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Evaluation of the immunomodulatory effect of *Bougainvillea xbuttiana* (Var. Orange) on the cytokine production induced by *Botrhops jararaca* venom in macrophages

[Evaluación del efecto inmunomodulador de *Bougainvillea xbuttiana* (var. naranja) sobre la producción de citoquinas de macrófagos inducida pelo veneno de *Bothrops jararaca*]

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Abstract: To study the effect of 50% ethanol extract of *Bougainvillea xbuttiana* on the enzymatic activity, cell viability and cytokine production provoked by the venom of *Bothrops jararaca* in macro-phages. Three assays were used to study the effects of *B. xbuttiana* extract on the damage pro-duced by *B. jararaca*: Enzymatic activity was detected by measuring the proteolytic and phos-pholipase A2; macrophages cytotoxicity was determined by the MTT method; levels of cytokine were evaluated using ELISA and a biological assay. After treatment with 300 µg/mL *B. xbuttiana* extract for 30 min, the proteolytic and phospholipase A2 activities of the venom were reduced to 95 and 61%, respectively. In macrophages cultures treated with *B. xbuttiana* extract combined with venom, the production of TNF-α, IL-6 and IFN-γ was reduced, whereas IL-10 was potenti-ated. Our results support the potential effect of the *B. xbuttiana* extract as a complementary therapy against the toxicity caused by the venom of *B. jararaca* snakes.

Keywords: *Bothrops jararaca*; Venom; *Bougainvillea xbuttiana*; Cytokines; Macrophages

Resumen: Estudiar el efecto del extracto etanólico al 50% de *Bougainvillea xbuttiana* sobre la actividad enzimática viabilidad celular y producción de citoquinas provocada por el veneno de *Bothrops jararaca* en macrófagos Se utilizaron tres ensayos para estudiar los efectos del extracto de *B. xbuttiana* sobre el daño producido por *B. jararaca*: Se detectó actividad enzimática mediante la medición del proteolítico y fosfolipasa A2; la citotoxicidad de los macrófagos se determinó por el método MTT; Los niveles de citoquinas se evaluaron utilizando ELISA y un ensayo biológico. Después del tratamiento con 300 µg/mL de extracto de *B. xbuttiana* durante 30 min, las actividades proteolíticas y de fosfolipasa A2 del veneno se redujeron a 95 y 61%, respectivamente. En cultivos de macrófagos tratados con extracto de *B. xbuttiana* combinado con veneno, la producción de TNF-α, IL-6 e IFN-γ se redujeron, mientras que IL-10 se potenció. Nuestros resultados apoyan el efecto potencial del extracto de *B. xbuttiana* como terapia complementaria frente a la toxicidad provocada por el veneno de *B. jararaca*.

Palabras clave: *Bothrops jararaca*; Veneno; *Bougainvillea xbuttiana*; Citocinas; Macrófagos

INTRODUCTION

The envenoming caused by the *Bothrops* genus snakes is a public health problem in South America (Resiere *et al*., 2020). The severity of the clinical picture depends not only on the aggressor animal, but also on the victim´s age, weight, state of health, part of the body where bite occurred, depth of the bite, individual susceptibility, etc (Amazonas *et al.*, 2018, Del-Rei *et al*., 2019). Snake venom is a highly complex mixture containing various proteins, primary enzymes, as well as nucleotides, metal ions and pigments, which together cause ophidian toxicity. The venom has several actions, including: proteolytic (due to proteases, phospholipases A, L-amino-oxidase, hyaluronidase, nuclease and vasoactive substances releasing factors), coagulant (L-arginine esterase that transforms fibrinogen into fibrin) and vasculotoxic (metalloproteins that act on vascular endothelia). The high concentrations of these enzymes in the ophidian venom explain the haemorrhagic, local and systemic effects, as well as the haemostatic disturbances observed after bite. The general symptomatology of the toxicity induced by *Bothrops* sp. occurs in the originating local tissue and includes damage oedema, pain, haemorrhage, myonecrosis and infiltration of leukocytes, whereas systemic effects include coagulopathies, systemic haemorrhage, nephrotoxicity, and cardiotoxicity, which can lead to death (Williams *et al.*, 2019). These effects are primarily caused by metalloproteases, serinoproteases, phospholipases, disintegrins, lectins, among other proteins (Rashidi *et al*., 2020) and by release of endogenous mediators, such as histamine, kinins and prostaglandins, due to the action of venom components on mast cells, kininogens and phospholipids (Días *et al*., 2017; Péterfi *et al*., 2019). During the envenomation caused by *Bothrops* snake venom, bleeding can be particularly severe, resulting in non-coagulable blood. Furthermore, haemorrhages in the vessels contribute to potentially fatal haemorrhaging (Gutiérrez *et al*., 2016). Myotoxins with phospholipase A_2 structure may cause muscle necrosis. These myotoxins can affect the integrity of the plasma membrane of skeletal muscle through a mechanism that is not directly related to the hydrolysis of phospholipids. In addition, ischaemia is observed in the musculature as a consequence of drastic vascular damages (Péterfi *et al*., 2019).

In the literature, there are several studies showing that *Bothrops jararaca* venom is a complex mixture of components able to induce several changes in the inflammatory response (Menezes *et al*., 2019). These disproportionate differences are invariably associated with the release of immunological mediators. In the specific case of *B. jararaca* venom, several studies have shown that this venom is capable of inducing the increase of leukotriene B_4, thromboxane A_2, interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) (Menaldo *et al*., 2017; Boda *et al*., 2018). At the site of the snake bite, an intense inflammatory reaction occurs. After an injury, activated macrophages produce excessive levels of mediators that can lead to damage in host tissues (Moreira *et al*., 2020).

