

ANTI-INFLAMMATORY ACTIVITY OF *Blutaparon portulacoides* ETHANOLIC EXTRACT AGAINST THE INFLAMMATORY REACTION INDUCED BY *Bothrops jararacussu* VENOM AND ISOLATED MYOTOXINS BthTX-I AND II

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ABSTRACT: This article reports the anti-inflammatory effect of *Blutaparon portulacoides* (*B. portulacoides*), specifically the ethanolic extract of its aerial parts, on the edema formation and leukocyte influx caused by *Bothrops jararacussu* (*B. jararacussu*) snake venom and Bothropstoxin-I and II (BthTX-I and II) isolated from this venom as an alternative treatment for *Bothrops* snakebites. The anti-inflammatory effect of *B. portulacoides* ethanolic extract was compared with an animal group pretreated with dexamethasone. *B. portulacoides* ethanolic extract significantly inhibited paw edema induced by *B. jararacussu* venom and by BthTX-I and II. Also, results demonstrated that the extract caused a reduction of the leukocyte influx induced by BthTX-I. However, the extract was not capable of inhibiting the leukocyte influx induced by the venom and by BthTX-II. In conclusion, these results suggest that the ethanolic extract of this plant possess components able to inhibit or inactivate toxins present in *B. jararacussu* venom, including its myotoxins, responsible for the edema formation. However, the leukocyte migration caused by the venom and BthTX-II was not inhibited by the plant, probably due to the different mechanisms involved in the edema formation and leukocyte influx. This is the first report of *B. portulacoides* extract as anti-inflammatory against snake venoms and isolated toxins.

KEY WORDS: *Bothrops jararacussu*, myotoxins, inflammation, edema, *Blutaparon portulacoides*.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Plants have often been used by humans, sometimes successfully, against numerous diseases caused by different pathological agents. Pharmacological studies have demonstrated that the extracts and fractions from some of these plants used in traditional medicine possess anti-inflammatory, antiviral and antiophidian properties that constitute an alternative for ophidic accident treatment, displaying a large diversity of chemical compounds with several pharmacological activities of medical-scientific interest (1-7). The antiophidian activity of several plant species in general use in some Brazilian communities has been investigated (2, 8-15).

B. portulacoides, belongs to the Amaranthaceae family, Magnoliopsida class, Caryophyllales order, characterized by A. L. Jussieu in 1789. Herbaceous plants of annual and permanent growth are established predominantly in temperate, subtropical and tropical regions. In Brazil they occur mainly in the Atlantic bush (16). In folk medicine, *B. portulacoides* has been employed for the treatment of leucorrhea (17). In an experimental model, the crude extract presented trypanocidal and leishmanicidal activity *in vitro*, and also antimicrobial activity (18).

Snake venoms are complex mixtures of proteins including phospholipases A₂, myotoxins, hemorrhagic metalloproteases and additional proteolytic enzymes, cytotoxins, cardiotoxins, among others. The pathophysiology of snake envenomation involves a complex series of events that depend on the combined action of these venom components (19). The venom of the snake *B. jararacussu* causes symptoms similar those provoked by other *Bothrops* species including hemorrhage (4, 20, 21), myonecrosis (22, 23) and edema (24). The local effects caused by *B. jararacussu* venom are due to, at least in part, its myotoxin content (25). Two myotoxins with PLA₂ structure, BthTX-I and BthTX-II, have been isolated and characterized from *B. jararacussu* snake venom (26, 27). These proteins can be classified into two categories: the Asp49 PLA₂, catalytically active, and Lys49 PLA₂, devoid of significant catalytic activity upon artificial substrate (28-30). BthTX-I (Lys 49) induces several pharmacological effects which include edema, mastocyte degranulation, irreversible blockade of muscle contraction, liposome disruption, and cytotoxicity upon muscle and endothelial cells (26, 27, 31). BthTX-II (Asp 49) induces edema and leukocyte migration (32, 33).

In this study, we examined the anti-inflammatory activity of *B. portulacoides* ethanolic extract against edema formation and leukocyte influx induced by *B. jararacussu* snake venom and its main isolated myotoxins.

