



Research Article



Anti-Inflammatory and Antithrombotic Properties of *Jatropha Elliptica*, a Plant from Brazilian Cerrado Biome

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Abstract

Purpose: The aim of this study was to evaluate the antithrombotic and anti-inflammatory properties of *J. elliptica*.

Methods: Phytochemical screening and thin-layer chromatography (TLC) assays were performed on *J. elliptica* hydroalcoholic extract (TE) in order to observe its main constituents. The antithrombotic activity of TE was evaluated by the *in vitro* neuromuscular blockade caused by *Bothrops jararacussu* venom (Bjssu), in a mouse phrenic nerve-diaphragm model (PND). A quantitative histological study was carried out to observe a possible protection of TE against the venom myotoxicity. The anti-inflammatory activity was also evaluated in two models, Bjssu-induced paw edema, and carrageenan-induced neutrophils migration in the peritoneal cavity.

Results: TLC analysis revealed several compounds in TE, such as saponins, alkaloids, and phenolic constituents. TE was able to neutralize the blockade and the myotoxicity induced by venom, when it was pre-incubated for 30 min with venom. In addition, it showed anti-inflammatory activity, inducing less neutrophils migration and reducing paw edema.

Conclusion: *J. elliptica* showed both antithrombotic and anti-inflammatory properties.

Introduction

The "Cerrado" (tropical savanna) is the second larger biome in Brazil, covering 25% of Brazilian territory. It is characterized by a continuous herbaceous stratum connected to an arboreal stratum, with variable density of woody species. As an ecosystem with limited resources, their native plants can produce and accumulate more bioactive compounds. Some woody species and their permanent parts are most commonly used in traditional medicine.¹

Rhizomes of *Jatropha elliptica* (Pohl) Oken. (Euphorbiaceae), also known as "batata-de-teiu", contains diterpenes, esters, coumarins, terpenoids, and steroids.² In Brazilian communities of Cerrado, *J. elliptica* is popularly used to treat snakebite, and its rhizomes are prepared by infusion and given orally. They are infused or macerated in hydroalcoholic beverages and used to treat severe itches, syphilis, and snakebites.³ The pharmacological properties including anti-inflammatory activity of genus *Jatropha* was reviewed by Sharma and Singh.⁴ Other species from *Jatropha* genus, such as *Jatropha gossypifolia* inhibited

enzymatic and biologic activities of the *Bothrops jararaca* venom in both *in vitro* and *in vivo* assays.⁵

The clinical signs and symptoms of *B. jararacussu* envenomation were detailed by Milani et al.⁶, and are similar to other *Bothrops* species causing ecchymosis, bleeding, skin infection with abscess and local inflammation. This venom has been used to test anti-inflammatory and anti-myotoxic properties, as well as the activity of many substances against *in vitro* neuromuscular paralysis.

As *J. elliptica* is popularly used to treat snakebites, we hypothesized that this plant would have an antivenom activity. Therefore, the aim of the present study was to observe the ability of a hydroalcoholic extract, obtained from *J. elliptica* roots, to neutralize the *in vitro* neurotoxicity and myotoxicity caused by *B. jararacussu* venom. In addition, the anti-inflammatory activity was verified through paw-edema and neutrophils migration in mouse models. Thin-layer chromatography (TLC) and other chemical analyses were carried out to observe the main chemical constituents of this extract. (Graphical Abstract)

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Materials and Methods

Plant material collection

Roots of *Jatropha elliptica* were collected in 2013, in Santa Tereza city (S 10° 19.207' and W 047° 47.954'), at Tocantins state (TO), Brazil. The ethnobotanical study was carried out in the *Quilombola Kalunga do Mimoso* Community of Arraias city - TO, Brazil. The plant exsiccate was deposited in the Tocantins Federal University (UFT) Herbarium in Porto Nacional - TO, Brazil, voucher specimen #10.681, according to the *International Code of Botanical Nomenclature (ICBN)*.