Studies related to venomous animals constitute an interesting source of investigation, both to know the pathological effects caused by the venom and to achieve different therapeutic potential of its constituents (De Souza *et al.*, 2018). In this context, the use of medicinal plants has been practiced for many generations. In addition, plants are natural sources for the development of many valuable medical contributions (Monroy & Monroy, 2006). Natural resources are the largest reservoir of pharmacologically active compounds, and isolated secondary metabolites such as phenols, flavonoids, alkaloids, steroids, could be important in the development of new therapeutics (Figueroa *et al*., 2014; Abarca-Vargas *et al*., 2016). Therefore, plant species can be beneficial for the treatment of different diseases. These compounds act through the formation of complexes, and unions have demonstrated hydrogen bridge between phenolic groups and amides of protein chains. They have anti-inflammatory, anti-hepatotoxic, anti-arrhythmic, hypocholesterolaemic, anti-allergic, anti-tumour, and other properties. The most decisive activity from the point of anti-venom is the inhibition of enzymes. There are many investigations reporting the use of plants with anti-ophidic properties. A considerable number of studies have described immunomodulatory properties in various medicinal plants (Jorge *et al*., 2019). Some plants are able to inhibit both cellular and humoral responses, while others are capable of stimulating the immune response.

Mankind has successfully used plants against numerous diseases caused by different pathological agents. Among plants used globally in medicine, several species have verified anti-snake venom

activity, shielding people and animals towards snakebite (Riera-Borrull *et al*., 2017; Jorge *et al*., 2019; Resiere *et al*., 2020). Several compounds, such as different flavonoids with anti-inflammatory activity, had been discovered in large quantities in one-of-a-kind plants (Veronese *et al*., 2005; Arteaga Figueroa *et al*., 2017; Jorge *et al*., 2019). For toxicity provoked by the *Bothrops* genus, the handiest tolerable treatment is application of specific anti-venom. When the anti-venom is administered immediately after the bite, neutralization of the systemic effects is successful in most cases. Neutralizing local damage is a difficult task. For some cases of snakebite, lack of neutralization of the local effect precedes perpetual sequelae (Wen, 2000; Jorge *et al*., 2019, Pinto *et al*., 2019). However, to deal with these effects, new pharmacological techniques have to be introduced to relieve the consequences of *Bothrops* envenomation.

Bougainvillea xbuttiana is a member of the Nyctaginacea family that is broadly dispersed throughout Mexico. This plant is used in traditional Mexican medicine to deal with one-of-a-kind illnesses (Gomes *et al*., 2010; Petricevich & Vera, 2016; Abarca-Vargas *et al*., 2016). However, studies evaluating the anti-inflammatory activity of this plant species are scarce (Arteaga Figueroa *et al*., 2017). The predominant goal of this study was to evaluate the effect of 50% ethanol extract of *B. xbuttiana* Orange on cytokine production caused by *B. jararaca* venom in macrophages.

MATERIAL AND METHODS

Chemicals, reagents, and buffers

Ethanol, actinomycin D, *O*-phenylenediamine (OPD), 4-nitro-(3-octanoyloxy)-benzoic, azocasein, phosphorric acid, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), RPMI-1640 medium (with L-glutamine and sodium bicarbonate, liquid, sterile-filtered), and 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich Chemical Co. (Toluca, Mexico). Fetal calf serum was acquired from Gibco Life Technologies Corporation (Grand Island, NY, USA). Kits for cytokine quantification (capture and detection antibodies and recombinant cytokines) were purchased from BD Biosciences Pharmingen (USA), and recombinant TNF was purchased from Boehringer Mannheim (Mannheim, Germany).

Plant material and extraction

The specimens of the *B. xbuttiana* plant were collected in Cuernavaca (Morelos, Mexico) and identification of specimens was accomplished by Herbarium HUMO CIByC-UAEM and catalogued with the voucher number 23683. The extraction method is reported in detail in the patent MX/a/2011/813522 (Figueroa *et al*., 2014). In brief, *B. xbuttiana* bracts dried at room temperature (100 g) were ground and the extract was obtained by exhaustive extraction in 50% ethanol. The chromatographic GC-MS profile of the extract obtained in these conditions revealed the presence of 9 compounds such as: 2-propenoic acid, 3-(2-hydroxyphenyl), (E)-and-hexadecanoic acid, 3-*O*-methyl-D-glucose, tetradecanoic acid, 1-nonadecene, isopropyl palmitate, diisooctyl maleate, 1,2-benzenedicarboxylic diisooctyl ester, and squalene (Arteaga Figueroa *et al*., 2017).

B. jararaca venom

Lyophilized venom of *B. jararaca* was obtained through collaboration with Dr. Ronaldo Zucatelli Mendonca (Instituto Butantan, SP-Brazil). Venom was maintained at -20°C, reconstituted in RPMI-1640 medium at the time assays were performed.

Enzymatic activity: Proteolytic activity

The effects *of B. xbuttiana* Orange on the proteolytic activity of *B. jararaca* venom was assessed using azocasein (Moura *et al*., 2010). Briefly, various amounts of venom (15 µg/mL) were pre-incubated with *B. xbuttiana* Orange ethanolic extract (100, 200 or 300 µg/mL) for 30 min at 37°C. In separate tubes, either *B. jararaca* venom 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 was evaluated for 90 min at 37°C and was interrupted by the addition of 400 µL of 20% trichloroacetic acid. After incubation at 25°C for 30 min, tubes were centrifuged at 71,680 x g at 25°C for 10 min. Next, 1.0 mL of the supernatants were eliminated, and pellets were re-suspended in 500 µL NaOH (2 M). After 10 min, the final solution was analyzed by spectrophotometry at an absorbance of 450 nm. Results are indicated as percent activity reduction of the mean ± SD of the mean (n=4) samples.

Phospholipase A_2

The effect of the 50% ethanol extract of *B. xbuttiana* Orange on PLA_2 activity in total *B. jararaca* venom was measured according to the protocol adapted for microplates, described by Albano *et al.* (2013). 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 chromogenic substrate [4-nitro-(3-octanoyloxy)-benzoic acid 10 mM], 20 µL of VBj (40 µg) and 20 µL of ethanolic extract of *B. xbuttiana* Orange (100, 200 and 300 µg) dissolved in a 10% PBS-DMSO 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 *B. jararaca* venom and *B. xbuttiana* Orange ethanolic extract) and the blank extract (in which all components were added except for the venom). Absorbance was determined at 425 nm at 10 min intervals using a BioRad microplate reader.