MATERIALS AND METHODS

Plant Material

Aerial parts of *B. portulacoides* (Amaranthaceae) were collected at Restinga de Maricá, Rio de Janeiro, RJ, Brazil, in December 2002 and identified by Prof. Dr. Josafá Carlos de Siqueira (PUC-Rio, Rio de Janeiro, RJ). A voucher specimen was deposited at the Herbarium of the Department of Biology, Ribeirão Preto School of Philosophy, Sciences and Literature, University of São Paulo, SP, Brazil (registration number SPSFR 02961).

Preparation of Plant Extract

The powdered, air-dried aerial parts of the plant (1000 g) were extracted exhaustively by maceration at room temperature with hexane and ethanol successively, in the powder/solvent mass ratio of 1:20 (mass/volume). The biomass was filtered from the extracts and the solvents were removed under vacuum in a rotary evaporator (below 40°C), to obtain the hexanic (yield = 6 g) and ethanolic (yield = 90 g) crude extracts.

Venom and Myotoxins

Lyophilized crude venom of *Bothrops jararacussu* were supplied by Dr. José Carlos Cogo, Serpentarium of CEN (Nature Center of Study), UNIVAP, São José dos Campos, SP, Brazil. Myotoxins bothropstoxins-I (BthTX-I) and bothropstoxin-II (BthTX-II) were supplied by Dr. Andreimar M. Soares, from the University of São Paulo, USP, Ribeirão Preto, SP, Brazil. BthTX-I and II were isolated and purified as previously described (26, 27, 34).

Animals

All animal care was in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and approved by the Committee for Ethics in Animal Research of UNIVAP, under number A117/2007/CEP. Male, Swiss mice (22-25 g) (45 days old) were used through the experiment, randomly divided into six

groups of five animals each. Animals were kept in plastic cages, with water and food *ad libitum*, and maintained under controlled temperatures (26°C) and on a 12 hour light/dark cycle.

Dose-response Curve

To determine the optimum dose of *B. portulacoides* the animals received intraperitoneal (i.p.) injections of *B. portulacoides* ethanolic extract at the doses 100, 250 or 500 mg/kg (constant volume of 100 µL), one hour before venom (0.1 mg/kg) and the edema was measured.

Evaluation of Paw Edema

Fifty microliters of sterile saline containing 0.1 mg/kg of *Bothrops jararacussu* venom or 0.4 mg/kg of myotoxins (BthTX-I and BthTX-II) was injected in the subplantar region of the right hind paw in a total volume of 50 µL. The left hind paw received an equal volume of sterile saline alone and served as control. The volumes of both hind paws were measured by plethysmometry (Plethysmometer model 7140®, Ugo Basile, Italy) before and 15, 30 minutes, 1, 2, 4 and 6 hours after venom or myotoxins administration according to the method describe by Van Arman *et al.* (35). The edema was expressed as the percentage increase in the volume of the treated (right) paw relative to that of control (left) paw at each time interval. As anti-inflammatory control we used dexamethasone (1 mg/kg).

Evaluation of Leukocytes Influx in the Peritoneal Cavity

Bothrops jararacussu venom (0.2 mg/kg) or myotoxins (0.8 mg/kg BthTX-I or 0.4 mg/kg BthTX-II) dissolved in 1 ml of sterile saline were injected i.p.. Groups of animals were killed six hours after the injections and the inflammatory exudates were withdrawn after washing the peritoneal cavity. Leukocytes were harvested by washing cavities with 2 mL of saline containing heparin (5 U/mL). Aliquots of the washes were used to determine total cell counts in a Neubauer chamber after dilution in Turk solution (0.2% crystal violet dye in 30% acetic acid). Differential leukocyte counts were performed on stained Instant Prov.

Statistical Analysis

Mean and standard deviation were calculated for each group. To establish whether the difference between the mean values of any two experimental groups was significant the Student's t-test was performed, using a statistical significance level of $p < 0.05$. When more than two groups were compared a two-way analysis of variance was applied, followed by the Tukey-Kramer test.