Preparation of extract

Herbal preparations were prepared in accordance to European Medicines Agency guideline.⁷ The collected sample was meticulously washed, dried at 37°C for 48 hours. Then it was powdered, ground in a mill, and macerated (531 g during 3 days) in 2 L of 70% ethanol. The suspension was percolated (protected from light) at 20 drops/min, resulting in a 20% (m/v) hydroalcoholic extract.

J. elliptica or "Teiu" extract (TE) was concentrated under reduced pressure and lyophilized, providing 15 g (20% extraction yield). It was stored at room temperature, protected from light and humidity, until the assays were performed.

Pharmacognostic Study

Cyanogenic glycosides detection

Picrate papers [filter paper saturated with 0.05 M aqueous picric acid, previously neutralized with NaHCO₃ 10% (w/v)] were used for this test.⁸ Color changes from yellow to reddish-brown indicated the enzymatic release of HCN from the plant content. *Malus* sp. seeds were used as positive control in this assay.

Saponins detection

In a test tube, 100 mg of TE was added to 5 mL of purified water, and the tube was kept in intense agitation during 15 s. Then, the tube was maintained in resting during 15 s, and the result was registered. The test was considered positive when a persistent foam was formed on the liquid surface.⁹

Foam index determination

A TE solution (0.1g/100 mL of purified water) was distributed in 10 test tubes, in 1, 2, 3 mL and successively until reaching 10 mL. Each tube was adjusted to a final volume of 10 mL with purified water, and shaken for 15 s (twice). After 15 min of resting, the foam height was measured. The tube with remaining dense foam of 1 cm height was registered. The foam index (FI) was calculated as $FI = 1000/A$, being "A" the volume (in mL) of TE of the higher foam height registered.¹⁰

Hemolytic activity

According to the Brazilian Pharmacopeia,¹⁰ a diluted series of 0.1% TE was prepared using phosphate buffer

pH 7.4, and a 2% sheep-blood suspension. A saponin-rich quillaja (*Quillaja saponaria*) extract (Sigma-Aldrich, Saint Louis, MO, USA), containing 8 to 10% triterpenoid saponin, was used as positive control. After dilution, all tubes were carefully inverted, avoiding the foam formation. They were inverted again after 30 min, and read for hemolysis after resting for 6 h at 37°C.

Alkaloid acquisition

A solution of 0.1 g TE dissolved in 10 mL of 70% ethanol was basified with 10% NH₄OH to pH 9.0, and partitioned four times with 3 mL of ether. The fractions were evaporated, the residue was re-suspended in 5 mL of 1% H₂SO₄, and basified to pH 9.0 with 10% NH₄OH. This entire process was repeated; the residue was re-suspended in 1mL 1% H₂SO₄, and submitted to the following assays:

Precipitation assay: The reaction was carried out with 2 drops of Dragendorff's and Mayer's precipitation reagents. Orange and white color precipitates respectively were considered positive results.¹¹

Thin layer chromatography (TLC): Aliquots of TE and the following standards (all from Sigma-Aldrich, 1 mg/mL, solubilized in methanol): β-sitosterol, gallic acid, tannic acid, chlorogenic acid, caffeic acid, rutin, quercetin, and apigenin were spotted onto 0.3 mm-thick silica gel plates (Merck, Germany).⁸ The TLC system consisted of ethyl acetate-formic acid-acetic acid-water (100:11:11:24, v/v) as mobile phase. It was visualized by using ammonium vapor and NP/PEG, which consisted of 5% (v/v) ethanol NP (diphenylboric acid 2-aminoethyl ester, Sigma), and 5% (v/v) ethanol PEG 4000 (polyethylene glycol 4000, Synth, Brazil). Visualization was performed under UV light at 365 nm. A second plate was visualized with aluminum chloride. The presence of the phytochemical groups was investigated by comparison with the standards. The retention factor (Rf) of each standard was compared with spots exhibited by TE sample.

Determination of Flavonoids and Polyphenols

Flavonoid content: Flavonoids were determined as previously described by Camargo *et al.*,¹² using quercetin as standard. The percentage of flavonoids (%) was calculated from a standard curve of quercetin (0, 4, 8, 12, and 16 µg/mL) prepared in methanol.