Animals

BALB/c female mice strain weighing 15-20 g were acquired from Bioterio-Instituto Nacional de Salud Publica (Cuernavaca, México). Animals were sustained and used in accordance with strict ethical conditions according to international recommendations. For the development of the present study, experiments were approved by the Committee of Experimental Animal Administration of Facultad de Medicina with protocol number 005/2016 (Arteaga Figueroa *et al*., 2017).

Peritoneal macrophages collection

Groups of 6 female mice from BALB/c strain were sacrificed and peritoneal cells were collected by peritoneal lavage as detailed described by Arteaga Figueroa *et al.* (2017). The cell suspension was centrifuged and recovered cell pellets were adjusted to a concentration of 1×10^6 cells/mL in RPMI-1640 with 10% FCS followed by distribution in 96-well microplates and incubated at 37°C and 5% CO_2 for 2 h. After this time, non-adherent cells were removed and adherent cells were exposed to different amounts (100, 200 and 300 µg/mL) of *B. xbuttiana* Orange ethanolic extract (alone), or 100 µg/mL of *B. jararaca* venom (alone) or combined *B. xbuttiana* Orange ethanolic extract (100, 200 and 300 µg/mL) + *B. jararaca* venom (100 µg/mL) and cultured under the same conditions for the indicated experimental times.

Viability assay

Macrophages cultures submitted to different treatments were incubated for various time periods. Media were discarded and replaced with fresh media and different amounts of *B. xbuttiana* Orange ethanolic extract (alone), or *B. jararaca* venom (alone) or combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom, and cultures without samples were named as negative controls. All cells were maintained in the same conditions and were subjected to 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays at different points (Fadilah, 2019). In brief, after treatment, medium containing the extract or venom alone or combined extract + venom was replaced with medium containing 1 mg/mL MTT and incubated for 4 h at 37°C. The supernatant was discarded and formed formazan purple crystals were solubilized in absolute ethanol for 15 min. Absorbance was determined at 570 nm, and values of absorbance for the negative control (absence of ethanolic extract or venom) was considered as 100% cell viability. Values of treated cells were calculated as a percentage of the negative controls.

Cytokines production: ELISA assay

IL-1β, IL-4, IL-6, IL-10 and IFN-γ cytokines produced by macrophages from BALB/c mice treated with *B. xbuttiana* Orange ethanolic extract (alone) or *B. jararaca* venom (alone), or combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom were determined by ELISA assay (Koelman *et al*., 2019). All assays were evaluated in accordance with the manufacturer´s recommendations. The minimum levels of each cytokine in this study was 1 pg/10^6 cells for IL-1β and IL-4, and 10 pg/10^6 cells for IL-6, IL-10 and IFN-γ.

Biological assay

The presence of TNF-α in supernatants of macrophages exposed to *B. xbuttiana* Orange ethanolic extract (alone) or *B. jararaca* venom (alone), or combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom were determined by biological assay (De Silva *et al*., 2016). The percent cytotoxicity of TNF on L929 cells was calculated by the formula: $(A_{control} - A_{sample}/A_{control}) \times 100$. TNF activity is expressed as pg/mL as determined by a

comparison to a standard made with mouse recombinant TNF.

Statistical analyses

Values were expressed as the mean ± SD. The data were analyzed by one-way ANOVA and comparisons were made with the statistical program GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, United States). The differences between the means were considered significant at $p<0.05$ and $p<0.01$.

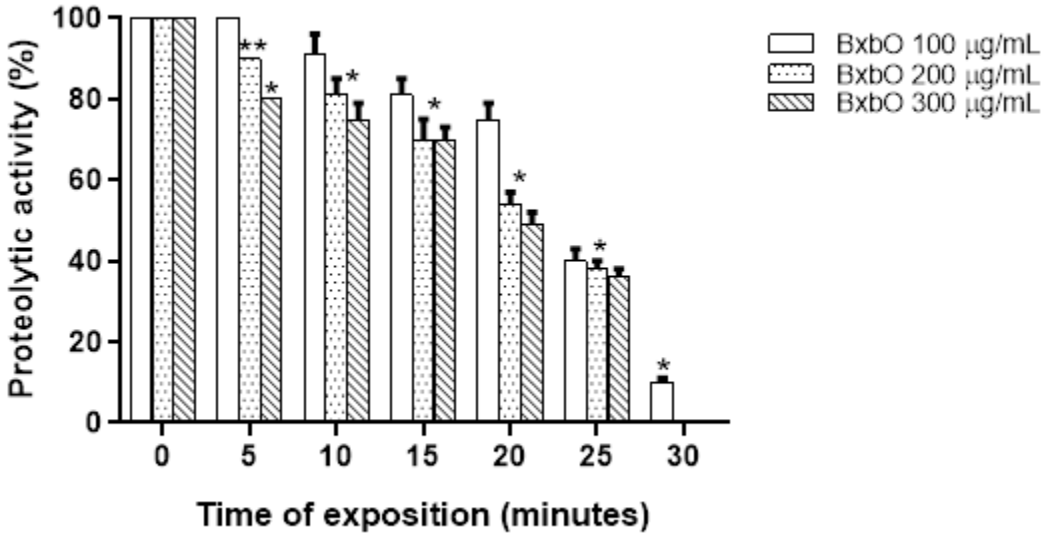

Figure No. 1
Effect of *B. xbuttiana* Orange extract on proteolysis induced by *B. jararaca* venom
***B. xbuttiana* Orange = BxbO extract 100, 200 and 300 µg/mL were incubated for 30 min at room temperature with 15 µg/mL *B. jararaca* venom. Data were reported as mean ± SD (n=4). *$p<0.001$ and **$p<0.01$ for the difference between treated groups and the venom**

RESULTS

Effect of 50% ethanolic extract of Bougainvillea xbuttiana on enzymatic activity: Proteolytic activity

To determine the neutralizing effect of 50% ethanolic extract of *B. xbuttiana* Orange against enzymatic activity, two assays were performed. The proteolytic activity of *B. jararaca* venom was determined using the hydrolysed azocasein assay. Different amounts (100, 200 and 300 µg/mL) of *B. xbuttiana* Orange extract inhibited proteolysis caused by *B. jararaca* venom (Figure No. 1). *B. xbuttiana* Orange extract decreased proteolytic activity of *B. jararaca* venom in a concentration- and time-dependent manner. At concentrations of 100 and 200 µg/mL, *B. xbuttiana* Orange ethanolic extract caused a mild reduction in the proteolytic effects of *B. jararaca* venom. However, 300 µg/mL of *B. xbuttiana* Orange ethanolic extract caused a 52% reduction in *B. jararaca* venom proteolytic activity at 30 min (data not shown). Maximal inhibition of proteolytic activity of *B. jararaca* venom at 30 min was obtained with 50% ethanol extract of *B. xbuttiana* Orange at concentrations of 100, 200 and 300 µg/mL, with proteolytic activities of 10%, 0% and 0%, respectively.

