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ORIGINAL ARTICLE

Anti-Inflammatory Effect of *Bougainvillea × buttiana* (Var. Orange) Extract in Experimental *Bothrops jararaca* Envenomation

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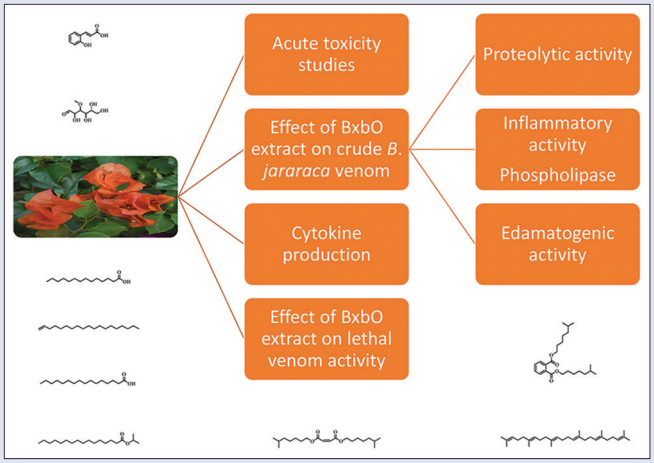
ABSTRACT

Background: *Bougainvillea x buttiana* is a plant used in folk Mexican medicine to treat different inflammatory diseases. **Objective:** In this study, the anti-inflammatory effect of *B. x buttiana orange* extract (BxbO) was evaluated on edema formation, cytokine production, and lethality in mice in response to venom of *Bothrops jararaca* (VBj) snake. **Materials and Methods:** The BxbO extract was tested *in vitro* to determine its effect on phospholipase A_2 (PLA_2) and *in vivo* for the formation of edema, the paw edema model was used, as well as the toxicity of the extract and the production of cytokines. Lethality induced by VBj, the survival percentage, was calculated. **Results:** BxbO extract significantly inhibited *in vitro* PLA_2 and *in vivo,* blocked the edema formation and cytokine production, and prevented lethality induced by VBj. The constituents of BxbO may bind to components of VBj, such as PLA_2, thereby blocking the proteolytic action of the venom. In animals treated with BxbO extract injected 1 h after the venom injection, no difference was observed in the cytokine secretion. In contrast, for all mice treated with BxbO extract for 1 h before VBj administration or together with VBj, the pro-inflammatory cytokine secretion in the serum was attenuated and an exacerbated production anti-inflammatory cytokine. In the presence of the BxbO extract injected 1 h before the VBj injection or together with the VBj injection, mortality was significantly lower. **Conclusion:** Altogether, our results show that BxbO extract can inhibit the local and systemic activities of VBj. However, new studies are still required to identify the interaction mechanisms between bioactive compounds and cellular components.

Key words: *Bothrops jararaca venom*, *Bougainvillea x buttiana*, cytokines, phospholipase

SUMMARY

- *B. x buttiana* is a plant used in traditional Mexican medicine to treat inflammatory diseases, in this work the anti-inflammatory effect was determined *in vitro* by evaluating the effect on PLA_2 and *in vitro* the formation of edema and cytokine production, as well as the decreased lethality by injection of *Bothrops jararaca* venom.



Abbreviations used: TNF-α: Tumor necrosis factor; IL-1β: Interleukin-1 beta; IL-6: Interleukin 6; IFN-γ: Interferon-gamma; BxbO: Extract from *Bougainvillea x buttiana;* VBj: *Bothrops jararaca* venom.

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INTRODUCTION

According to data from the World Health Organization, an estimated 2,500,000 cases of snake poisoning occur per year, resulting in approximately 125,000 deaths and several cases ensuing in severe sequelae.[1] The *Bothrops* genus (Family Viperidae, subfamily Crotaline) contains 20 species that have been classified so far. Serpents of the same species but of different geographic areas may present venoms with different composition and toxicity. Victims from *Bothrops* snake accidents generally manifest both local effects, consisting of edema, ecchymoses, blisters, necrosis, local hemorrhage, and inflammation, as well as signs of systemic poisoning, which include spontaneous bleeding, blood incoagulability, arterial hypotension, acute myocardial damage, pulmonary edema, and acute renal failure. Local necrosis is one of the extreme effects that is associated with bothropic envenomation, as this effect may necessitate limb amputation.[2]

Snake venom contains complex components that exert a series of effects, most of which are biologically active toxic and non-toxic

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proteins.[3] *Bothrops* sp. venoms contain various compounds that are responsible for their biological activity, including enzymes, such as phospholipase A_2 (PLA_2), metalloproteases, L-amino acid oxidases, serinoproteases, and compounds without enzymatic activity, including nerve growth factors, lectin Type C, natriuretic peptides, cysteine-rich proteins, and myotoxins.[4] In addition, PLA_2 has been shown to have several pharmacological activities, which include neurotoxic, myotoxic, edematogenic, hypotensive, platelet aggregation, anticoagulant, and cardiotoxic activities.[1,2,5]

