Pharmacological Properties of Vochysia Haenkeana (Vochysiaceae) Extract to Neutralize the Neuromuscular Blockade Induced by Bothropstoxin-I (Lys49 Phospholipase A2) Myotoxin

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Introduction

In Brazil, Bothrops snakes comprise more than 30 species distributed throughout the country and they are responsible for approximately 70% of snakebites every year; the World Health Organization (WHO) has considered snakebites as a neglected tropical disease due to the numerous cases and difficulties in specific regions to reach antivenom therapy.¹⁻⁵ Bothrops venoms induce severe local and systemic damages due to their high enzymatic action basically mediated by proteases and phospholipases A₂ (PLA₂).⁶,⁷ In envenomation by Bothrops venoms, the local effects

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Abstract

Purpose: Bothrops snakes are responsible for more than 70% of snakebites every year in Brazil and their venoms cause severe local and systemic damages. The pharmacological properties of medicinal plants have been widely investigated in order to discover new alternative treatments for different classes of diseases including neglected tropical diseases as envenomation by snakebites. In this work, we have investigated the ability of Vochysia haenkeana stem barks extract (VhE) to neutralize the neuromuscular effects caused by Bothropstoxin-I (BthTX-I), the major phospholipase A₂ (PLA₂) myotoxin from B. jararacussu venom.

Methods: The biological compounds of VhE were analysed under thin layer chromatography (TLC) and its neutralizing ability against BthTX-I was assessed through twitch-tension recordings and histological analysis in mouse phrenic nerve-diaphragm (PND) preparations. The antimicrobial activity of VhE was assessed against S. aureus, E. coli and P. aeruginosa strains. The aggregation activity of VhE was analysed under protein precipitation assay.

Results: VhE showed the presence of phenolic compound visualized by blue trace under TLC. VhE abolished the neuromuscular blockade caused by BthTX-I applying the pre-toxin incubation treatment and partially neutralized the BthTX-I action under post-toxin incubation treatment; VhE contributed slightly to decrease the myotoxicity induced by BthTX-I. The neutralizing mechanism of VhE may be related to protein aggregation. VhE showed no antimicrobial activity.

Conclusion: V. haenkeana extract which has no antimicrobial activity exhibited neutralizing ability against the neuromuscular blockade caused by BthTX-I and also contributed to decrease its myotoxicity. Protein aggregation involving phenolic compounds may be related in these protective effects.
are marked by intense necrosis accompanied by edema, equimosis and acute inflammatory activity. In addition, local infections caused by gram-negative anaerobic bacteria derived from oral flora of snakes are considered an important clinical complication in victims of snakebites.\textsuperscript{4,8} Snakebites are conventionally treated through antivenom serum therapy; however, based on popular practices, the pharmacological properties of medicinal plants have been widely investigated in order to discover new alternative treatments for different classes of diseases including neglected tropical diseases as envenomation by snakebites.\textsuperscript{9} Recent investigations have shown that plant extracts exhibit antimicrobial and antiophidian activities.\textsuperscript{10-12}Bothrops jararacussu snake, popularly known as Jararacuçu, is widely distributed in Southeast region of Brazil\textsuperscript{9} and its venom is composed by enzymatic and non-enzymatic proteins, carbohydrates, peptides, lipids, biogenic amines and inorganic components, similarly to other Bothrops venoms.\textsuperscript{13} Bothropstin-I (BthTX-I) is a non-enzymatic Lys49 PLA\textsubscript{2} isolated from B. jararacussu venom which induces irreversible neuromuscular blockade in vertebrate neuromuscular preparations in vitro characterized by intense myonecrosis, increase in creatine kinase release, muscle contracture and membrane depolarization.\textsuperscript{14-17}Vochysia haenkeana (Vochysiaceae), plant popularly known in Brazil as “escorrega-macaeo”, “pau-amarello” and/or “cambarazinho”, comes from semidecidual broadleaf forest common in Mato Grosso do Sul, Mato Grosso and Goiás Brazilian States; V. haenkeana exhibits a great variety of secondary metabolites such as tannins, saponins, phenolic compounds, flavonoids and coumarins and it has been cited in ethnobotanical studies related to treatment of respiratory diseases.\textsuperscript{18-20} However, its pharmacological activities have been poorly investigated.\textsuperscript{21}In this work, we have assessed the neutralizing ability of V. haenkeana hydroalcoholic extract against the main PLA\textsubscript{2}-myotoxin (BthTX-I) from B. jararacussu venom in mouse nerve-diaphragm preparations and its antimicrobial activity against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa strains.