Phospholipase A_2

The capacity of 50% ethanol extract of *B. xbuttiana* Orange to reduce the phospholipase activity of *B. jararaca* venom is shown in Figure No. 2. *B. xbuttiana* orange extract reduced the phospholipase activity of *B. jararaca* venom in a concentration- and time-dependent manner. The percent reduction of phospholipase activity of the *B. jararaca* venom caused by the presence of the *B. xbuttiana* Orange extract at amounts of 100, 200 and 300 µg/mL during

the 30-minute reaction mL was significantly reduced (p<0.001). At these concentrations of *B. xbuttiana* Orange ethanolic extract resulted in a 40%, 60% and 61% reduction of PLA_2 activity in *B. jararaca* venom, respectively.

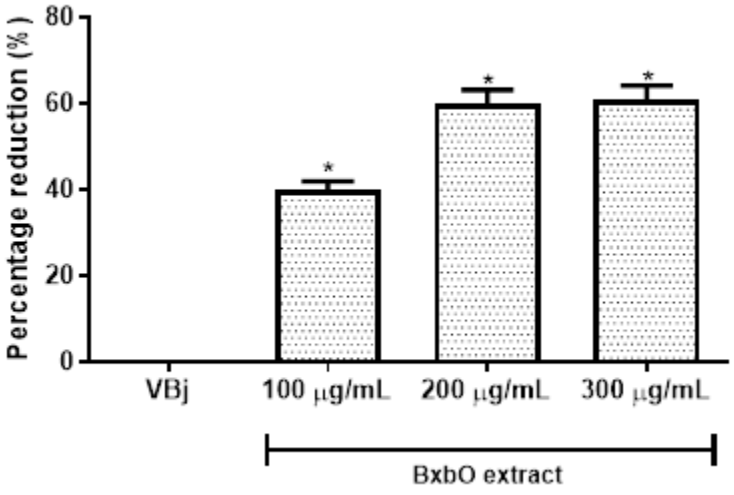

Figure No. 2
Effect of *B. xbuttiana* Orange extract on PLA_2 activity of *B. jararaca* venom
Reduction percentage obtained in the *B. xbuttiana* Orange = BxbO extract 100, 200 and 300 µg/mL incubated for 30 min at 25°C with 40 µg/mL *B. jararaca* venom. Data were reported as mean ± SD (n=4). *p<0.001 for the discrepancy between treated groups and the venom

Effect of 50% ethanol extract of B. xbuttiana Orange or B. jararaca venom or combined B. xbuttiana Orange ethanolic extract + B. jararaca venom on macrophages viability

The effect of ethanol extract of *B. xbuttiana* Orange alone, or *B. jararaca* venom alone, or combined *B. xbuttiana* Orange + *B. jararaca* venom on viability of macrophages was evaluated by measuring viability percentage. As shown in Figure No. 3A, *B. xbuttiana* Orange ethanolic extract induced dose-dependent inhibition in cell viability. In the first 24 h, the lowest viability percentages of 80% were obtained in macrophages cultures exposed to 300 µg/mL *B. xbuttiana* Orange ethanolic extract. In cultures of macrophages exposed for 48 h, viability percentages were 78%, 75% and 70% for 100, 200 and 300 µg/mL *B. xbuttiana* Orange ethanolic extract, respectively. For macrophages exposed to 100, 200 and 300 µg/mL *B. xbuttiana* Orange ethanolic extract for 72 h, the percentages of viability were 68%, 65% and 65%, respectively. In cultures of macrophages exposed to ethanol extract of *B. xbuttiana* Orange for 96 h, the viability was approximately 68%, 62% and 58% in response to *B. xbuttiana* Orange treatment of 100, 200 and 300 µg/mL, respectively. Viability percentages for macrophages treated for 120 h with 100, 200 and 300 µg/mL were 67%, 60% and 55%, respectively.

The effect of *B. jararaca* venom alone on viability of macrophages was determined by measuring viability percentages. Macrophages treated with *B. jararaca* venom exhibited a decrement on cell viability in a dose-dependent manner (Figure No. 3B). In the experimental conditions used for the development of this study, throughout the entire evaluated period, the lowest percentages of viability were obtained in cultures of macrophages treated with 150 µg/mL of *B. jararaca* venom. In cultures of macrophages exposed for 24 h, viability percentages were 68% and 62% for 50 and 100 µg/mL *B. jararaca* venom, respectively. The viability percentage in macrophages exposed to *B. jararaca* venom decayed with increasing incubation time. With respect to macrophages cultures with 50 and 100 µg/mL of VBj, the viability percentages obtained were greater than 50% for 72, 96 and 120 h.