Polyphenol content: Polyphenols were determined by a modified method by Hagerman.¹³ The percentage of polyphenols was determined by a calibration curve (5, 10, 15, 20, 25, 30, 35, and 40 µg/mL) of gallic acid (Sigma).

Pharmacological Assays

Crude snake venom

Bothrops jararacussu venom was collected from two adult specimens kept in Nature Studies Centre's Serpentry of University of Vale do Paraiba (Univap), SP, Brazil. The venom was lyophilized and maintained at 4 to 8 °C until the use, being certified by Professor

Dr. José Carlos Cogo from University Camilo Castelo Branco, Unicastelo.

Animals

Male Swiss mice (26-32 g) were supplied by Anilab (Animais de Laboratório, Paulínia, SP, Brazil). The animals were housed at 25 ± 3 °C on a 12 h light/dark cycle and had access to food and water *ad libitum*. The protocol was approved by the institutional Committee for Ethics in Research from Federal University of São Carlos (#070/2012, approved in 2012, April). The experiments were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation and with the guide for the care and use of laboratory animals, Eighth Edition.¹⁴

Venom neutralization assays

Mouse phrenic nerve-diaphragm muscle (PND) preparation

The phrenic nerve-diaphragm was obtained from mice anesthetized with halothane (Cristália, Brazil) and killed by exsanguination. The PND myographic recording was performed according to Farrapo et al.¹⁵ PND was allowed to stabilize for at least 20 min before the following experiments.

A concentration-response curve of TE was carried out using 500 µg (n=8); 750 µg (n=6); 1000 µg (n=4); and 2000 µg (n=2). Control formulation was the Tyrode solution. The 500 µg concentration was further assayed with 40 µg/mL Bjssu venom (n=4), using two models: 1) pre-incubation during 30 min, prior to add into the organ bath (n=5); 2) TE was added after 10 min of venom (n=7).

Quantitative histological study

In order to observe the effect of TE in the myotoxicity (edema, intense myonecrosis characterized by atrophy of the muscle fibers, hyaline aspect, sarcolemmal disruption and lysis of the myofibrils) of Bjssu venom, three resulting preparations from each pharmacological set (Bjssu venom and preincubation) were analyzed by a quantitative morphometric method, and expressed as myotoxicity index (MI), *i.e.*, the percentage of damaged muscle cells number divided by the total number of cells in three non-overlapping, non-adjacent areas of each preparation.¹⁶

Anti-inflammatory study

The paw edema and the neutrophils migration in the peritoneal cavity were performed in mice in order to determine the TE's anti-inflammatory activity. TE was administered orally (V.O.) at doses of 250, 500 or 1000 mg/kg with a gavage needle. For both tests, six animals were used per group (n=6). Phosphate buffered saline (PBS pH 7.4) with 5% dimethylsulfoxide (DMSO) was used to dilute the extract.

Bothrops jararacussu-induced paw edema

The paw edema method proposed by Winter et al.¹⁷ was performed using Bjssu venom as the phlogistic agent. First, different doses of Bjssu venom were tested to determine the venom concentration producing similar edema to carrageenan, without causing pronounced bleeding. Formulations consisting of 0.05 mL of 0.9% NaCl with 0.25, 0.5, 1.0 or 2.0 µg of Bjssu were injected into the left hind paw of mice. The contralateral paw received the same volume of sterile saline as a control. A carrageenan group was used as positive control to the venom groups. The edema was measured with a plethysmometer (model 7140, Ugo Basile, Italy) before and at 30 min, 1 h, 2 h, 3 h, 4 h and 5 h after carrageenan or venom injections. Differences between left and right hind-paws volumes were considered as a measure of the edema (mL).

To determine the anti-inflammatory activity of TE, 0.5 µg of the venom was injected into the hind paw. TE (250, 500 and 1000 mg/kg) or vehicle (PBS - 10 mL/kg) were administered orally. As a positive control, dexamethasone (2 mg/kg) was injected intraperitoneally 30 min before the subplantar injection of the phlogistic agent.