Materials and Methods

Reagents and BthTX-I

All salts for the physiological solution were of analytical grade. BthTX-I was provided by Dra Adélia Cristina Oliveira Cintra from São Paulo University (USP, Ribeirão Preto, SP, Brazil).

Animals

Male Swiss mice (25–30 g) obtained from Multidisciplinary Center for Biological Investigation (CEMIB/Unicamp) were housed at a maximum of 10 mice per cage at 23 °C on a 12 h light/dark cycle. The animals had free access to food and water ad libitum.

Plant material

The hydroalcoholic extract from stem barks of V. haenkeana was provided by Dr Márcio Galdino dos Santos from Tocantins Federal University (UFT, Palmas, TO, Brazil). The full description about the origin of the extract has been shown elsewhere.\textsuperscript{18} The plant exsiccate was deposited in the Herbarium of the Tocantins Federal University (UFT, Porto Nacional, TO, Brazil) as voucher specimen #10.074 by Solange de Fátima Lolis according to the International Code of Botanical Nomenclature (ICBN).

Solubilisation of V. haenkeana extract

In order to find out the ideal solvent for V. haenkeana extract (VhE) without affecting the basal twitch responses recorded in mammalian nerve-muscle preparations, polyethylene glycol 400 (PEG 400, Synth\textsuperscript{®}), dimethyl sulfoxide (DMSO, Sigma\textsuperscript{®}) and ethanol (Synth\textsuperscript{®}) were selected to be tested. Ethanol (30 µL) showed be the best solvent to solubilize VhE; ethanol 70 % did not change the twitch responses in control experiments recorded from mouse phrenic nerve-diaphragm (PND) preparations.\textsuperscript{22}

Thin layer chromatography (TLC)

For TLC, it was used aluminium plates coated with silica gel 60 (0.20 mm thick) containing the fluorescent indicator UV254 (Macherey-Nagel GmbH & Co., Bethlehem, PA, USA) and phytochemical standard in methanol (1 %) (Sigma-Aldrich Co., St. Louis, MO, USA) including quercetin (1), rutin (2), caffeic acid (3), tannic acid (4), coumarin (5), gallic acid (6) and VhE (7); a purposeful empty lane was maintained for observing the solvent race (8). The solvent system (mobile phase, 10 mL) consisted of ethyl acetate, formic acid, acetic acid and water (100:11:11:27), as described elsewhere.\textsuperscript{23} The chromatograms were initially stained with diphenylboric acid 2-aminooethyl ester solution (5 % in ethanol) (Sigma-Aldrich Co., St. Louis, MO, USA) followed by polyethylene glycol 4000 solution (5 % in ethanol) (Sigma-Aldrich Co., St. Louis, MO, USA), with visualization under UV light at 360 nm. The retention factor (Rf) and sample colours were visually compared to phytochemical standards.

Antimicrobial activity of VhE

The following bacterial strains were purchased from American Type Culture Collection (ATCC): Escherichia coli ATCC 25922, E. coli ATCC 10536, Pseudomonas aeruginosa ATCC 25922, S. aureus ATCC 29213, methicillin-resistant S. aureus (MRSA) ATCC 35951, and methicillin/oxacillin resistant S. aureus (MRS/ORSA) ATCC 43300. The cultures were stored at -80 °C in Tryptic soy broth (TSB; Difco Laboratories, Detroit MI, USA) containing 40 % (v/v) glycerol and were routinely cultured in Tryptic soy agar (TSA; Difco Laboratories) under aerobic conditions at 37 °C. Antimicrobial activity of the extract was tested by using the broth microdilution method according to the guidelines
from the Clinical and Laboratory Standards Institute (CLSI), protocol M07-A9. Briefly, four to five colonies were harvested from pure cultures growing on TSA and were used to prepare a bacterial inoculum. Colonies were transferred into tubes containing 5 mL of TSB and cultured at 37 °C until reaching turbidity equivalent to 0.1 at 660 nm (approximately 1.5 x 10^8 CFU/mL). Two-fold dilutions of the VhE (from 1000 to 0.4 µg/mL) were made in 96-well plates with 100 µL of Mueller Hinton Broth (MHB; Difco) per well. Then, the bacterial suspension (100 µL) was inoculated, and the plates were incubated for 24 h at 37 °C. The lowest concentration with any visible bacterial growth was taken as the minimum inhibitory concentration (MIC). In addition, bacterial growth was assessed by optical density measurement (660 nm).