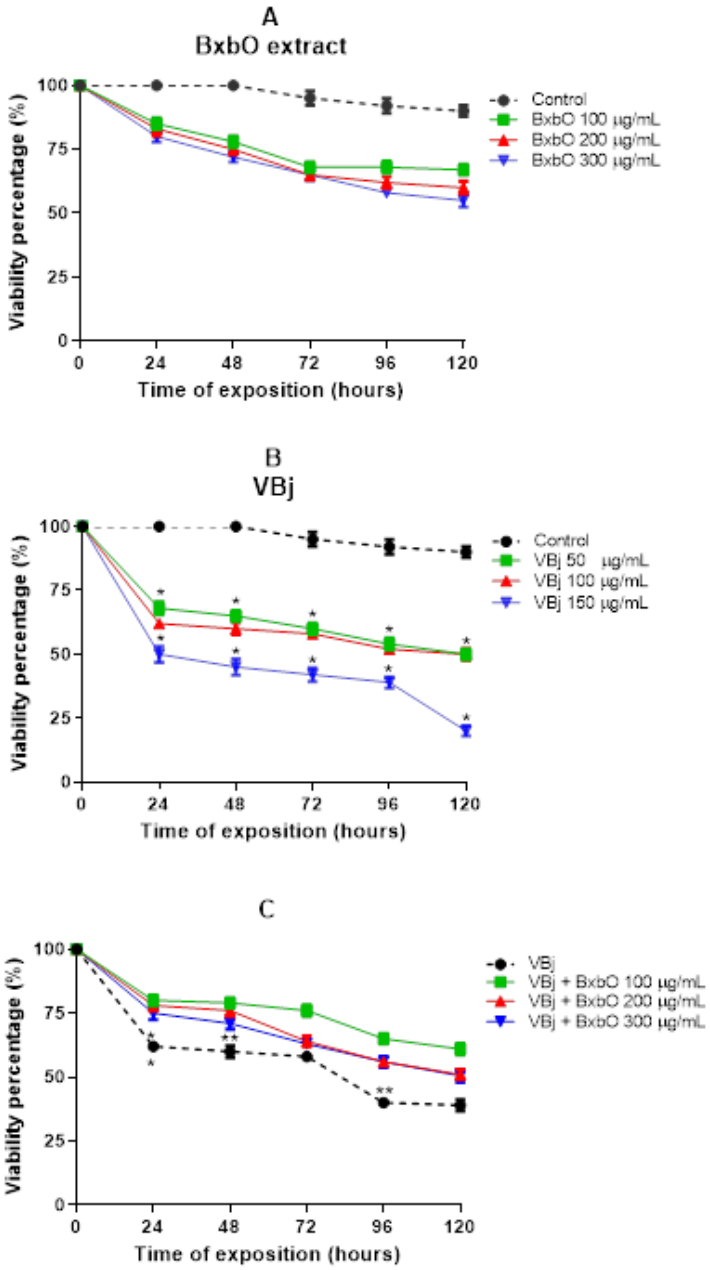

Figure No. 3
Effect of *B. xbuttiana* Orange alone, *B. jararaca* venom alone and *B. xbuttiana* Orange combined with *B. jararaca* venom on peritoneal macrophage viability
Peritoneal macrophages were obtained and exposed to different amounts of *B. xbuttiana* Orange = BxbO (3A), *B. jararaca* venom = BVj (3B) and combined *B. xbuttiana* Orange and *B. jararaca* venom (3C) as described in materials and methods. The percentage of viability was observed at 24, 48, 72, 96 and 120 h. Each point represents the mean value of samples from four experiments in different groups of five mice. Data reported mean ± SD (n=4). *p<0.001 and **p<0.01 for the discrepancy between treated groups and the venom

To establish optimal concentrations of *B. xbuttiana* Orange ethanolic extract and venom, macrophages cultures treated with 100 µg/mL *B. jararaca* venom exhibited percentages of viability greater than 50%. As such, we maintained this dose and combined it with different concentrations of ethanol extract of *B. xbuttiana* Orange (Figure No. 3C). Results achieved show that the extract had a cytoprotective effect on macrophages exposed to *B. jararaca* venom. Viability percentages obtained in macrophages cultures exposed to 100 µg/mL *B. xbuttiana* Orange ethanolic extract were between 80% and 61%, while in macrophages cultures treated with 200 and 300 µg/mL *B. xbuttiana* Orange, viability percentages were between 78% to 51% and 75% to 50.5%, respectively. These kinetics assays of macrophage viability indicate that the *B. xbuttiana* Orange extract demonstrate a cytoprotective effect against the actions of the venom (Figure No. 3C).

Comparison between the effect of 50% ethanol extract of B. xbuttiana Orange, B. jararaca venom or combined B. xbuttiana Orange + B. jararaca venom on macrophage cytokine production

To understand the kinetics of pro-inflammatory cytokine production, macrophages cultures were stimulated with *B. xbuttiana* Orange ethanolic extract (100 µg/mL) and *B. jararaca* venom (100 µg/mL) alone or with combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom (100 µg/mL) for different time points. Figure No. 4 shows the effect of *B. xbuttiana* Orange ethanolic extract on the production of pro-inflammatory cytokines. In cultures of macrophages exposed to *B. jararaca* venom alone, the highest production of TNF-α, IL-1-β and IL-6 was obtained after 24 h of exposure. In contrast, in supernatants from macrophages cultures treated with combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom, production of pro-inflammatory cytokines was significantly lower compared to macrophages treated with *B. jararaca* venom alone ($p<0.001$) for TNF-α and IL-6 ($p<0.05$) and IL-1-β. In cultures of macrophages exposed to *B. jararaca* venom alone, the higher production of IFN-γ was observed with 72 h, decaying thereafter. Significantly lower levels of IFN-γ were obtained in macrophages cultures exposed to combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom ($p<0.001$) (Figure No. 4).

The kinetics of the generation of anti-inflammatory cytokines is defined in Figure No. 5. In macrophages cultures exposed to *B. xbuttiana* Orange ethanolic extract alone, the highest levels of IL-4 were detected at 24, 48 and 72 h of exposure, decaying thereafter. Significantly lower levels of IL-4 were detected in the cultures of macrophages treated with *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom combined ($p<0.001$), compared to cultures treated with *B. xbuttiana* Orange alone. In the presence of the extract alone or in the combined form with *B. jararaca* venom, IL-4 production was significantly increased compared to the results obtained from cultures of macrophages treated with *B. jararaca* venom alone ($p<0.001$). Levels of IL-10 increased with time of exposure in macrophages cultures exposed to *B. xbuttiana* Orange ethanolic extract alone or combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom (Figure No. 5). IL-10 production in macrophages treated with *B. xbuttiana* Orange ethanolic extract was significantly higher compared to cultures exposed to combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom ($p<0.001$). Curiously, levels of IL-10 were significantly higher in cultures of macrophages exposed to combined *B. xbuttiana* Orange ethanolic extract + *B. jararaca* venom than in macrophages treated with *B. jararaca* venom alone ($p<0.001$) (Figure No. 5).