Bothropic envenomation is characterized by an intense inflammatory process at the site of the venom injection, which is attributed to the action of toxins that act upon the endothelial cells of capillaries and venules, increasing their permeability. Certain components of the venom result in the release of histamine from mast cells, and PLA_2 induces the release of arachidonic acid from membrane phospholipids, initiating a pathway that leads to the synthesis of prostaglandin. In addition, proteases that act on plasma kininogens lead to the release of kinins (e.g., bradykinin), and components C3 and C5a of the complement system participate in the inflammatory reaction by causing an expansion in vascular permeability.[5] At the site of inoculation, there is an excessive inflammatory reaction that is recognized by firm edema, pain, flushing, and local hemorrhage; necrosis may also be observed.[6] The following inflammatory mediators have been identified: vasoactive amines, eicosanoids, platelet-activating factor, plasma kinins, complement system, free radicals, cytokines, and others. The type and relative quantity of each mediator varies depending on the developmental phase of the response and, in certain cases, depending on the type of stimulus that initiates the process. Rapid-acting mediators, which include vasoactive amines and eicosanoids, modulate the immediate response. Then, neosynthesized mediators, including leukotrienes and interleukins (ILs), are released and begin to manipulate the accumulation and activation of cells within the inflamed site. Once the leukocytes have reached the site of the lesion, they start to produce new mediators, which determine the evolution of the process.[7]

Prominent local edema is a common cause of snake envenomation. This intense edematogenic reaction frequently ends in ischemia and neural compression, which can potentially result in a compartmental syndrome, which can, in turn, cause permanent tissue failure, incapacitation, or amputation of the damaged limb due to necrosis.[1,2,8] However, in contrast to other local effects, which can be a result of the direct activity of specific toxins, the edematogenic effect is apparently the end result of the combined action of various toxins found in *Bothrops* venoms, which act rapidly on the connective and muscular tissues, leading to the release of numerous endogenous inflammatory mediators. Several studies have reported the inflammatory response produced by paw edema in mice induced by *B. jararaca* venom, and studies have demonstrated that the secretion of pro-inflammatory mediators, such as IL-6, (IL-1β), tumor necrosis factor α (TNF-α), and macrophagic inflammatory protein 2, is relevant to the development of acute inflammation.[5] The effect of venom on the modulation of endogenous mediators typically decreases the effectiveness of the antiophidic serum because although antivenom is capable of neutralizing the toxins, it cannot diminished, the inflammation generated by the chemical mediators released as a result of these toxins. For this reason, the antiophidic serum is not capable of reversing local tissue damage that is already established.[6,9]

Currently, serum therapy is the only specific treatment available to combat the toxic effects of snake envenomation.[9,10] The antiophidian serum is a neutralizing antibody formulation prepared from the serum of hyperimmunized animals against a specific toxin, a specific venom or, more commonly, a pool of various ophidian venoms.[11] Serum therapy provides the affected patient with high-affinity antibodies to ophidian venoms to assist with the removal of the toxins that are responsible for the toxicity of envenomation.[10]

The intention of this study is not to replace the use of serum therapy, but rather to use a plant extract as an alternative and/or complementary treatment, as medicinal plants are highlighted as an affluent source of natural inhibitors and pharmacologically active compounds. There are diverse communications of the applicability of medicinal plants for treating serpent bites worldwide.[12,13] The main advantages of using plants are as follows: plants are an inexpensive form of treatment, and they are easily accessible, stable at room temperature, and capable of neutralizing a wide spectrum of toxins.[12] Although many plants may not neutralize envenomation itself, they can be used to relieve some of the symptoms of snake bite poisoning, especially the local effects.[13] It has been reported that the tranquilizing, immunostimulating, and/or anti-inflammatory activity of certain medicinal plants may be of great value in relieving certain symptoms of poisoning.[13] We previously reported the anti-inflammatory and immunomodulatory effects of the *Bougainvillea x buttiana* extract, which is an endemic plant that is extensively used in traditional Mexican medicine for the treatment of various diseases.[14] Arteaga Figueroa *et al.* (2017)[15] reported the presence of nine compounds identified using gas chromatography mass spectrometry, in the BxbO extract. The compounds and their respective concentrations used in all assays are described in Table 1.

The current study was designed to determine the ability of the BxbO extract to neutralize the inflammation induced by venom of *Bothrops jararaca* (VBj) using the following three parameters: (a) proteolytic activity, (b) inflammatory process (phospholipase, edematogenic activity, and cytokine production), and (c) comparison of cytokine balance and survival rate.

MATERIALS AND METHODS

Reagents

Actinomycin D, ethanol, phosphoric acid, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), azocasein, naphthylenediamine, dimethylformamide, o-phenylenediamine, RPMI-1640 medium, and 4-nitro-(3-octanoyloxy)-benzoic acid) were acquired from Sigma-Aldrich Chemical Co. (Toluca, Mexico). Fetal bovine serum was Fetal calf serum was acquired from Gibco Life Technologies Corporation (Grand Island, NY, USA). L-929 cells were purchased from ATCC (Manassas, VA, USA). The kits including the monoclonal antibodies used in the ELISA assay were purchased from BD Biosciences Pharmingen (CA, USA).