**Mouse phrenic nerve-diaphragm (PND) preparation**

PND preparations were obtained from male Swiss mice killed with isoflurane (Fortval®. Vinhedo, SP, Brazil). The preparations were mounted under a resting tension of 5 g in a 5 mL organ bath containing aerated (95 % O_2/5 % CO_2) Tyrode solution (composition, in mM: NaCl 137, KCl 2.7, CaCl_2 1.8, MgCl_2 0.49, NaH_2PO_4 0.42, NaHCO_3 11.9 and glucose 11.1, pH 7.0) at 37 °C as described elsewhere.

The preparations were stimulated indirectly (5–7 V, 0.1 Hz, 0.2 ms) with supramaximal stimuli being delivered from a stimulator (Model ESF-15D, Ribeirão Preto, SP, Brazil) via bipolar electrodes positioned on the nerve. Muscle twitches were recorded using a force displacement transducer coupled to a two-channel Gemini recorder (both from Ugo Basile®, Varese, Italy). After stabilization for 20 min, VhE and BthTX-I (a single concentration per experiment) was added to the preparations and left in contact for 120 min or until complete blockade. For neutralization assays, we applied two types of protocols, as suggested elsewhere:

1) pre-toxin incubation: BthTX-I was maintained under incubation with VhE for 30 min before twitch tension experiments and 2) post-toxin incubation (VhE was added into the recording chamber 10 min later than BthTX-I).

**Protein precipitation measurement**

The protein precipitation induced by VhE extract was evaluated as previously described elsewhere. Albumin and BthTX-I (10 µg) was incubated separately with VhE (150 µg) following the protocols: 1) pre-toxin incubation with VhE: BthTX-I (50 µg/mL) was maintained under incubation with VhE (0.4 mg/mL) for 30 min at room temperature (23-25 °C) and then for 60 min at 37 °C; and 2) without pre-toxin incubation with VhE: BthTX-I (50 µg/mL) was maintained incubated with VhE (0.4 mg/mL) for 60 min directly at 37 °C. In both of protocols, the mixture was centrifuged at 5,000 rpm for 15 min and the protein concentration in supernatant was measured as essentially described elsewhere. The absorbance obtained was compared with tubes containing only protein (albumin or BthTX-I) to assess the % of protein precipitation. Ethanol (solvent for VhE) was tested alone to verify its influence on the protein precipitation. Tubes containing the same amount of VhE or ethanol, in absence of albumin or BthTX-I, were used as blanks.

**Quantitative histological study**

The preparations from the preincubation and post toxin assays were analysed by a quantitative morphometric method and compared to Tyrode control, VhE and BthTX-I. At the end of each experiment (after 120 min), three preparations of each group were fixed by a formalin 10 % solution, and processed by routine morphological techniques. Cross-sections (5 µm thick) of diaphragm muscle were stained with 0.5 % (w/v) hematoxylin-eosin, for microscopy examination. Tissue damage (edema, intense myonecrosis characterized by atrophy of the muscle fibers, hyaline aspect, sarcolemmal disruption and lysis of the myofibrils) was verified by three different trained people and it was expressed as a 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.

**Statistical analysis**

Changes in the twitch-tension responses of PND preparations were expressed as a percentage relative to baseline (time zero) values and morphological alterations measured based on myotoxicity index (MI, in %). The results were expressed as the mean ± SEM and statistical comparisons were done using Student’s t-test with p < 0.05 indicating significance. All data analyses were done using Microcal Origin 8 SR4 v.8.0951 (Microcal Software Inc., Northampton, MA, USA) software.

**Results and Discussion**

Based on popular medicinal practices, stem barks from *Vochysia haenkeana* (a plant variety of the Vochysiaceae family widely distributed in semideciduous broadleaf forest from west central region of Brazil) has been selected in this work to be investigated in terms of its neutralizing ability against the effects induced by snake venom and antimicrobial activity. *V. haenkeana* is popularly known as “escorrega-macaco” and shows long plum trunk and smooth barks.