Neutrophils migration in the peritoneal cavity

This test was carried out according to Denny et al.¹⁸ The neutrophil migration to the peritoneal cavity was stimulated by the intraperitoneal (I.P.) injection of carrageenan at 500 µg per mouse cavity. Vehicle (0.9% NaCl - I.P.) was used as negative control. TE at 250, 500, 1000 mg/kg and vehicle (PBS) were administered orally. Indomethacin (10 mg/kg), used as positive control, was administered I.P. 30 min before the carrageenan stimuli.

Four hours after carrageenan injection, all mice were killed, and their peritoneal cavity content was harvested by washing the cavity with 3 mL of PBS containing EDTA 1.2 mM. Right after the collection, all cells were counted in an optical microscope using a Neubauer chamber. Smears were prepared using a cytocentrifuge (Cytospin 3; Shandon Lipshaw, Pittsburgh, USA), and stained with a Panotic staining kit. The total leucocytes and neutrophils were also counted. The results were represented as number of neutrophils per cavity.

Statistical analysis

Data from neutrophils migration and neuromuscular blockade assay were verified using ANOVA and Tukey as post hoc test. For paw edema assay, ANOVA and Dunnett tests were used. For myographic and histological assays the *t*-Student test was used. Significance level was set at 5%.

Results and Discussion

Snake envenomation is still an important issue in many rural areas of tropical and subtropical countries. Some medicinal plants, such as *J. elliptica* are used for treating snakebites in folk medicine. We have

confirmed *in vivo* the anti-inflammatory and antithrotophic effects of “teiu extract” (TE) from *J. elliptica* rhizomes in the present study.

Pharmacognostic study

The extract was negative for cyanogenic glycosides and the foam index of saponin was 2500, i.e., 2.5 L of water is needed to dilute 1 g of extract to provide a 1 cm-height foam. The hemolytic test was negative for TE and positive to quillaja suspension. Supplementary Figure S1 shows the assays used to evaluate the TE content.

TE did not present cyanogenic glycosides or hemolytic activity, showing that this plant has a potential to be safe for human use. The foam formation is indicative of saponins, which are usually steroidal or triterpene glycoside compounds. Saponins have antimicrobial activity, causing cell rupture on microbial community. In addition, the extract showed alkaloids in its composition, a largest single class of secondary metabolites, which function is still obscure.⁸ In the alkaloids assay, both colors orange and white show alkaloids (Supplementary Figure S2.1, S2.2, and S2.3) in TE composition. Alkaloids were confirmed by TLC (Supplementary Figure S2.4), using atropine as positive control. Atropine and TE showed similar retention factors (Rf).

The total phenolic content in TE was of 14.5%, which was obtained by using the equation $y = 0.1031x + 0.0489$ ($R = 0.9986$), and gallic acid as standard. Flavonoids content was of 0.08% ($y = 0.0488x - 0.0025$, $R = 0.9998$; quercetin as standard). Supplementary Figure S3 shows the chromatographic profile of TE (lanes 3 and 9) compared to the following phytochemical standards: 1 - β -sitosterol ($R_f=0.9$); 2 - gallic acid ($R_f=0.92$); 4 and 7 - tannic acid ($R_f = 0.77$); 5 - chlorogenic acid ($R_f=0.6$ and 0.75); 6 - caffeic acid ($R_f=0.9$); 8 - rutin ($R_f=0.47$ and 0.67); 10 - quercetin ($R_f=0.88$); and 11 - apigenin (R_f =dragged spot). TE expressed more phenolic constituents (blue colors) than flavonoids (orange colors) in the solvent system used. The chromatographic fingerprint of TE was similar to the ones observed in literature.² The predominant constitution was phenolic acids (14.5%) and lower flavonoids content (0.08%) were not mentioned previously. Further studies are needed to explore the extract composition.

Venom neutralization assays

Figure 1 shows TE in concentrations of 500 μg ($n=8$), 750 μg ($n=6$), 1000 μg ($n=4$), and 2000 μg ($n=2$) added into the 5 mL bath containing the preparation. The 500 μg was selected as the concentration for TE in the remaining assays, since it induced fewer changes in the basal response, ideal for the venom neutralization assays. A facilitator effect (increased amplitude of muscular response) was observed in 750 μg TE.