Plant material, extraction, and identification

B. x buttiana (Variety Orange) bracts were authenticated in the HUMO herbarium from Centro de Investigación en Biodiversidad y Conservación (CIByC), UAEM voucher specimen (No. 23683). The extract of *B. x buttiana* (variety Orange) (BxbO) was maintained, processed, and standardized as defined in the patent (MX343163B).

Table 1: Compounds concentrations in *Bougainvillea x buttiana* extract

Compound	Concentrations (μg/g)
3-(2-hydroxyphenyl)-,(E)-2-propenoic acid	6.77
3-O-Methyl-d-glucose	6.29
Tetradecanoic acid	2.02
1-Nonadecene	1.63
n-Hexadecanoic acid	9.47
Isopropyl palmitate	4.22
Diisooctyl maleate	6.95
1,2-Benzenedicarboxylic acid, diisooctyl ester	6.74
Squalene	5.12

Acute toxicity studies

Groups of animals 15–20 g maintained under basic laboratory conditions were used for toxicity testing as described by Organisation for Economic Co-operation and Development Guideline number 425, 2008.[16] Groups of six mice were orally treated with aqueous and organic phases purified and dried from *B. x buttiana* extracts at one of four doses (5, 50, 500, and 2000 mg/kg). Animals were individually observed for the control of toxic symptoms such as locomotion, convulsions, and mortality for 72 h. All the observations of the different parameters were systematically recorded and maintained for each mouse individually. In mice injected with 500 and 2000 mg/kg remained under observation for 14 d for additional toxicity study.

Venom

Lyophilized VBj was obtained from Dr. R. Zucatelli Mendonça (Instituto Butantann, SP, Brazil) and stored at −20°C preceding to use in the subsequent experiments.

Animals

The female BALB/c mice weighing 15–20 g that were used in this study were acquired from the Bioterio del Instituto Nacional de Salud Publica (Cuernavaca, Morelos). For acclimatization, the animals were allocated in the Laboratorio de Inflamación y Toxicología (Facultad de Medicina UAEM) and maintained with free access to water and food under controlled illumination, humidity, and temperature, according to the national and international standards for animal experimentation. Management of animal use followed the principles and guidelines approved by the Guide for the Use of Laboratory Animals, while euthanasia followed the Euthanasia Practice Guidelines. All animal handling was performed according to the guidelines of the Committee on Ethics in the Use of Animals of Facultad de Medicina UAEM (CCUAL-FM-UAEM Protocol N° 005/2016). At the end of all animal experiments were exposed to carbon dioxide euthanasia and subsequently sacrificed by cervical dislocation.

Effect of BxbO extract on crude *Bothrops jararaca* venom

Proteolytic activity

The effect of BxbO extract on the proteolytic activity of VBj venom was observed using azocasein.[17] Briefly, various amounts of venom (15 μg/mL) were preincubated with BxbO extract (0.1–200 μg/mL) for 30 min at 37°C. In separate tubes, either VBj alone or a solution comprising (0.2% azocasein, 20 mM $CaCl_2$, and 0.2 M Tris-HCl pH 8.8) was added. The enzymatic reaction ran for 90 min at 37°C and was interrupted by the addition of 400 μL of 20% trichloroacetic acid. The tubes were allowed to stand at 25°C for 30 min and were then centrifuged at 20,000 rpm for 10 min at 25°C. Next, 1.0 mL of the supernatants were removed and mixed with 500 μL of NaOH (2M). After 10 min, the final solution was analyzed by spectrophotometry at an absorbance of 450 nm. The results were expressed as a percentage of proteolytic activity as the mean ± standard mean error, with $n = 4$.

Inflammatory activity

Phospholipase

The effect of the BxbO extract on the PLA_2 activity of the total *B. jararaca* venom was measured according to the protocol adapted for microplates, described by Cotrim *et al.*, 2011.[18] The assay mixture was prepared by combining 200 μL of a solution containing (10 mM Tris-HCl, 10 mM $CaCl_2$, and mM NaCl, pH 8.0), 20 μL of chromogenic substrate (4-nitro-(3-octanoyloxy)-benzoic acid 10 mM), 20 μL of VBj (40 μg), and 20 μL of BxbO (0.1–200 μg) dissolved in a 10% phosphate-buffered saline dimethyl sulfoxide solution to a final volume of 260 μL. Two blanks were used, including the general blank (in which all components of the reaction mixture were added, except for VBj and BxbO) and the blank extract (in which all components were added except for the venom). The absorbance was determined at 425 nm at 10 min intervals using a BioRad microplate reader.