*V. haenkeana* contains secondary metabolites such as tannins, saponins, phenolic compounds, flavonoids and coumarins. Here, we selected a sample *V. haenkeana* extract (VhE) to be subjected to Thin Layer Chromatography which revealed the presence of phenolic compounds characterized by a blue spot not matched with caffeic, tannic nor gallic acids; a weak yellow spot (indicated by an arrow) can be also seen in the chromatogram profile suggesting the presence of flavonoids not related to quercetin or rutin (Figure 1).
We have not observed the presence of coumarin in VhE under TLC system; our data were inconclusive to justify the involvement of this compound eventually present in VhE with treatment of respiratory diseases, as suggested by popular practices. However, it has already shown that commercial coumarin alone does not protect the neuromuscular blockade induced by B. jararacussu or Crotalus durissus terrificus venoms in vitro.

Orange and blue compounds visualized by TLC in plant extracts are suggestive of flavonoids and polyphenol compounds. Oshima-Franco and Dal Belo reviewed antineurotoxic polyphenol plants, such as: Camellia sinensis L., Casearia sylvestris Sw., Casearia gossypiosperma Briquet, Curcuma zedoaroides A. Cheveerach & T. Tanee, Dipteryx alata Vogel, Galactia glaucescens Kunth., Hypericum brasiliense Choisy, Jatropha elliptica (Pohl) Oken., Mikania laevigata Sch. Bip. ex Baker, Plathymenia reticulata Benth., Terminalia jagifolia Mart., and Vellozia flavicans Mart. ex Schult. As showed in this work, V. haenkeana also has in vitro antineurotoxic effect, by an in vitro interaction.

Vochysia haenkeana has been poorly studied and its pharmacological properties are still unknown and need to be further explored. It has been shown that VhE does exhibit antitumoral activity in rats with induced Erlich tumor. In addition, our data have shown that VhE also does not promote antimicrobial action against S. aureus, E. coli and P. aeruginosa strains. Investigations about antimicrobial activities of plant extracts associated to local effects induced by snake venoms show potentially useful to indicate alternative methods to treat snakebites since infections caused by bacteria from snake’s mouth is often associated to snakebites.

Before subjecting VhE to neutralization assays in PND preparations, we have verified whether the extract by itself could cause changes in twitch responses; a concentration-response experiment was carried out using 0.2, 0.4 and 0.8 mg of VhE/mL; the concentrations of 0.2 and 0.4 mg/mL did not cause changes in PND preparations during 120 min incubation (p > 0.05) whereas 0.8 mg of VhE/mL induced a slight decrease in twitch tension from 50 min incubation (p < 0.05 compared to Tyrode solution alone). We have selected the VhE concentration of 0.4 mg/mL to be used in those protocols with neutralization of BthTX-I-induced neuromuscular blockade (50 μg/mL; concentration enough to produce complete neuromuscular blockade in PND preparations) (Figure 2).

BthTX-I induces irreversible neuromuscular blockade and myonecrosis in vitro similarly to effects caused by crude venom of Bothrops jararacussu from where it comes from. The ability of this toxin to reproduce the neuromuscular effects seen with crude venom becomes it the main myotoxin from B. jararacussu venom. We have applied two different protocols to assess the neutralizing ability of VhE: pre-toxin incubation (BthTX-I was maintained under incubation for 30 min with VhE prior experiments in PND preparations) and post-toxin incubation (VhE was added into the record chamber 10 min after toxin addition). Under pre-toxin incubation, VhE neutralized completely the neuromuscular blockade caused by BthTX-I; on the other hand, VhE was not able to avoid the blockade by BthTX-I when added after toxin although it has been noticed a slight attenuation over 120 min (Figure 3).

Oshima-Franco et al. studied the presynaptic nature of BthTX-I. Thus, the preincubation of toxin with VhE observed in pre-toxin experiments can be related to ability of VhE to avoid the presynaptic trigger of myotoxin; which, in turn, once triggered VhE was unable of repairing (post-toxin experiments).

Table 1 compares the level of myotoxicity induced BthTX-I to those ones reached by VhE under pre- and post-toxin incubation protocols. The pool of BthTX-I used in this work caused low myotoxicity when compared to other data shown in previous investigations, where 20 μg of toxin/mL induced 67 ± 2.3% of damage cell in PND preparations. Here, we have used 50 μg of

Figure 1. Thin layer chromatography (TLC) of phytochemical standards (quercetin (1), rutin (2), caffeic acid (3), tannic acid (4), coumarin (5), gallic acid (6), VhE (7) and solvent alone (8)). Solvent: ethyl acetate, formic acid, acetic acid and water (100:11:11:27, respectively). Stain: NP – diphenylboric acid 2-aminoethyl ester, PEG – polyethylene glycol 4000. Arrow: yellow spot weekly revealed indicating an eventual presence of flavonoids in VhE.