RESULTS

Effects of *B. portulacoides* Ethanolic Extract on Edema Formation Induced by *Bothrops jararacussu* Venom

Intraplantar injection of *B. jararacussu* venom (0.1 mg/kg) caused time-dependent paw-volume increases. Edema formation peaked one hour after injection of *B. jararacussu* venom (Figure 1).

To determine the optimum extract dose for inhibiting edema induced by *B. jararacussu* venom, animals were pretreated with 100, 250 or 500 mg/kg, i.p., of *B. portulacoides* one hour before the injection of 0.1 mg/kg of venom (Figure 1). The dose of 100 mg/kg showed a small, non-significant reduction of edema. The extract doses of 250 and 500 mg/kg showed a significant reduction of the edematogenic effect ($*p < 0.05$) in comparison to the edema induced by the venom. *B. portulacoides* at 500 mg/kg inhibited the edema formation by 53%, 36%, 35%, 37% and 39%, 30 minutes, 1, 2, 4 and 6 hours, respectively, whereas the dose of 250 mg/kg, in the same periods of time, presented reductions of 13%, 20%, 19%, 20.6%, 28.5%. These results were comparable with dexamethasone. From these findings, a dose of 500 mg/kg i.p. of *B. portulacoides* was used in the subsequent studies.

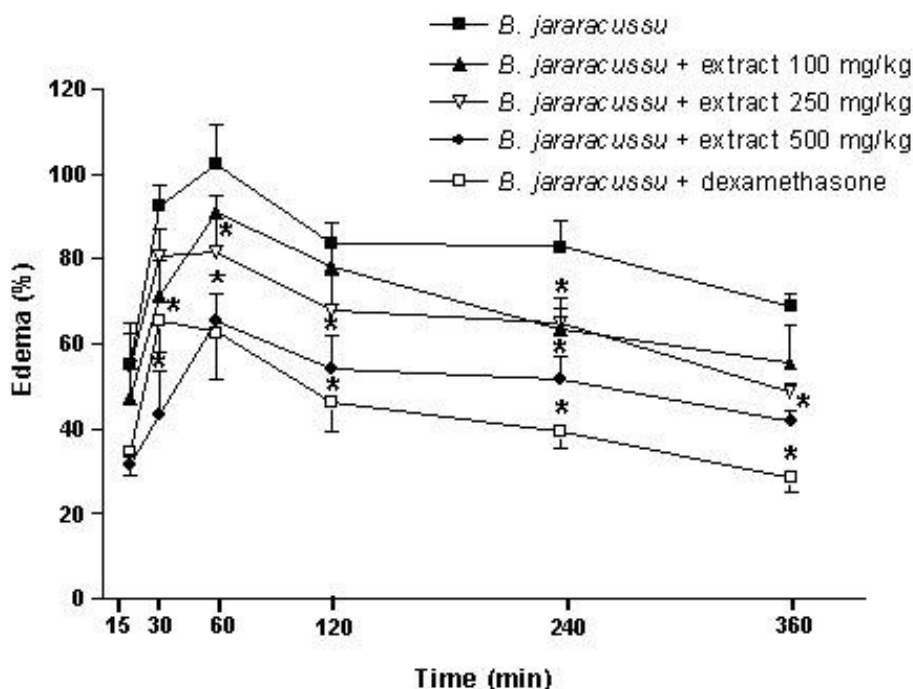


Figure 1. Antiedematogenic effect of the ethanolic extract from the *B. portulacoides* aerial parts on paw edema induced by *B. jararacussu* venom. Mice injected with *B. jararacussu* venom (0.1 mg/kg), were pretreated with the extract (100, 250, 500 mg/kg, i.p.) one hour before the venom. The other animal group was pretreated with dexamethasone (1 mg/kg, i.p.). Edema was evaluated plethysmographically at various time intervals (15, 30 minutes, 1, 2 4 and 6 hours) after venom injection and was expressed as the percentage of increase in the volume of the right as compared to the left footpad. Results are presented as mean \pm SEM ($n = 5$). * $p < 0.05$.