Effect of concentration in combined 50% ethanol extract of B. xbuttiana Orange + B. jararaca venom administration on macrophages cytokine production

Since we verified decreased levels of pro and/or anti-inflammatory cytokines in cultrures of macrophages exposed to combined *B. xbuttiana* Orange ethanolic extract (100 µg/mL) + *B. jararaca* venom (100 µg/mL), we also decided to determine the best performance of the extract on the production of cytokines through the use of different concentrations of *B. xbuttiana* Orange ethanolic extract combined with *B. jararaca* venom. To know this effect, different concentrations of *B. xbuttiana* Orange ethanolic extract combined with *B. jararaca* venom (100 µg/mL) were used. The effect of concentration of *B. xbuttiana* Orange ethanolic extract combined with (100 µg/mL) of *B. jararaca* venom is shown in Figure No. 6. In cultures of macrophages exposed to increasing concentration of the extract combined with *B. jararaca* venom, the production of all the pro-inflammatory cytokines was significantly diminished

compared to those results achieved in macrophages cultures exposed to *B. jararaca* venom alone (p<0.001). In contrast, in cultures of macrophages treated with different amounts of *B. xbuttiana* Orange ethanolic extract, levels of IL-4 and IL-10 cytokines were significantly higher compared to those observed in *B. jararaca* venom -treated macrophages (p<0.001) (Figure No. 6). For both the decrease in the production of pro-inflammatory cytokines and the increase in levels of anti-inflammatory cytokines we verified a response dose-dependent manner.

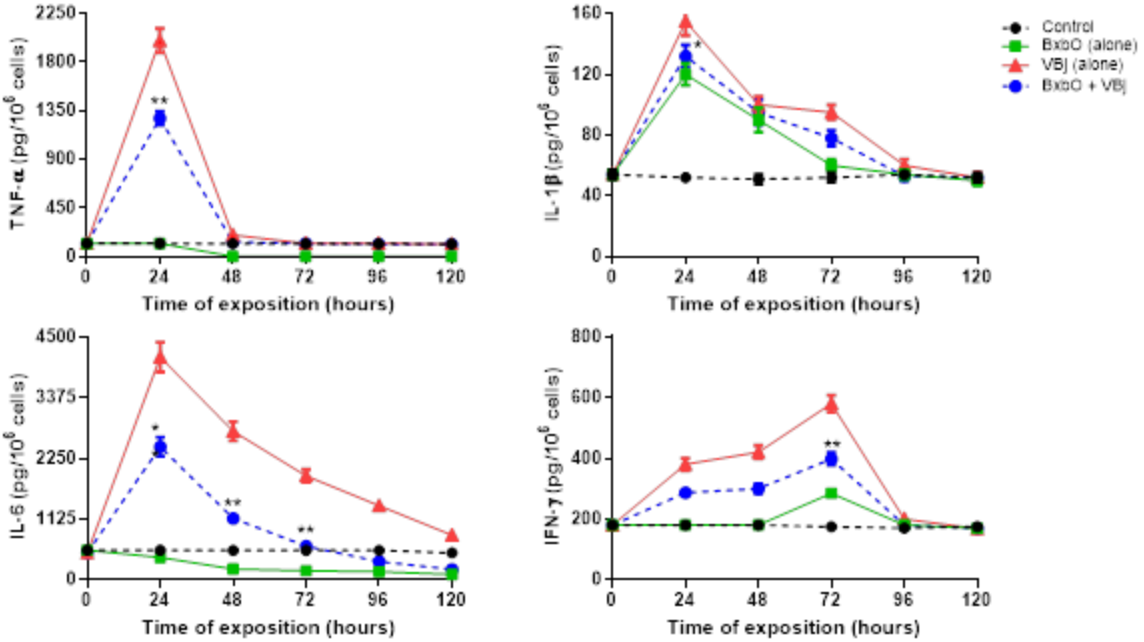

Figure No. 4
Effect of *B. xbuttiana* Orange, *B. jararaca* venom and combined *B. xbuttiana* Orange + *B. jararaca* venom on peritoneal macrophage proinflammatory cytokines
Peritoneal macrophages were obtained and exposed to (100 µg/mL) of *B. xbuttiana* Orange = BxbO, or (100 µg/mL) of *B. jararaca* venom = BVj and combined *B. xbuttiana* Orange + *B. jararaca* venom (100 µg/mL) as described in materials and methods. The production of pro-inflammatory cytokines was observed at 24, 48, 72, 96 and 120 h. Each point represents the mean value of samples from four experiments in different groups of five mice

DISCUSSION

For the treatment of snake bites, administration of the anti-venom therapy is indicated, which will neutralize the primary effects of the envenomation, which presents some limitations, such as reduced efficacy against local effects, risk of immunological reactions, high cost and difficulty in access in some regions (Scheske *et al*., 2015, Salvador *et al*., 2019). However, there is no known treatment for the most frequent secondary complications of snake bites. According to the literature, different types of medicinal plants with anti-ophidian properties have been extensively studied (Alves *et al*., 2019). Special attention is concentrated on the main bioactive compound, which is capable of neutralizing the toxicological effects of venomous snakes (Carvalho *et al*., 2013; Alves *et al*., 2019). Isolation of the bioactive compound involves extensive and labourious work, using different amounts of solvents and specific techniques. The pharmacological activity of a plant extract should be substantiated by the presence of its bioactive compound. Plants extracts

can be used as a wealthy source of bioactive compounds and isolated secondary metabolites such as phenols, flavonoids, alkaloids, steroids, *etc*, which may be important for the development of new therapies, new synthetic drugs, *etc*, capable of neutralizing local inflammation caused by *Bothrops* venom (Carvalho *et al*., 2013; Alves *et al.*, 2019).