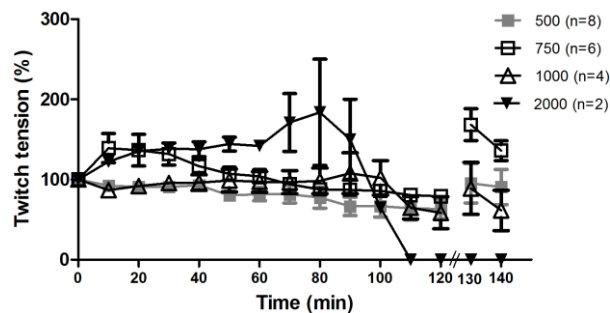


Figure 1. Contractile response of PND incubated with TE without venom

Contractile response (mean \pm SEM) of stimulated PND preparations incubated with different TE concentrations and without Bjssu venom. The number of experiments (n) is shown in the legend of figure.

Figure 2 shows the contractile responses of two models. The pre-incubation model ($86 \pm 14\%$) showed a better protective effect than the post venom model ($64 \pm 10\%$).

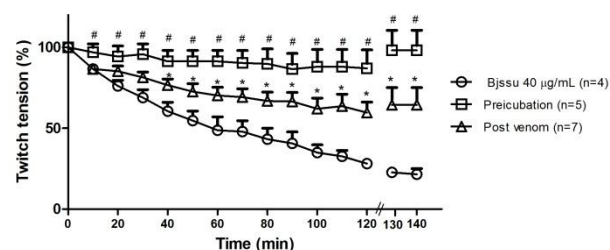


Figure 2. Effect of TE on contractile response of PND in pre-incubated and post venom models

Contractile responses (mean \pm SEM) of stimulated PND preparations in pre-incubated (30 min) and post venom models. Statistically significant differences ($p < 0.05$) between post venom model with Bjssu venom alone (*), and pre-incubation model with both Bjssu venom alone and post venom model (#) were indicated. The number of experiments (n) is shown in the legend of figure.

Myographic assays showed that the pre-incubation model was more advantageous than post-venom model, since it permitted to TE constituents to neutralize the venom constituents *in vitro* before exposing the phrenic nerve preparations. Besides, it showed the ability of the TE to avoid paralysis evolution, suggesting antithrotophic properties, such as *Casearia gossypiosperma*,¹² *Vellozia flavicans*,¹⁹ and phenolics from *Dipteryx alata*.²⁰

Some constituents of our *J. elliptica* extract, such as flavonoids (apigenin, quercetin, and rutin), probably have a role in the protection against the paralysis induced by Bjssu, although they were found in a very small content (0.08%). Previously, quercetin showed some protection against the neuromuscular blockade of Bjssu in the same experimental conditions.²¹ In contradiction, another study demonstrated that a commercial rutin formulation did not protect against the paralysis induced by either Bjssu or bothrospoxin-I, which could be related to a large amount used (180 mg/mL).²² Considering these previous studies, we believe that some phenolic compounds of *J. elliptica* extract may have the

effects observed in the present study; however, further studies are needed to prove this hypothesis.

A high extract concentration (2000 µg) produced an intense neurotransmitter release before the complete blockade. From the extract constituents, saponins could be the best candidate to produce this effect, since its neuroprotective effect was previously demonstrated.²³ These authors proposed several possibilities for explaining the neuroprotective role of saponins, from which at least three of them can be shared against the venom: modulation of neurotransmitter (intense acetylcholine release followed by a possible depletion); 2) attenuation of Ca²⁺ influx; and 3) inflammatory inhibition.

The myotoxic index (MI) of venom alone was 44.4% ± 0.86 (n=3), which showed characteristic damage, including myonecrosis, edema, membrane rupture, and presence of ghost cells. In the pre-incubated preparations, MI was reduced to 16.8% ± 0.55 (n=3), showing that TE protected (p<0.05) against the myotoxic effect of Bjssu. Irrespectively of its constituent, *J. elliptica* extract expressively decreased almost three times the cell damage induced by Bjssu, by an unclear mechanism.