Edematogenic activity

The paw edema model described by Albano *et al.* (2010)[18] was used to evaluate the *in vivo* inflammatory activity of *B. jararaca* venom. Four groups of five female BALB/c mice were used to generate a paw edema model by intraplantar injection in the right hind paw. The following four treatment protocols were used: (a) VBj (20 μg/50 μL) alone; (b) VBj (20 μg/50 μL) 1 h before the oral injection of BxbO extract (200 μg/100 μL); (c) VBj (20 μg/50 μL) 1 h after the oral injection of BxbO extract (200 μg/100 μL); and (d) VBj (20 μg/50 μL) i.p. injected incubated together the oral injected with BxbO extract (200 μg/50 μL). The paw thickness was measured before the experimental injections (baseline volume) and at the indicated time intervals (0, 30, 60, 90, and 120 min) using a digital caliper. The percentage of edema was calculated based on the increase in paw thickness, which was estimated by the subtraction of the basal volume.

Cytokine production

To evaluate the effect of BxbO on cytokine production caused by VBj, the mice were separated into distinct groups. Four treatment protocols were used: (a) VBj 1.5 LD_{50} (110 μg/mouse) i.p. injection; (b) VBj 1.5 LD_{50} (110 μg/mouse) i.p. injection 1 h before the oral injection of BxbO extract (200 μg/100 μL); (c) VBj 1.5 LD_{50} (110 μg/mouse) i.p. injection 1 h after the oral injection of BxbO extract (200 μg/100 μL); and (d) VBj (20 μg/50 μL) i.p. injected and oral injection of BxbO extract (200 μg/50 μL). For each group at 0, 2, 4, 8, 12, and 24 h after treatment, the retro-orbital bleeding blood was recovered from the plexus retro-orbital, and the serum collection was maintained at −20°C until the samples were analyzed for mediator quantification. The samples from the mouse serum of each treatment were used to evaluate the levels of IL-1β, interferon-gamma (IFN-γ), IL-10, and IL-6. Each cytokine was evaluated using an ELISA kit, according to the manufacturer's instructions (Pharmingen). The minimal level of detection for all cytokines was 10 pg/mL. To quantify the levels of TNF-α, the L929 cell line was used in accordance with the method described by Ruff and Gifford.[19]

Effect of BxbO extract on lethal venom activity

To verify the effect of the extract on lethal venom activity, the following four treatments were designed: (a) VBj 1.5 LD_{50} (110 μg/mouse) i.p. injection; (b) VBj 1.5 LD_{50} (110 μg/mouse) i.p. injection 1 h before the oral injection of BxbO extract (200 μg/100 μL); (c) VBj 1.5 LD_{50} (110 μg/mouse) i.p. injection 1 h after the oral injection of BxbO extract (200 μg/100 μL); and (d) VBj (20 μg/50 μL) i.p. injected together with the oral injection of BxbO extract (200 μg/50 μL). Each group included 10 mice. The survival analysis was recorded every 12 h for 144 h, by the following formula: % Survival rate = (Number of animals survivors/Total number of animals) × 100

Statistical analysis

The results of these tests, expressed as the mean ± standard deviation, were subjected to one-way analysis of variance, followed by a multiple comparison test of Bonferroni to establish differences between the concentrations used in each test and the results obtained from the mixture of each extract with the poison. Differences were considered

to be significant at $P < 0.05$. GraphPad Prism Version 6 software (San Diego, CA, USA) was used to prepare the graphs and perform the statistical analysis.

RESULTS

Extract toxicity and compounds

For studies of acute toxicity, the mice treated with 1000 mg/kg of BxbO did not show mortality or physical changes eyes, nasal respiratory rate, circulatory signs, or autonomic effects. Since none of the described toxic signs or symptoms or mortality was observed in the animals at the above-mentioned dose, 5 up to 15 mg/kg body weight of extract were selected for the evaluation of anti-inflammatory activity (data not shown).

Effect of BxbO extract on crude *Bothrops jararaca* venom

Proteolytic activity of Bothrops jararaca venom

To determine the neutralizing effect of BxbO extract against the proteolytic activity of VBj, hydrolysed azocasein was used. As shown in Figure 1, the BxbO extract (0.1–200 μg/mL) inhibited the proteolysis induced by VBj. The BxbO extract decreased the proteolytic activity of VBj in a concentration- and time-dependent manner. At concentrations of 0.1 and 1 μg/mL, the BxbO extract caused a mild reduction in the proteolytic effects of VBj. However, 10 μg/mL of the BxbO extract caused a 52% reduction in the VBj proteolytic activity at 30 min. The maximal inhibition of the proteolytic activity of VBj at 30 min was obtained with BxbO extract at concentrations of 100 and 200 μg/mL, with proteolytic activities of 10% and 0%, respectively.

Phospholipase activity of Bothrops jararaca venom

The use of plant extracts with antiphospholipase activity has been widely studied. The capacity of the extract to reduce the phospholipase activity of VBj is shown in Figure 2a. The BxbO extract reduced the phospholipase activity of VBj in a concentration- and time-dependent manner. The phospholipase activity of VBj in the presence of BxbO extract at concentrations of 0.1, 1, and 10 μg/mL was mildly altered during the 30 min of the reaction [Figure 2a]. However, when the concentration of the BxbO extract was elevated to 100 or 200 μg/mL, the phospholipase activity of the VBj was significantly reduced ($P < 0.001$). Figure 2b shows the percentages of reduction of the phospholipase activity of the VBj caused by the presence of the BxbO extract at amounts of 0.1, 1, 10, 100, and 200 μg/mL during the 30-min reaction. At concentrations of 100 and 200 μg/mL, the BxbO extract resulted in a 40% and 60% reduction of the phospholipase activity of the VBj, respectively.