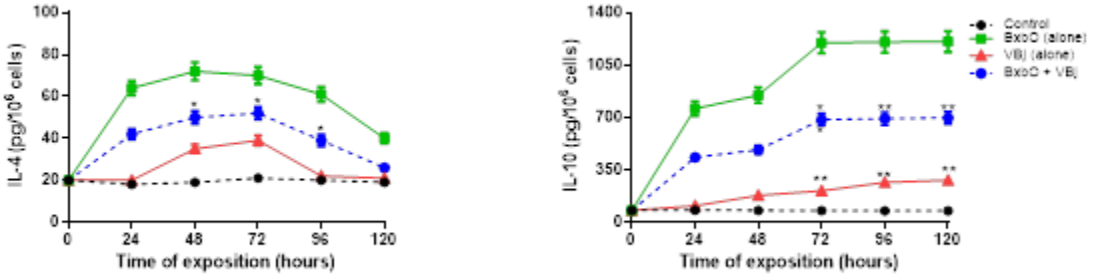

Figure No. 5
Effect of *B. xbuttiana* Orange, *B. jararaca* venom and combined *B. xbuttiana* Orange + *B. jararaca* venom on peritoneal macrophage anti-inflammatory cytokines
Peritoneal macrophages were obtained and exposed to (100 µg/mL) of *B. xbuttiana* Orange, or (100 µg/mL) of *B. jararaca* venom and combined *B. xbuttiana* Orange + *B. jararaca* venom as described in materials and methods. The production of proinflammatory cytokines was observed at 24, 48, 72, 96 and 120 h. Each point represents the mean value of samples from four experiments in different groups of five mice

The venom of *B. jararaca* consists of toxins that are capable of triggering bleeding and adverse coagulation. Moreover, these toxins cause a dangerous imbalance of the reduction-oxidation cellular reactions. Snake venoms of the *Bothrops* genus are constituted by of mixtures of proteins and components of low molecular weight and the primary function of the distinct compounds is to act as buffers or selective endogenous inhibitors for some enzymes (Péterfi *et al*., 2019).

Proteins present in the venom are responsible for almost all pathophysiological symptoms that characterize venom toxicity, including toxins, non-toxic proteins and enzymes such as hydrolases, proteinases, hyaluronidase, phospholipase, etc (Boda *et al*., 2018; Péterfi *et al*., 2019). In this study, *B. xbuttiana* Orange was able to reduce proteolytic activity and PLA_2. Distinct studies have shown that bioactive compounds from plants have significant inhibitory properties against snake venom enzymatic toxins such as PLA_2 and hyaluronidases (Santhosh *et al*., 2013; Jorge *et al*., 2019). However, these compounds may be responsible for inhibition of toxins present in snake venom, acting directly as enzymatic inhibitors and chemical inactivators, or indirectly as immunomodulators, by interacting with biological targets (Santhosh *et al*., 2013; Jorge *et al*., 2019). Among compounds detected in the extract from *B. xbuttiana*, it is possible to highlight the presence of individual compounds with anti-ophidic properties.

The importance of inhibitors, especially in the treatment of pro-inflammatory diseases, is favoured by acting on the inhibition of PLA_2s that produce pro-inflammatory effects of eicosanoids, mediators of the cyclooxygenase 2 (COX-2) pathway (Galvão *et al*., 2018). In this way, the use of PLA_2 inhibitors, as a therapeutic strategy in the treatment of inflammation and tissue damage, is of great importance, since it provides control of PLA_2 activity, facilitating the treatment of pathological states related to inflammatory processes (Galvão *et al*., 2018). Hydrolysis of the phospholipid ester bonds of the membrane by PLA_2 and consequent release of fatty acids are the initial steps of inflammation. The results obtained in this work demonstrate that the extract reduced inflammation caused by *B. jararaca* venom through inhibition of PLA_2 activity.