Anti-inflammatory study

Figure 3 describes the variation of paw volume or ΔV (volume of paw edema at each time-interval minus the volume of paw at baseline) after 5 h of injection of Bjssu venom or carrageenan. All Bjssu venom doses were able to induce similar or more pronounced paw edema than carrageenan. The maximum edema was obtained after 2 h of injection for all doses. In doses of 0.5 µg and 0.25 µg, Bjssu venom induced more similar effects to carrageenan than the other doses (p> 0.05). In fact, 0.5 µg of Bjssu venom produced more pronounced edema than carrageenan, 2 h after injection, while 0.25 µg induced less edema at 3 h (p<0.05). Higher venom doses (1 and 2 µg) induced 2 to 3 times higher paw edema than carrageenan, and caused a visible and

diffused hemorrhage (p>0.05). Therefore, 0.5 µg of Bjssu venom was chosen for the hind-paw edema assay in order to avoid under-estimated inflammatory effects of the venom. Bjssu at 0.5 µg induced similar paw edema, without a prominent hemorrhage, when compared to 50-µg carrageenan. *Bothrops* venoms are known to cause edema, local pain, erythema hemorrhage, and necrosis.²⁴

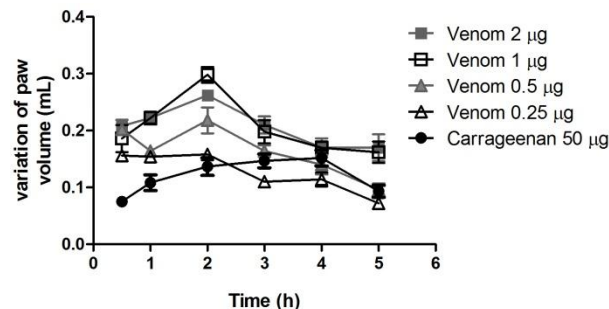


Figure 3. Dose-response of hind-paw edema induced by venom

Dose-response (mean ± SD) profile of hind-paw edema (ΔV) induced by *B. jararacussu* venom. Comparisons were performed between carrageenan and each dose of venom (ANOVA/Dunnet; n=5).

Table 1 shows the ΔV (in percentage) after TE (V.O.) and dexamethasone (I.P.) administration. Both substances reduced the paw edema after 1 h of administration, when compared to a negative control group (NaCl 0.9%, V.O.). In addition, dexamethasone and TE (in all doses) induced maximal edema inhibition between 3 to 5 h after injection in comparison to negative control (p<0.05). TE 1000 mg/kg and dexamethasone induced similar anti-inflammatory effect at 3, 4, and 5 h time-intervals (p<0.05). The edema caused by Bjssu is induced by prostanoids production and neutrophils migration.²⁵ Our results showed that *J. elliptica* extract, in all concentrations tested, reduced the paw edema similarly to dexamethasone for at least 5 hours.

Table 1. Effect (mean±SD of ΔV in mL, % of reduction) of TE and dexamethasone on paw edema induced by Bjssu venom.

Groups	Dose (mg/kg)	Mean and Standard deviation of ΔV (mL) and percent of reduction of paw edema (induced by 0.5 µg of Bjssu)					
		30 min	1 h	2 h	3 h	4 h	5 h
Control	---	0.13 ± 0.03	0.12 ± 0.03	0.10 ± 0.02	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.02
Dexamethasone	2 mg/kg (i.p.)	0.12 ± 0.04 (10.0%)	0.07 ± 0.03* (39.5%)	0.05 ± 0.02* (51%)	0.04 ± 0.02* (57%)	0.03 ± 0.02* (60%)	0.03 ± 0.02* (66%)
TE	250 mg/kg	0.12 ± 0.04 (13.7%)	0.06 ± 0.03* (53.5%)	0.05 ± 0.03* (45.6%)	0.05 ± 0.02* (44.6%)	0.04 ± 0.01* (52%)	0.04 ± 0.02* (54%)
TE	500 mg/kg	0.13 ± 0.03 (2.5%)	0.07 ± 0.02* (35%)	0.07 ± 0.02* (34%)	0.06 ± 0.01* (39.5%)	0.05 ± 0.02* (40%)	0.05 ± 0.02* (44%)
TE	1000 mg/kg	0.12 ± 0.02 (12.5%)	0.05 ± 0.03* (53.4%)	0.06 ± 0.01* (42%)	0.03 ± 0.02* (66%)	0.04 ± 0.03* (52%)	0.03 ± 0.03* (66%)