According to the two above-mentioned experiments that were performed to analyze the proteolytic and phospholipase activity, the most effective concentration of the BxbO extract was determined to be 200 μg/mL; therefore, this concentration was used in the subsequent assays.

Effect of BxbO extract on edema formation induced by venom of *Bothrops jararaca*

The effect of BxbO extract on the inflammatory activity of VBj was evaluated using the paw edema model. Various amounts of the extract, when injected independently, induced edema in mice (data not shown). A subplantar injection of *B. jararaca* venom (20 μg/50 μL) induced an increment in paw volume of 45%–50% at 30–120 min posttreatment [Figure 3]. In the mice treated with 20 μg/50 μL of VBj 1 h before the oral injection of the BxbO extract (200 μg/100 μL), no difference in edema volume was observed when compared with the mice that were injected with that VBj alone [Figure 3]. In contrast, in the mice that were treated with VBj 1 h after the oral injection of the extract of BxbO or VBj together with BxbO extract in all the intervals of times studied in this study, the volume of the edema caused by the venom was significantly reduced ($P < 0.001$) [Figure 3].

Effect of BxbO extract on cytokine production induced by venom of *Bothrops jararaca*

The effect of the extract on the secretion of cytokines induced by VBj injection was evaluated in the serum from mice treated with the

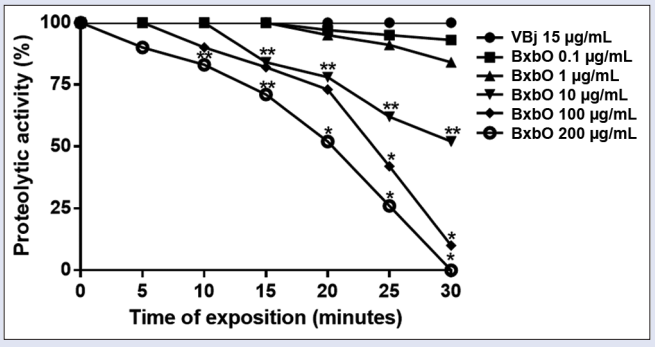

Figure 1: Effect of BxbO extract on proteolysis induced by venom of *Bothrops jararaca*. BxbO extract 0.1, 1, 10, 100, and 200 μg/mL were incubated for 30 minutes at room temperature with 15 μg/mL venom of *Bothrops jararaca*. Data reported mean ± standard deviation ($n = 4$). *$P < 0.001$ and **$P < 0.01$ for the difference between treated groups and the venom

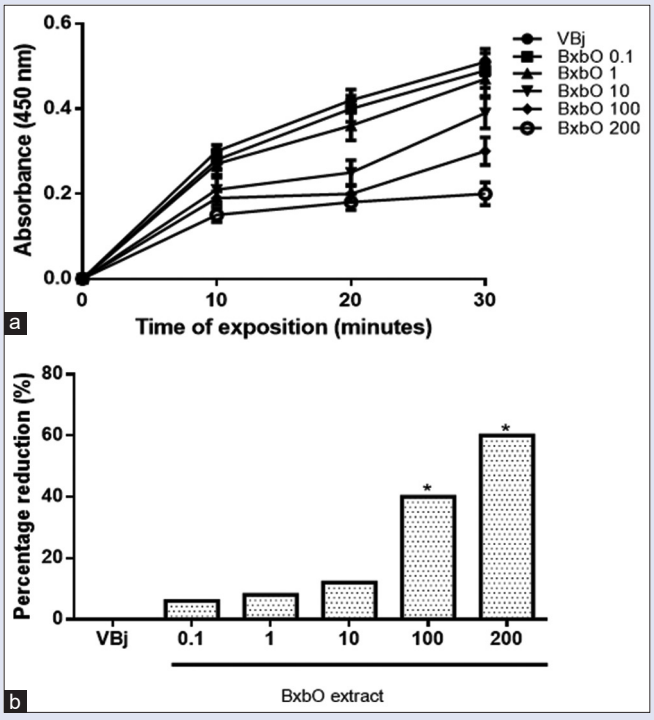

Figure 2: Effect of BxbO extract on phospholipase activity of venom of *Bothrops jararaca*. (a) Absorbance obtained in the BxbO extract 0.1, 1, 10, 100, and 200 μg/mL incubated for 30 minutes at 25°C with 40 μg/mL venom of *Bothrops jararaca*. (b) Reduction percent. Data reported mean ± standard deviation ($n = 4$). *$P < 0.001$ for the discrepancy between treated groups and the venom