Effects of *B. portulacoides* Ethanolic Extract on Edema Formation Induced by Bothropstoxin I and II

The edema formation was evaluated after the intraplantar injection of BthTX-I or BthTX-II isolated from the *B. jararacussu* venom at the dose of 0.4 mg/kg or saline (control). The kinetics of edema formation was evaluated 15, 30 minutes, 1, 2, 4 and 6 hours after the BthTX-I or BthTX-II injection. BthTX-I provoked edema formation that started 15 minutes after its injection and was elevated for 6 hours. The edema induced by BthTX-II had started by 15 minutes after its intraplantar application, followed by a peak at one hour and diminution in subsequent hours (Figure 2). The

potential of the antiedematogenic effect of the extract, applied through the intraperitoneal (i.p) route, demonstrated that the BthTX-I extract caused a significant reduction of the edematogenic effect from 30 minutes of its administration, producing reductions of 48.2%, 38.9%, 36.9%, 45.1% and 45.0% at 30 minutes, 1, 2, 4 and 6 hours, respectively (Figure 2A). For BthTX-II, the significant reduction of the edematogenic effect had started by 15 minutes, yielding respective reductions of 50.1%, 37.9%, 61.8%, 36.1%, 43.1%, 28.2% at 15 minutes, 30 minutes, 1, 2, 4 and 6 hours (Figure 2B).

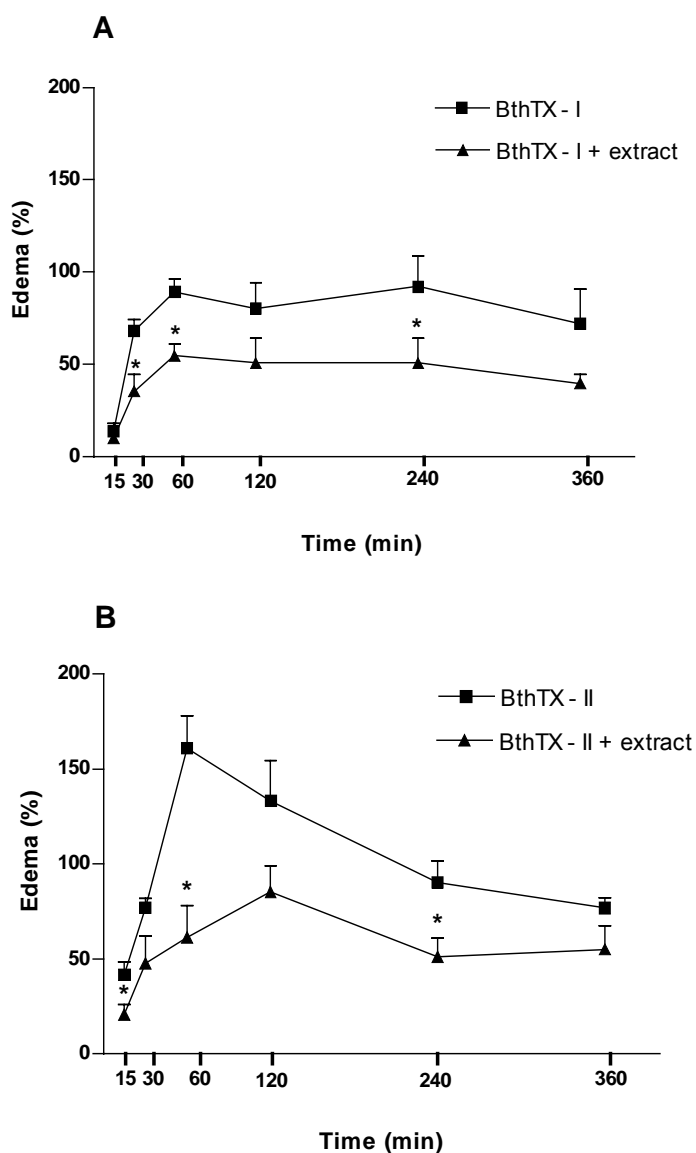


Figure 2. Effects of ethanolic extract from the *B. portulacoides* aerial parts on edema formation induced by BthTX-I and BthTX-II. (A) BthTX-I and (B) BthTX-II. Mice were pretreated with the *B. portulacoides* ethanolic extract (500 mg/kg, i.p.) one hour

before BthTX-I or II injection. Then, after extract administration groups of mice were injected in the right paw with BthTX-I or BthTX-II (0.4 mg/kg) and saline (control) in the left paw. Edema was evaluated plethysmographically at various time intervals after myotoxin injection and expressed as the percentage increase in the volume of the right footpad as compared to the left footpad. Results are presented as mean \pm SEM ($n = 5$). * $p < 0.05$.

