for 30 minutes at 37°C and the anti-phospholipase A$_2$ potential of the extracts was measured after 18 hours of plate incubation at 37°C. This experiment was done in triplicate. Crude venom and phosphate buffered saline (PBS) were used as controls.

Fibrinogenolytic Activity

The fibrinogenolytic activity was evaluated as previously described (20). Bovine fibrinogen (40 μg) was incubated at 37°C for one hour with 10 μg of *B. jararacussu* snake venom preincubated with the extracts (1:30, w/w) for 30 minutes at 37°C and the reaction was stopped with 25 μL of 0.5M Tris-HCl buffer (pH 6.5) containing 2% (w/v) SDS, 3.5% (v/v) β-mercaptoethanol and 0.05 % (w/v) bromophenol blue. The samples were analyzed by 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Edema-Inducing Activity

Edema was evaluated by subplantar injection of BthTX-I (10 μg) in the right paw of male Swiss mice (18-25 g, n = 5). Inhibition studies were

performed after preincubation of venom/toxin with the extracts at a 1:30 (w/w) ratio. Control animals received an injection of PBS under identical conditions. The progression of edema was evaluated by measuring the paw with a low-pressure pachymeter (Mitutoyo, Japan) at four time intervals (0, 30, 60 and 180 minutes) upon and after injection (21). Activity was expressed as the mean of percent edema values induced by the snake venoms in the absence and presence of the plant extracts and their fractions.

Myotoxic Activity

Male Swiss mice (18-25 g, n = 5) were injected intra-muscularly (IM) in the right gastrocnemius muscle with samples containing doses of 10 µg of BthTX-I or *B. jararacussu* snake venom. Following incubation of *S. erecta* methanolic extract and fractions with the crude venoms or isolated toxins for 30 minutes at 37°C, the mixtures of myotoxin/inhibitors (1:30, w/w) were then evaluated. Controls received venom/toxin. Mice were bled from the tail three hours after injections and blood was collected into heparinized capillary tubes. Plasma creatine kinase (CK) activity was determined using the kit CK-UV (Bioclin, Brazil) (22). The myotoxic activity was expressed in U/L.

Hemorrhagic Activity

Hemorrhagic activity was assessed according to the method of Gutiérrez *et al.* (23). The minimum hemorrhagic dose (MHD) of *B. jararacussu* snake venom (10 µg) was determined by intradermal (ID) injection into marked positions on the shaved dorsal skin of mice (18-25g, n = 5). The inhibition of hemorrhagic activity was assayed by ID injection of venom preincubated with the extracts (30 minutes at 37°C, 1:30, w/w) in the back of mice. After three hours, mice were sacrificed with CO_2, the dorsal skin was removed and the diameter of the hemorrhagic lesion on the inner surface of the skin was measured. Hemorrhagic activity was expressed as the mean of the hemorrhagic halos (in mm). Crude venom was used as control.

Coagulant Activity

A minimum coagulant dose (MCD) was defined as the amount of *B. jararacussu* venom that clots 200 µL of bovine plasma in 120 seconds (24). Briefly, 200 µL aliquots of plasma (n = 3) were incubated with 20 µg of venom preincubated with the extracts (30 minutes at 37°C, 1:30, w/w) and clotting times were recorded. Control tubes included plasma with PBS plus calcium, extracts/fractions alone and with venom. Coagulant activity was expressed as the mean coagulation time (in minutes) induced by the snake venom in the absence and presence of extracts and fractions.

Statistical Analysis

The statistical significance of differences between groups was evaluated using one-way analysis of variance (ANOVA). A p-value < 0.05 was considered significant. Data are shown as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

The fractionation of methanolic extract of *S. erecta* (EFMeOH) on Sephadex LH20 resulted in four main fractions: SF1 (18.2%), SF2 (42%), SF3 (19.6%) and SF4 (16.2%). These fractions were subjected to phytochemical screening tests that revealed the presence of saponins, terpenes, flavonoids and tannins, respectively (results not shown).