BxbO extract 1 h before or 1 h after VBj or together with BxbO extract. The treatment with VBj caused a rapid elevation of cytokines, such as IL-1, IL-6, TNF-α, and the IL-10, concomitant with the development of envenomation. The kinetics of the cytokine production are shown in Figure 4. The cytokines that were produced most abundantly as a result of VBj alone were IL-6 at 4 h, IL-1β and TNF-α at 18 h, and IL-10 at 24 h. For the mice treated with BxbO extract injected 1 h after the venom injection, a similar cytokine secretion pattern was observed [Figure 4]. In contrast, for all mice treated with BxbO extract for 1 h before the administration of VBj or together with VBj, the cytokine secretion in the serum was significantly attenuated ($P < 0.01$ and $P < 0.001$) [Figure 4]. IL-10 production was detected between 12 and 24 h after the VBj injection [Figure 4]. Similar results were obtained in sera from mice treated with BxbO extract 1 h after the venom injection. However, for the animals treated with BxbO extract 1 h before the VBj injection or along with the VBj injection, the IL-10 production was significantly increased ($P < 0.001$) [Figure 4].

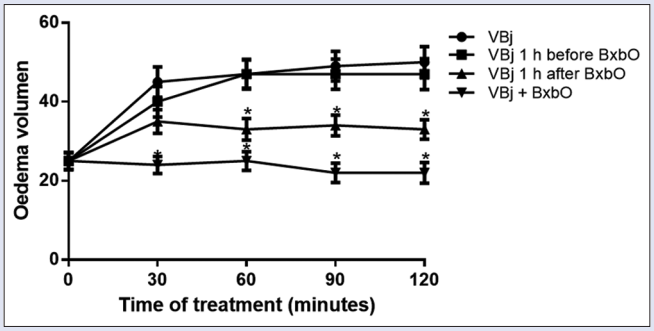

Figure 3: Effect of BxbO extract on edematogenic activity of venom of *Bothrops jararaca*. The effect of BxbO extract on edematogenic activity of venom of *Bothrops jararaca* was evaluated by use of the paw edema model. Groups of mice were treated with 20 μg/50 μL venom of *Bothrops jararaca* and BxbO extract (200 μg/mL) as described in Materials and Methods. Data reported mean ± standard deviation ($n = 4$). *$P < 0.001$ for the difference between treated groups and the venom

Effect of BxbO on balance of cytokines induced by venom of *Bothrops jararaca*

To assess the relationship between pro-inflammatory cytokines and anti-inflammatory cytokines after the VBj injection and to search the effect of the extract on the attenuation of these cytokines, the cytokine ratios were calculated. The relationship between the proportions of IL-1β/IL-10, IL-6/IL-10, and TNF-α/IL-10 was calculated and compared with lethality. In the mice treated with VBj or BxbO extract, the cytokine ratios were measured at 0, 2, 4, 8, 12, 18, and 24 h. The severity score of the envenomation was found to be associated with cytokine ratios that were elevated immediately after the VBj injection [Figure 5]. In contrast, the treatment with BxbO extract 1 h before the VBj injection or along with the VBj injection caused a significant decrease in these cytokine ratios [Figure 5].

Effect of BxbO extract on deaths of mice treated with venom of *Bothrops jararaca*

When we compared the survival rate with the cytokine ratios, the highest number of deaths of the mice treated with VBj coincided with the maximum imbalance of cytokines [Figure 6]. In the presence of the BxbO extract injected 1 h before the VBj injection or together with the VBj injection, mortality was significantly lower in comparison with the groups treated with venom alone ($P < 0.001$).

DISCUSSION/CONCLUSION

The search for alternative and complementary treatment to traditional serotherapy and a safe antidote against snake venoms has been long. Therapy with antivenom not only fails to efficaciously neutralize the local effects of envenomation but also presents problems in its use.[20] Given the precarious distribution and the difficulties of access of anti-venom, many populations are obliged to seek therapeutic alternatives for this type of injury, usually in the field of herbal medicine.[12,21,22] The plant