Effects of *B. portulacoides* Ethanolic Extract on Leukocyte Migration Induced by *Bothrops jararacussu* Venom

The leukocyte migration into the peritoneal cavity was evaluated six hours after i.p. injection of 0.2 mg/kg of the *B. jararacussu* venom. As shown in Figure 3 A, i.p. injection of *B. portulacoides* followed by i.p. injection of *B. jararacussu* caused accumulation of $8,430 \pm 1037 \times 10^3/\text{mL}$ leukocytes, which did not differ from that of venom alone ($7,900 \pm 604 \times 10^3/\text{mL}$). Differential leukocyte counts are displayed in Figures 3B and 3C. An influx of polymorphonuclear (PMN) and mononuclear (MN) cells was induced by the venom (PMN: $6020 \pm 768 \times 10^3/\text{mL}$; MN: $1879 \pm 340 \times 10^3/\text{mL}$). PMN and MN influxes caused by *B. jararacussu* were not inhibited by the *B. portulacoides* extract (PMN: $6,541 \pm 948 \times 10^3/\text{mL}$; MN: $1,741 \pm 531 \times 10^3/\text{mL}$) when compared with venom alone.

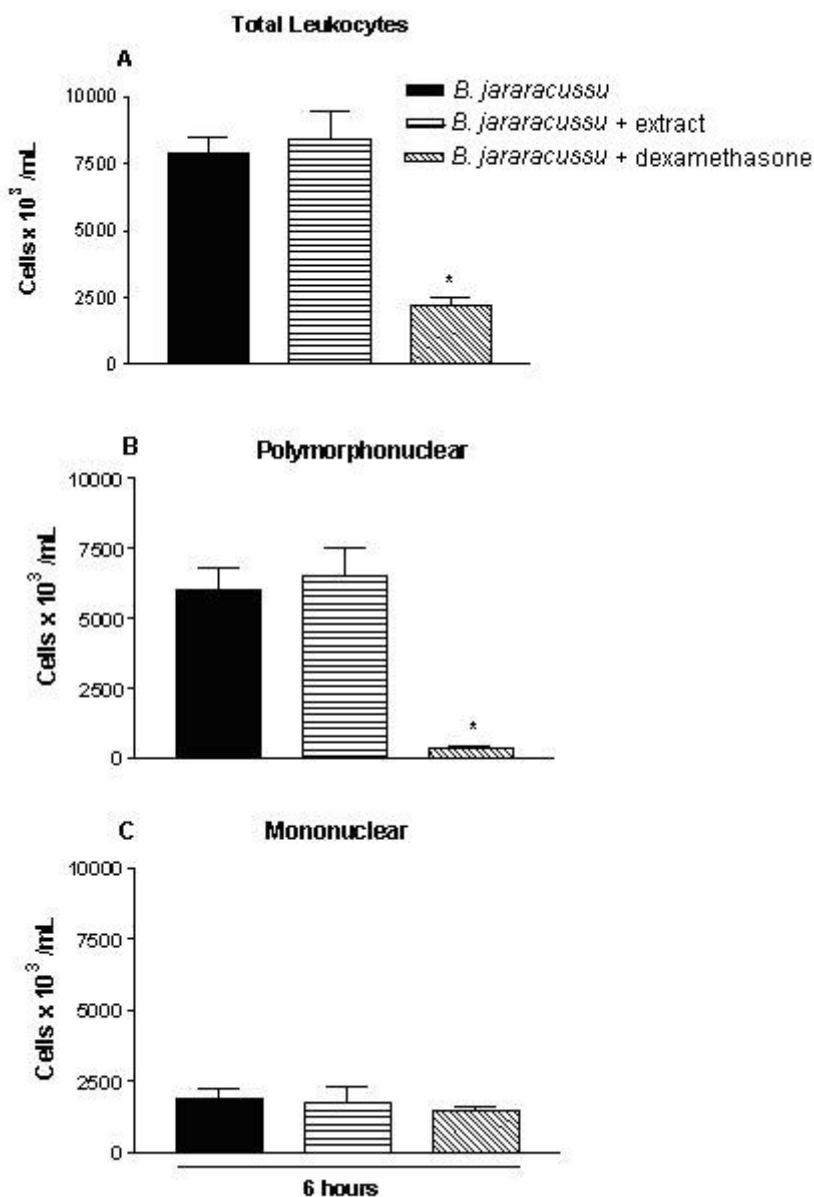


Figure 3. The effect of *B. portulacoides* ethanolic extract on leukocyte influx into the peritoneal cavity induced by *B. jararacussu* venom. Mice injected with *B. jararacussu* venom (0.4 mg/kg, i.p) dissolved in 1 mL of sterile saline and were pretreated with the extract (500 mg/kg, i.p.). Another group was pretreated with dexamethasone (1 mg/kg i.p.). Total leukocytes (A), PMN cells (B) and MN cells (C). Mice were killed after six hours and inflammatory exudates were withdrawn after washing the peritoneal cavity. Results are presented as mean \pm SEM ($n = 5$). * $p < 0.05$.

Effects of *B. portulacoides* Ethanolic Extract on Leukocyte Migration Induced by BthTX-I and II

Leukocyte migration into the peritoneal cavity was evaluated six hours after i.p. injection of BthTX-I or BthTX-II (0.8 mg/kg of BthTX-I or 0.4 mg/kg of BthTX-II). Figure 4A demonstrates the number of total leukocytes in the peritoneal cavity of the animals that had received BthTX-I or BthTX-II injection and those pretreated with 500 mg/kg of the extract. BthTX-I and BthTX-II caused a significant and similar leukocyte influx into the peritoneal cavity. The extract treatment significantly inhibited the induced leukocyte influx caused by BthTX-I. However, the leukocyte influx induced by BthTX-II was not inhibited by the extract. The differential leukocyte counts in the peritoneal cavity six hours after the i.p BthTX-I or BthTX-II injection are shown in Figures 4B and 4C. Figure 4B shows an important PMN cell influx, with predominance of neutrophils, after the injection of both myotoxins. The *B. portulacoides* extract inhibited PMN influx induced by BthTX-I, but not by BthTX-II (Figure 4B). Figure 4C shows the number of MN leukocytes, BthTX-II provoked significantly higher influx of these cells when compared with the BthTX-I.