Phospholipases A_2 (PLA_2s) constitute one of the widely distributed enzyme groups that hydrolyze glycerophospholipids at the sn-2 position of the glycerol backbone, thus releasing lysophospholipids and fatty acids. Snake venom PLA_2s are known to induce various pathological effects in experimental animal models. PLA_2 activity induced by *B. jararacussu* snake venom (Figure 1 – A) and myotoxin BthTX-II (Figure 1 – B) were inhibited at different levels by the methanolic extract of *S. erecta* and its fractions. For example, the PLA_2 activity induced by BthTX-II was almost completely inhibited by the action of fractions SF3 and SF4. The SF3 fraction is rich in flavonoids, compounds known to possess anti-inflammatory activity, while the SF4 fraction is rich in tannins, a class of compounds that can precipitate proteins. The fact that the fractions SF3 and SF4 have shown a greater efficiency in the inhibition of BthTX-II-induced PLA_2 activity and lesser action against the PLA_2 activity induced by crude venom may be related to the presence of other toxic components in the venom, which are also responsible for this activity. Several exogenous agents from medicinal plants such as flavonoids, aristolochic acid and coumestan are
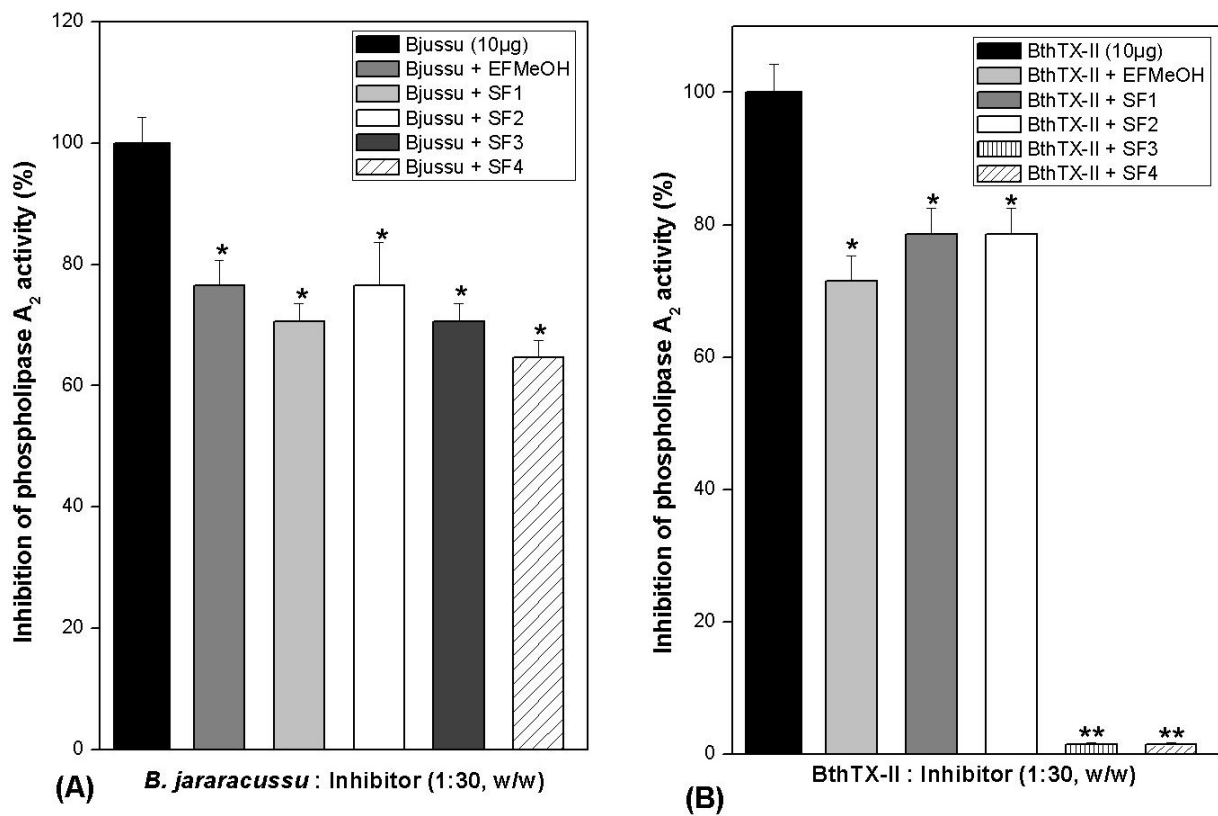
Figure 1. Inhibition of the PLA_2 activity by the methanolic extract of *S. erecta* and its fractions at 1:30 ratio (venom/toxin:inhibitor, w/w). (**A**) Effect of extracts on the PLA_2 activity induced by *B. jararacussu* crude venom (10 µg). (**B**) Effect of extracts on the PLA_2 activity induced by BthTX-II, an Asp49-PLA_2 (10 µg). The total activity of each venom or PLA_2 alone was considered to be 100%. Results are expressed as the mean ± SD (n = 3). * p < 0.05 and ** p < 0.01, when compared to control activity.
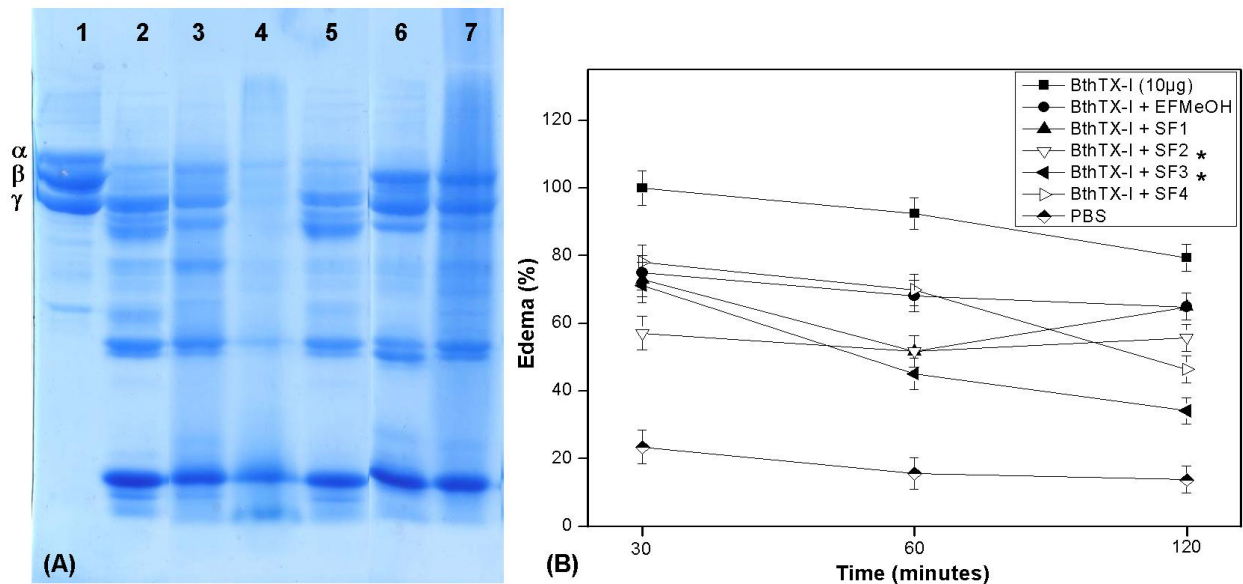
Figure 2. (**A**) SDS-PAGE of the proteolytic activity of *B. jararacussu* venom with and without *S. erecta* extracts upon bovine fibrinogen. Lanes: **1**, fibrinogen control (40 µg); **2**, fibrinogen + Bjussu; **3**, fibrinogen + Bjussu + EFMeOH; **4**, fibrinogen + Bjussu + SF1; **5**, fibrinogen + Bjussu + SF2; **6**, fibrinogen + Bjussu + SF3; **7**, fibrinogen + Bjussu + SF4. The *B. jararacussu* snake venom (10 µg) was preincubated with the extracts for 30 minutes at 37°C (1:30, w/w). (**B**) Inhibition of the edema-inducing activity of BthTX-I by methanolic extract of *S. erecta* and its fractions. BthTX-I (10 µg) was preincubated with the extracts for 30 minutes at 37°C (1:30, w/w). Results are expressed as mean ± SD (n = 5). * p < 0.05, when compared to control activity.
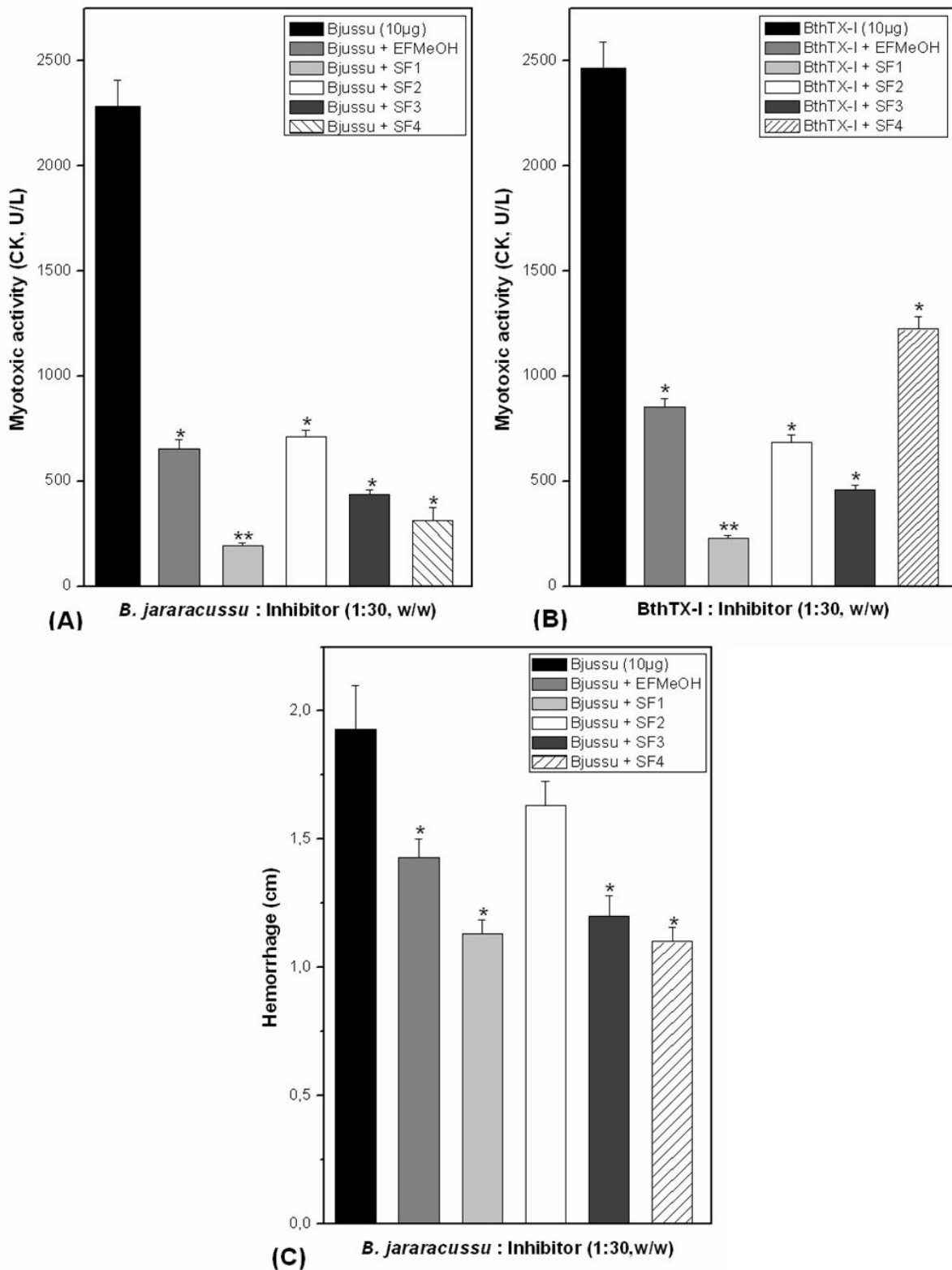
Figure 3. (**A**) Inhibition of the myotoxic activity induced by *B. jararacussu* crude venom (10 μg) by the methanolic extract of *S. erecta* and its fractions. (**B**) Inhibition of the myotoxic activity induced by myotoxin BthTX-I (10 μg) by the methanolic extract of *S. erecta* and its fractions. (**C**) Inhibition of the hemorrhagic activity of *B. jararacussu* crude venom (10 μg) by the methanolic extract of *S. erecta* and its fractions. Venom/ toxin was preincubated with the extracts for 30 minutes at 37°C (1:30, w/w). Results are expressed as mean ± SD (n = 5). * $p < 0.05$ and ** $p < 0.01$, when compared to control activity.