Figure 2. Neuromuscular responses of VhE (0.2–0.8 mg/mL) on mouse phrenic nerve-diaphragm (PND) preparation maintained under indirect stimulation. The points are the mean ± SEM (n = 5). *p < 0.05 compared to Tyrode control. VhE: Vochysia haenkeana extract.
toxin/mL to obtain complete muscle paralysis. Under pre- and post-toxin incubation protocols, VhE contributed to decrease in ~50 % the cell damage caused by BthTX-I.

Figure 3. Effect of VhE on the neuromuscular blockade induced by BthTX-I on mouse phrenic nerve-diaphragm (PND) preparations maintained under indirect stimulation. Note that VhE prevented the BthTX-I blockade-induced under pre-toxin incubation. The points are the mean ± SEM (n = 5–7). *p < 0.05 compared to Tyrode control. *p < 0.05 compared to BthTX-I alone. BthTX-I: Bothropstoxin-I; VhE: Vochysia haenkeana extract. Arrow indicates the exact moment in which VhE was added for post-toxin incubation protocols.

![Graph showing effect of VhE on neuromuscular blockade](image)

Table 1. Morphological analysis of diaphragm muscle expressed as Myotoxicity Index (MI, in %)

| Muscles resulting from Pharmacological assays | MI (%) |
|---------------------------------------------|--------|
| Tyrode control                              | 8.04 ± 5 |
| VhE                                         | 7.85 ± 5 |
| BthTX-I                                     | 26.7 ± 16 |
| Pre-toxin incubation                        | 14.4 ± 10 |
| Post-toxin incubation                       | 14.8 ± 11 |

The neutralizing ability of VhE against the neuromuscular blockade caused by BthTX-I in vitro may be related to its capacity to induced protein aggregation as seen in our experiments in protein precipitation assay (Figure 4). The incubation with VhE promoted the precipitation of BthTX-I reducing the toxin-concentration in 70.5 ± 7.1 %. The incubation of VhE with albumin has also been evaluated as positive control showing 79.4 ± 6.7 % of precipitation. Ethanol (solvent for VhE) alone was assessed under protein precipitation assay as negative control in order to refute its influence in that protein aggregation seen with VhE; it did not promote protein aggregation in both of compounds albumin and BthTX-I.

Figure 4. Protein precipitation induced by VhE. Note that VhE caused significant precipitation of BthTX-I and albumin; ethanol (used for solubilizing the VhE) had no activity on these effects. *p < 0.05. VhE: V. haenkeana extract.

![Graph showing protein precipitation](image)

The pharmacological action of plant extracts to neutralize the neuromuscular activity of snake venoms and their toxins in vitro shows still unknown but it is frequently associated with protein precipitation, proteolytic degradation, enzyme inactivation, metal chelation and antioxidant action. Flavonoids and tannins are the main components related with those activities and both of them were previously observed in VhE. Although we have not found evidence for tannic acid in this extract, responsible for antiophidian mechanism of plant extracts as suggested by Melo et al., the protein precipitation promoted by VhE may be related to its neutralizing effect against BthTX-I.

**Conclusion**

V. haenkeana stem barks extract abolished the neuromuscular blockade caused by BthTX-I under pre-toxin incubation treatment; however, it was not efficient to neutralize the toxin-induced neuromuscular blockade under post-toxin treatment. In both of treatments VhE contributed to decrease the myotoxicity caused by toxin. These effects may be related to protein aggregation involving phenolic compounds which represent the major constituent of VhE, as shown by TLC. VhE showed no antimicrobial activity against S. aureus, E. coli and P. aeruginosa strains.

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Ethical Issues
This study was approved by the institutional Ethics Committee on Animal Use (CEUA/UNISO, protocol no. 054/2015) and the experiments were carried out according to the guidelines established by the Brazilian Society of Laboratory Animal Science (SBCAL).

Conflict of Interest
The authors declare that they have no competing interests.