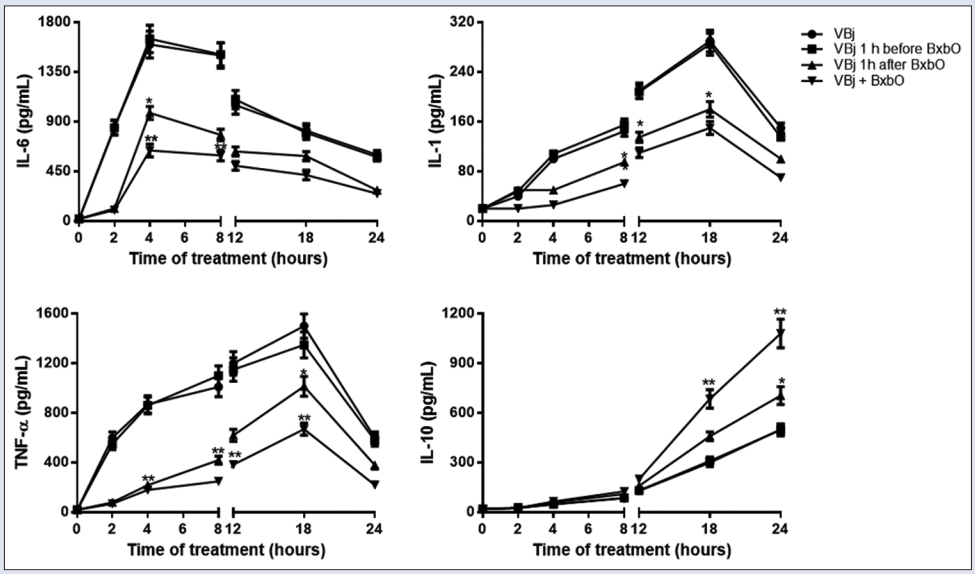

Figure 4: Effect of BxbO extract on levels of cytokine induced by venom of *Bothrops jararaca*. The effect of BxbO extract on cytokine production induced by venom of *Bothrops jararaca* was evaluated in serum from mice exposed to an indicated treatment. Groups of mice were treated with 1.5 LD_{50} of venom of *Bothrops jararaca* and BxbO extract (200 μg/mL) as described in Methods. Data reported mean ± standard deviation ($n = 4$). *$P < 0.01$ and **$P < 0.001$ for the variation among treated groups and the venom

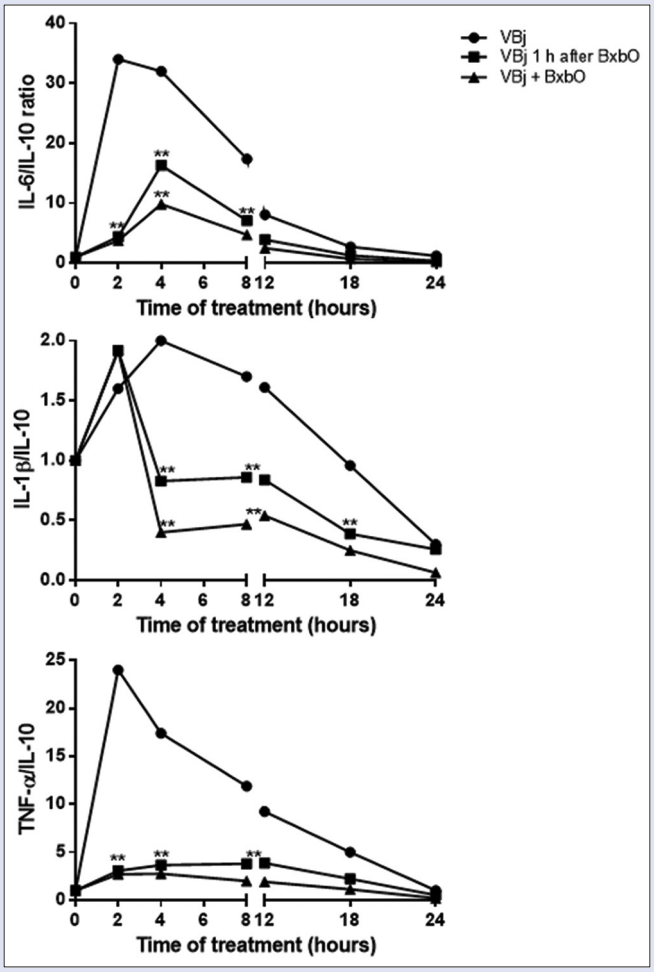

Figure 5: Effect of BxbO extract on cytokine ratios induced by venom of *Bothrops jararaca*. The ratio was evaluated in serum from mice exposed to an indicated treatment. Groups of mice were treated with 1.5 LD_{50} of venom of *Bothrops jararaca* and BxbO extract (200 μg/mL) as described in Methods. Data reported mean ± standard deviation ($n = 4$). *$P < 0.01$ and **$P < 0.001$ for the variation among treated groups and the venom

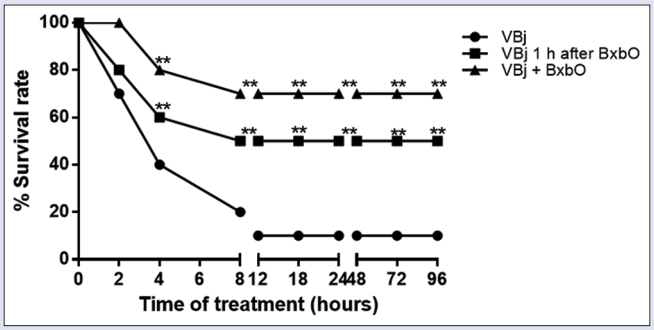

Figure 6: Effect of BxbO extract on lethality induced by venom of *Bothrops jararaca*. The effect of BxbO extract on cytokine production induced by venom of *Bothrops jararaca* was evaluated in groups of mice exposed to an indicated treatment. Groups of mice were treated with 1.5 LD_{50} of venom of *Bothrops jararaca* and BxbO extract (200 μg/mL) as described in Methods. The animals were observed up to 96 hours and each 12 hours survival was recorded. Data reported mean ± standard deviation ($n = 10$). *$P < 0.01$ and **$P < 0.001$ for the variation among treated groups and the venom

kingdom has contributed alternatives to therapy with antivenom in the form of folk medicines that suppress the envenomation symptoms, but like preparations are not extensively recognized for use in the practice medicine.[21,22] Extracts of medicinal plants are widely used to treat snake accidents, primarily by populations in regions where access to serum therapy is deficient or nonexistent. Expanding upon traditional knowledge, scientific evidence has appeared on the anti-snake venom properties of various extracts.[21,22] In this context, several species of plants with anti-snake venom properties have been studied to validate traditional knowledge and identify the substances with pharmacological activity that are able to neutralize the local and systemic effects of snake envenomation. Plant extracts may contain various chemical components, such as alkaloids, tannins, flavonoids, triterpenes, and lignins, which have the capacity to inhibit snake venom and neutralize enzymes or chemicals.[22,23]