Table 1. Inhibition of the coagulant activity of *B. jararacussu* venom (20 µg) by the methanolic extract of *S. erecta* and its fractions, preincubated at 1:30 (w/w) ratio for 30 minutes at 37°C

Samples	Coagulant activity (min)	Sample	Coagulant activity (min)
B. jararacussu	1 min 20 s ± 0.03	PBS + Ca+2	5 min 15 s ± 0.03
B. jararacussu + EFMeOH	1 min 30 s ± 0.02	EFMeOH	2 min 43 s ± 0.04
B. jararacussu + SF1	1 min 50 s ± 0.03 *	SF1	1 min 42 s ± 0.02
B. jararacussu + SF2	1 min 36 s ± 0.03	SF2	2 min 30 s ± 0.02
B. jararacussu + SF3	> 50 min **	SF3	> 50 min
B. jararacussu + SF4	> 50 min **	SF4	> 50 min

Results are expressed as mean ± SD (n = 3). * $p < 0.05$ and ** $p < 0.01$, when compared to control activity.

also known to inhibit PLA_2 activity (3, 25, 26).

Some compounds from snake venoms show large diversity in their proteolytic activity and fibrinogen is one of their main substrates. These proteases can convert fibrinogen into fibrin. Once again, fractions SF3 and SF4 were capable of preventing the complete degradation of the fibrinogen chains (Figure 2 – A). The fractions effectively antagonized the defibrinogenating activity induced by *B. jararacussu* snake venom. Previously studied plant extracts also revealed efficient results in the protection of fibrinogen, including *Bauhinia forficata* (27), *Citrus limon* and *Ficus nymphaeifolia* (4, 9).

The formation of mast-cell-mediated edema in the presence of PLA_2s is the result of enzymatic activity. Nevertheless, venom PLA_2s with no enzymatic activity activate mast cells by an independent mechanism of catalytic activity (28). In assays of the edema-inducing activity (Figure 2 – B), the methanolic extract of *S. erecta* and its fractions inhibited BthTX-I diminishing the formation of edema. Other plant extracts also inhibited the formation of edema-inducing activity, such as *Mandevilla velutina*, *Cordia verbanacea*, *Mikania glomerata* and *Blutaparon portulacoides* (29-32).

Local tissue damage induced by *Bothrops* venoms is caused by hemorrhage, proteolysis, myonecrosis and edema. Myotoxic activity is the result of the action of hemorrhagic metalloproteases or myotoxic PLA_2s (33). The myotoxicity induced by *B. jararacussu* snake venom (Figure 3 – A) and its isolated myotoxin, BthTX-I (Figure 3 – B), was significantly decreased

by preincubation with the methanolic extract of *S. erecta* and its fractions. Some medicinal plants with antiophidian activity also showed promising results in the inhibition of myonecrosis induced by snake venom, including *Calendula officinalis* (34) and *Tabernaemontana catharinensis* (35).

The methanolic extract of *S. erecta* and its fractions decreased the hemorrhagic activity induced by *B. jararacussu* venom (Figure 3 – C). One of the most beneficial first-aid treatments in viperid snakebites is the neutralization of hemorrhagic symptoms. These results suggest an interaction between the extract components and metalloproteases, which inhibited their hemorrhagic activities. Antiophidian plants with antihemorrhagic properties have been identified in different studies as *Calendula officinalis*, *Baccharis trimera*, *Mikania glomerata*, *Casearia sylvestris* and *Eclipta alba* (26, 31, 34, 36, 37).

Bothrops venom usually produces hemorrhages due to a considerable degradation of fibrinogen and other clotting factors which prevents clot formation (1). The methanolic extract of *S. erecta* and its fractions were able to delay the clotting time of citrated plasma after addition of the venoms at a 1:30 ratio, but fractions SF3 and SF4 were more efficient in that, since they considerably inhibited the coagulant activity of *B. jararacussu* venom (Table 1). Results with fractions SF3 and SF4, rich in flavonoids and tannins, showed that these compounds act as powerful inhibitors of the hemorrhagic and clotting activity, probably due to interaction with metalloproteases and thrombin-like enzymes, respectively. Studies of several plants (*Heliconia curtispatha, Pleopeltis*

percussa, Brownea rosademonte, Bixa orellana, Trichomanes elegans, Struthanthus orbiculareis and *Casearia sylvestris*) describe the inhibitory effect of all or part of the coagulant activity of snake venoms from *B. asper, B. jararacussu, B. pirajai, B. neuwiedi, B. moojeni* and *C. d. terrificus* (9, 38, 39).

CONCLUSION

Ophidian accidents caused by *Bothrops* snakes are characterized by prominent local tissue damage due to myonecrosis, hemorrhage and edema, and unfortunately antivenoms are unable to antagonize the myotoxic effects of phospholipase myotoxins or the hemorrhagins in the venom (1). Vegetal extracts have served as alternatives for treatment or as an additional therapy for local tissue damage, since they are sources of chemical compounds with several pharmacological activities of medical-scientific interest. Folk medicine shows several examples of claimed medicinal plants including the popular use of *S. erecta* for treating inflammatory and ulcerative diseases. Possibly its antiophidian activity is due to the presence of phenolic compounds such as flavonoids and tannins. These compounds present the ability to inhibit toxic activities of *B. jararacussu* snake venom and the isolated myotoxins BthTX-I and II. Other studies were also able to demonstrate the role of phenolic compounds in inhibiting the effects of snake venoms (40-42). Furthermore, possible isolated compounds can be used as molecular models of inhibitors for the development of new therapeutic agents in treatment of ophidian accidents. This extract is a promising source of natural inhibitors, such as flavonoids and tannins, which probably act by forming complexes with metal ions and proteins that inhibit the action of serine proteases involved in blood coagulation disturbances and metalloproteases responsible for hemorrhagic processes and enzymatic activity of phospholipases A_2.

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