Mean and Standard deviation of ΔV represent the mean difference of paw volume between the basal measure and measures at each time point (30 min, 1, 2, 3, 4 and 5 h), n = 5. Percent of reduction of paw edema (previously induced by 0.5 µg of Bjssu) was calculated considering the paw volume for each group in comparison to the control group at each time interval. The symbol * represent significant differences when comparing each group to control (p<0.05; ANOVA - Dunnett's test).

Figure 4 shows TE (irrespectively of dose) and indomethacin reduced (p<0.05) the neutrophils migration

when compared to negative control (vehicle). No significant differences (p>0.05) among indomethacin

(positive control), carrageenan (control group), and TE groups (250, 500, and 1000 mg/kg) were observed regarding neutrophils migration. In a dose dependent manner, *J. elliptica* extract was also able to reduce migration of leucocytes induced by carrageenan similarly to indomethacin. The probable constituents responsible for the extract anti-inflammatory properties were the polyphenols, since these compounds previously showed potential anti-inflammatory properties.²⁶ Thus, in both inflammatory models the extract showed activity similar to the classical anti-inflammatory agents.

There are many questions for the snakebite managing: the high incidence in several countries, the unsatisfactory emergency health services, the difficult transportation of antiserum and patients, the difficult antiserum handling, the adverse reactions against the antiserum, a lot of clinical complications and the high mortality rates.²⁷ Thus, the bioprospection of new complementary agents for antiserum therapy, such as medicinal plants used in folk medicine, could be essential keys.

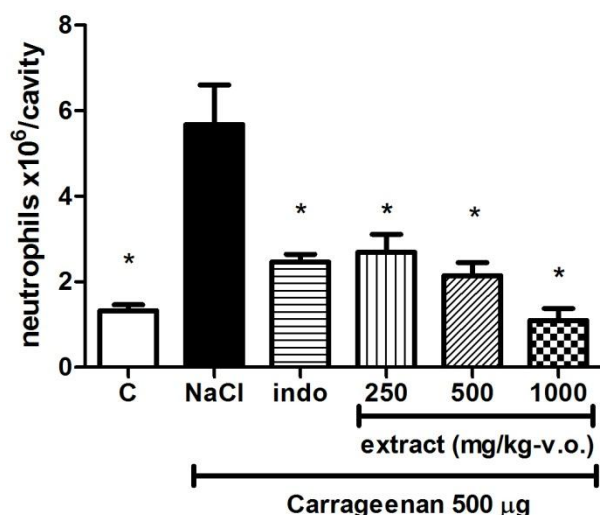


Figure 4. Effect of TE on neutrophils migration

Neutrophils migration (mean ± SD) towards the peritoneal cavity induced by carrageenan injection (i.p.). Control (C) is the intra-peritoneal injection of 0.9% NaCl (no carrageenan). NaCl is the negative control, i.e., 0.9% NaCl administered orally after carrageenan intra-peritoneal injection. Positive control (indo) is indomethacin administered at 10 mg/kg (i.p.). * - statistical significant differences when compared to NaCl group (ANOVA/Tukey, $p < 0.05$; $n = 6$).

Conclusion

J. elliptica extract, which was rich in phenolic compounds, showed medicinal properties as anti-inflammatory, being able to counteract the *in vitro* neurotoxicity and myotoxicity of *B. jararacussu* venom.

Acknowledgments

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Ethical Issues

The ethical issue has been stated on page 575, under the section "Animals".

Conflict of Interest

The authors declare no conflict of interests.

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