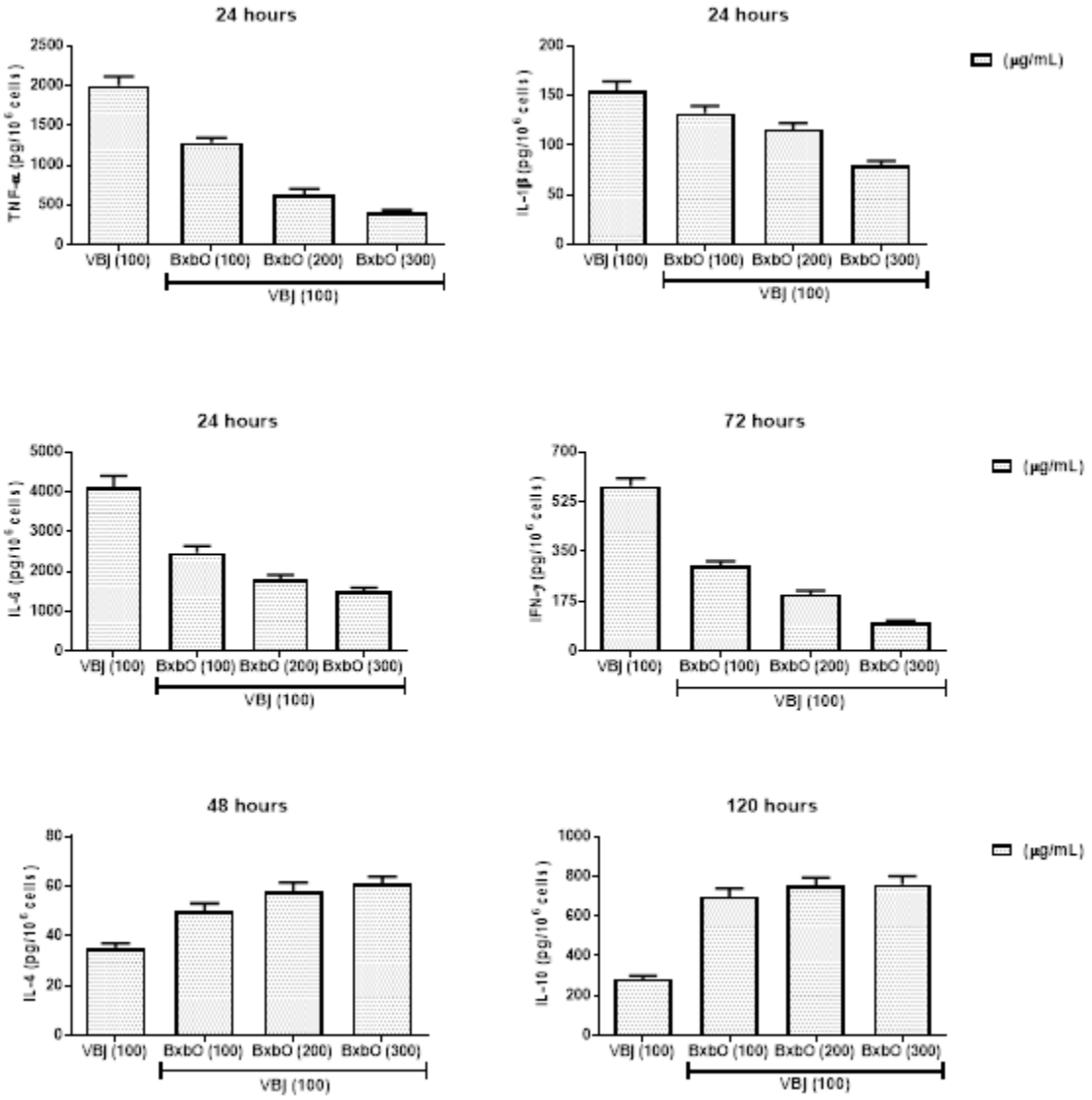

Figure No. 6
Effect of different amounts of *B. xbuttiana* Orange combined *B. jararaca* venom on peritoneal macrophage cytokines
Peritoneal macrophages were obtained and exposed to 100 µg of *B. jararaca* venom and combined *B. xbuttiana* Orange (100, 200 or 300 µg) + *B. jararaca* venom (100 µg) as described in materials and methods. The production of TNF-α, IL-1β and IL-6 was observed at 24, for IFN-γ at 72 h, while IL-4 and IL-10 for 48 and 120 h, respectively. Each bar represents the mean value of samples from four experiments in different groups of five mice

The description of the compounds found in the 50% ethanol extract of *B. xbuttiana* Orange was previously shown and is constituted by 2-propenoic acid, 3-(2-hydroxyphenyl), (E)- and-hexadecanoic acid, 3-*O*-methyl-D-glucose, tetradecanoic acid, 1-nonadecene, isopropyl palmitate, diisooctyl maleate, 1,2-benzenedicarboxylic diisooctyl ester, and squalene (Arteaga Figueroa *et al*., 2017). This

analysis was conducted for better comprehension of the chemical compounds presented in the *B. xbuttiana* Orange ethanolic extract. We have previously shown that the *B. xbuttiana* Orange ethanolic extract has anti-inflammatory, antioxidant, and immunomodulatory activity (Arteaga Figueroa *et al*., 2017).

Activation of macrophages through contact with pathogens releases cytokines and chemokines, which are the main primary components of the innate immune response (Arteaga Figueroa *et al*., 2017). Many more recent therapies have directly used cytokines to target cytokines. In the envenomation process by *Bothrops* snakes, pro-inflammatory cytokines such as TFN-α, IL-6, IL-1β and IFN-γ, play an important role. High levels of these pro-inflammatory cytokines lead to a systemic inflammatory response syndrome, as is the case with septic shock. In our study, results revealed that *B. xbuttiana* extract significantly reduces levels of pro-inflammatory cytokines such TNF-α, IL-6, IL-1β and IFN-γ in a dose-dependent manner in peritoneal macrophages exposed to *B. jararaca* venom. At this time, of all compounds present in the *B. xbuttiana* Orange ethanolic extract, two of them, 1-nonadecene and diisooctyl maleate, have not been previously identified in the literature for their immunological effects. When we consider the individual activity of the components present in the extract, tetradecanoic acid has no effect on IL-1 production (Nagasaki *et al*., 2012). In relation to *n*-hexadecanoic acid and isopropyl palmitate, different studies have shown that these components stimulate the production of TNF-α (Samokhvalov *et al*., 2008; Riera-Borrull *et al*., 2017).

In this work, we observed that the 50% ethanol extract of *B. xbuttiana* potentiated the production of both IL-4 and IL-10 in a dose-dependent manner in macrophage cultures treated with *B. jararaca* venom. This increase may be due to the presence of 2-propenoic acid, 3-(2-hydroxyphenyl) *n*-hexadecanoic acid and squalene, which individually have anti-inflammatory activities (Samokhvalov *et al*., 2008; Prieur *et al*., 2011; Cardeno *et al*., 2015). Based on the physical adsorption, non-specific phenolic compounds can interact with lipids in the cell membrane in a non-specific manner with respect to squalene in the literature, its antioxidant, detoxifying and immunostimulatory effects have been described (Shimizu *et al*., 2019).

Squalene possesses cytoprotective and selective effects, protecting normal cells against the toxicity from chemotherapeutic agents, and is considered a strong antioxidant because of its ability to exchange electrons without being exposed to molecular perturbations, protecting immune cell biomembranes against oxidative stress at the time phagocytosis processes (Fernando *et al*., 2018; Thirunavukkarasu *et al*., 2018).

This sudy analyzed *B. xbuttiana* ethanolic extract, confirmed its capability to inhibit both proteolytic activity and phospholipase A_2 activity of *B. jararaca* venom. This extract has also been shown significantly decrease the secretion of pro-inflammatory cytokines and increase the levels of anti-inflammatory cytokines. The extract exhibited an important cytoprotective effect, due to its antioxidant activity. The compounds present in this extract may be key for the dynamics of the inhibition of *B. jararaca* venom. For this reason, further studies on the isolation, structural characterization, and mechanism of action for this plant must be carried out in the future. Together with the results obtained during this study, we conclude that *B. xbuttiana* ethanolic extract may represent a complementary treatment of *B. jararaca* venom. In addition, *B. xbuttiana* Orange ethanolic extract is easily accessible as it is a species that can be found in many urban areas, which would facilitate its acquisition and use.

CONCLUSIONS

Our results showed that the 50% ethanolic extract of *B. xbuttiana* Orange was able to reduce the toxicity caused by the venom of *B. jararaca* snakes in macropages.

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