The enzyme PLA_2 from snake venoms exerts diverse functions, including cardiotoxicity, myotoxicity, edema, hypotension, and anticoagulation, among others.[6,8] In this study, BxbO extract inhibited the proteolytic and PLA_2 of VBj. These inhibitions may be related that the phytocompounds present in the extract of BxbO have synergistic and additive effects. This could create variations in the mode of action depending on their concentration. Among the 9 compounds found in the BxbO extract, 4 of them, such as 3-(2-hydroxyphenyl)-, (E)-2-propenoic acid; 3-O-methyl-glucose, *n*-hexadecanoic acid, and squalene, are able in the modulation of the immune response.[24,25] That is, some compounds could be bind to the active site, or a coordination site, leading to a decrease or inhibition of the enzyme activity. These results obtained are in accordance with other communications which described that various extracts from plants exhibited the capacity to neutralize different venoms.[12,25]

Bothropic venom can induce pathophysiological changes, and the primary effects of venom are caused by inflammation and tissue injury. The diffusion of inflammatory mediators leads to inflammatory activities in reaction to damage. We recently reported that the oral administration of BxbO extract resulted in anti-inflammatory activity, reducing the paw edema promoted by carrageenan.[13,14,15] Our results have demonstrated that BxbO extract neutralizes the toxic activities of *B. jararaca* venom *in vitro* and *in vivo*. BxbO extract was shown to be capable of suppressing the proteolysis induced by venom. Reduction of edema may be the end result of the inhibition of the enzymatic components of the venom. The potential of BxbO extract to inhibit the PLA_2 activity of VBj was confirmed, and this action may be associated with the reduction of the edema. In addition, other substances are capable of inhibiting PLA_2 activity and the inflammatory edema caused by bothropic poisons.[26]

Envenomation typically ends in excessive inflammatory and edema reactions, and PLA_2 enzymes associate with other enzymes. In this study, the serum concentrations of pro- and anti-inflammatory cytokines were significantly elevated in the mice treated with VBj. Our results showed that the BxbO extract significantly inhibited the secretion of pro-inflammatory cytokines and elevated the production of anti-inflammatory cytokines induced by VBj. These reductions on cytokine production can be related to the compounds present in extract that was demonstrated to exert anti-inflammatory activities by downregulation of TNF-α, IL-1, IL-6, and IFN-γ levels in murine model.[27] In this study, the mice injected with venom exhibited an increase in the production of pro-inflammatory cytokines, demonstrating a shift to a high basal Th2 response. IL-10 levels contributed more to the specific difference in basal cytokine response. IL-1β, IL-6, and TNF-α were also the strongest predictors of envenomation. The high ratios of IL-1β/IL-10, IL-6/IL-10, and TNF-α/IL-10 were associated with the severity of the poisoning and, consequently, lethality. However, it is important to note that a significant

reduction in the IL-1β/IL-10, IL-6/IL-10, and TNF-α/IL-10 ratios was detected in animals treated with the BxbO extract 1 h before the VBj injection or along with the VBj injection. These results indicate a positive effect of the BxbO extract on the balance of pro- and anti-inflammatory cytokines. Taken together, these results indicate a positive effect of the BxbO extract on the balance of pro- and anti-inflammatory cytokines. The positive or negative effects of inflammatory cytokines are dependent on many factors, such as the type of cytokine produced, the functional status and the type of cells stimulated, and the concentration and duration of cytokine exposition. In our experiments, the preincubation of BxbO extract with VBj averted the death of 70% of the animals, and the oral pretreatment with BxbO extract 1 h before the VBj injection decreased the lethality >60%. The ability of the BxbO extract to reduce VBj lethality can be seen as the sum of the neutralization of each toxic effect. However, the lethality potency test is the standard for assessing the efficacy of potential antiophidic substances.[23]

Our results demonstrated that the extract of *Bougainvillea x buttiana* (Variety: Orange) had a considerable inhibitory effect on the inflammatory activity induced *in vitro* and *in vivo* by *B. jararaca* venom. This extract was demonstrated to inhibit the following activities in *B. jararaca* venom: PLA_2 activity, edematogenic activity, cytokine secretion, and lethality. These findings suggest that BxbO extract can serve as a complementary treatment for victims bitten by *B. jararaca*.

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Conflicts of interest

There are no conflicts of interest.

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