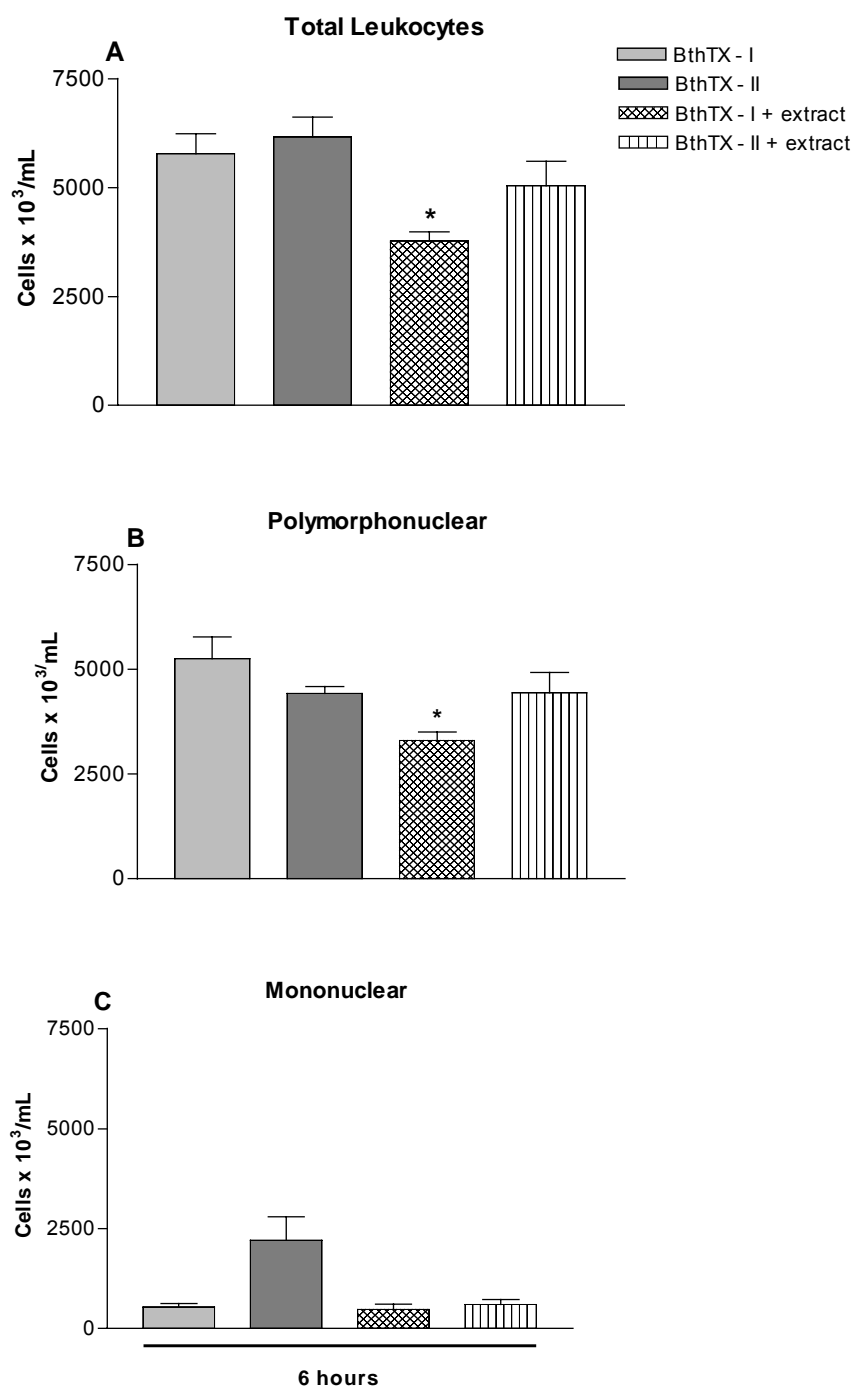


Figure 4. The effect of *B. portulacoides* ethanolic extract on leukocyte influx into the peritoneal cavity induced by two myotoxins isolated from *B. jararacussu* venom. Mice were pretreated with the extract (500 mg/kg) one hour before injection of BthTX-I or BthTX-II (0.8 mg/kg of BthTX-I and 0.4 mg/kg of BthTX-II). Mice were killed after six hours and inflammatory exudates were withdrawn after washing the peritoneal cavity. Total leukocytes (**A**), PMN cells (**B**) and MN cells (**C**). Results are presented as mean \pm SEM ($n = 5$). * $p < 0.05$.

DISCUSSION

Clinical investigations have demonstrated the efficacy of antivenoms in neutralizing life-threatening systemic effects associated with snakebites (36, 37). However, bothropic antivenom does not effectively neutralize the local effect induced by *Bothrops* venom (38, 39). Some alternatives have been proposed as adjuvants of the antivenoms, including those based on the use of plant extract.

Vegetal extracts constitute an excellent alternative source of novel antiophidian agents. In many countries, vegetal extracts have been traditionally used in the treatment of envenomations caused by snakebites (12-15, 40). Therefore, several plants have already demonstrated antivenom activity (2, 7, 11, 41). However, in most cases, scientific proof of their antiophidian activity is still needed.

Envenomation by the *B. jararacussu* species leads to signs and symptoms similar to those provoked by other *Bothrops* species and emphasis has been placed on acute alterations such as hemorrhage, edema and necrosis (42). The present study showed that the venom induces paw edema that peaks one hour after venom injection. This result corroborates previous studies showing the ability of *Bothrops* venom to induce edema (24, 38, 43).

B. portulacoides it is known popularly as “capotiraguá” and in folk medicine this plant has been employed to treat leucorrhoea. In a previously phytochemical study the following compounds were isolated and identified within the hexane and ethanol extracts of *B. portulacoides* aerial parts: a methylenedioxyflavonol, the isoflavone irisone B and the steroids stigmasterol, sitosterol and campesterol (44). In an experimental model, the crude extract and some isolated compounds presented trypanocidal and leishmanicidal activity *in vitro*, and also antimicrobial activity (18).

In our experimental model, *B. portulacoides* ethanolic extract showed anti-inflammatory effect against edema formation induced by crude venom and myotoxins, which were similar to dexamethasone. This result is similar to that found by Ticli *et al.* (24), using the same experimental model and venom, but with *Cordia verbenacea* methanolic extract. Also, Borges *et al.* (41) evidenced the ability of *Casearia sylvestris* extract (Flacourtiaceae) to inhibit edema-inducing activity of both whole venom and myotoxin II of *Bothrops moojeni*. Several reports have shown that plant-derived compounds, that act on arachidonic acid metabolism, lead to a marked inhibition of edema. Some of them have been characterized as inhibitors of

cyclooxygenase and lipoxygenase activities (2). Additionally, some plants active against the lethal effect of snake venoms also act against the edema induced by venoms (6, 45).

In the present study, we also evaluated the capacity of *B. portulacoides* ethanolic extract to inhibit leukocyte migration to the peritoneal cavity induced by the venom or by studied myotoxins. Neutrophils play a vital role in host defense. They are usually the first cell type to reach the injury site and predominate numerically in a recent lesion. Neutrophil attraction and migration are effected by chemoattractants generated in the injured area (46). PMN and MN infiltration into the location of *Bothrops* inoculation was previously reported in the literature (47-51).

Our results demonstrated that *B. jararacussu* venom was capable of inducing a marked influx of leukocytes to the injection location with predominance of neutrophils. These data corroborate the results of Barbosa *et al.* (52) that showed the capacity of the *B. jararacussu* venom to recruit leukocytes to gastrocnemius muscle. We also evaluated the capacity of the myotoxins to induce an influx of leukocytes. The results had demonstrated that both BthTX-I, devoid of enzymatic activity and BthTX-II, enzymatically active, were able to induce leukocyte influx to the location of its injection. The literature reports the presence of infiltrated PMN and MN after the injection of myotoxic PLA₂ from the venoms of *Bothrops nummifer* (53) and *Bothrops jararacussu* (54) in skeletal muscle of mice, and after intrapleural administration of myotoxins isolated from *Bothrops jararacussu* venom and *Bothrops pirajai* (32). Thus, it is possible to suggest that the PLA₂ contained in *B. jararacussu* venom contributes to the leukocyte migration evoked by *B. jararacussu* venom.

The results also show that the *B. portulacoides* extract at the concentration of 500 mg/kg did not reduce the leukocyte migration induced by the venom or by BthTX-II, whereas *B. portulacoides* extract at 500 mg/kg was capable of reducing the number of total leukocytes and polymorphonuclear cells induced by BthTX-I. It is possible that, in some sense, PLA₂ activity is important for leukocyte migration, probably by inducing a more powerful release of inflammatory mediators necessary for leukocyte migration. In this aspect, the literature shows that PLA₂ Asp49 is more powerful than the Lys49 in inducing inflammatory mediators such as IL-1, IL-6, TNF- α , beyond activating important adhesion molecules for the migration of leukocytes (55).

From these investigations, it may be concluded that *B. portulacoides* ethanolic extract showed anti-inflammatory effect against *B. jararacussu* venom-induced edema formation. In addition, *B. portulacoides* ethanolic extract also inhibited inflammatory effects induced by isolated myotoxins from this venom, an action in which BThTX-I and II may participate. However, the migration induced by the venom and myotoxins was not inhibited by the extract, probably due to the fact that different mechanisms are involved in this event and in edema formation.

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