References
1. Bemarde PS. Anfíbios e répteis: introdução ao estudo da herpetofauna brasileira. Curitiba: Analisbooks; 2012.
2. Motta YP. Aspectos clinico, laboratorial e histopatológico da intoxicação experimental pelos venenos das serpentes Bothrops jararaca e Crotalus durissus terrificus em ratos Wistar tratados com antiveneno e Mikania glomerata. Botucatu: Universidade Estadual Paulista (UNESP); 2008.
3. Palacio TZ. Isolamento e caracterização bioquímica e funcional de um inibidor de metaloproteases presente no soro de serpente Bothrops alternatus. Ribeirão Preto: Universidade de São Paulo; 2014.
4. Cardoso JLC, Fan HW, Malaque CMS, Haddad Jr V. Animais peçonhentos no Brasil, Biologia, clinica e terapêutica dos acidentes. 2nd ed. São Paulo: Servier; 2009.
5. Incidência de casos de acidentes por serpentes por 100.000 habitantes entre. Brasil: Grandes Regiões e Unidades Federadas; 2000 – 2013.
6. Silveira ACP. Atividade antimicrobiana da BthTX-I e seu uso como melhorador de desempenho alternativo na avicultura. Uberlândia: Universidade Federal de Uberlândia; 2013.
7. Sacoman TM. Efeito da interação entre Bothrotoxina-I e Crotapotina na junção neuromuscular de camundongos “in vitro”: estágio em iniciação científica. Brazil: Universidade Estadual Paulista; 2008.
8. Martins MS. Efeito da fitoterapia na injuria renal aguda induzida pela peçonha de Bothrops jararaca. Dissertação apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção de título de Mestrado em Ciências. São Paulo: 2013.
9. Rodrigues L, Cunha DB, Leite GB, Borja-Oliveira CR, Cintra ACO, Rodrigues-Simioni L, et al. Ação da heparina contra a atividade neurotóxica da miotoxina bothrotoxina-I. Saude Rev 2004;6(4):19-29.
10. Moura VM, Mourão RHV, Santos MCD. Acidentes ofídicos na Região Norte do Brasil e o uso de espécies vegetais como tratamento alternativo e complementar à soroterapia. Scientia Amazonia 2015;4(1):2-12.
11. Vilar JC, Carvalho CM, Furtado MFD. Ofidismo e plantas utilizadas como antiofídicas. Biol Geral Exper 2005;6(1):3-36.
12. Colaço RCO, Junior-Rocha DS, Silva MG, Cogo JC, Oshima-Franco Y, Randazzo-Moura P. Propriedade antiofídica do extrato metanólico de Mikania laevigata sobre as ações biológicas induzidas pelo veneno de Philodryas olfersii na junção neuromuscular. REU 2010;36(2):105-13.
13. Elflio- Esposito SL, Hess PL, Moreno AN, Lopes Ferreira M, Ricart CAO, Souza MV, et al. A C-type lectin from Bothrops jararacus venom can adhere to extracellular matrix proteins and induce the rolling of leukocytes. J Venom Anim Toxins incl Trop Dis 2007;13(4):782-99.
14. Dos Santos JI, Cardoso FF, Soares AM, dal Pai Silva M, Gallacci M, Fontes MR. Structural and functional studies of a bothropic myotoxin complexed to rosmarinic acid: new insights into Lys49-PLA(2) inhibition. Plos One 2011;6(12):e28521. doi: 10.1371/journal.pone.0028521
15. Oshima-Franco Y, Leite GB, Belo CA, Hyslop S, Prado-Franceschi J, Cintra AC, et al. The presynaptic activity of bothrotoxpin-I, a myotoxin from Bothrops jararacus snake venom. Basic Clin Pharmacol Toxicol 2004;95(4):175-82. doi: 10.1111/j.1742-7843.2004.pto_950405.x
16. Rostelato-Ferreira S, Leite GB, Cintra AC, Cruz-Höfling MA, Rodrigues-Simioni L, Oshima-Franco Y. Heparin at low concentration acts as antivenom against Bothrops jararacus venom and bothrotoxpin-I neurotoxic and myotoxic actions. J Venom Res 2010;1:54-60.
17. Heluany NF, Homsi-Brandeburgo MI, Giglio JR, Prado-Franceschi J, Rodrigues-Simioni L. Effects induced by bothrotoxin, a component from Bothrops jararacus snake venom, on mouse and chick muscle preparations. Toxicol 1992;30(10):1203-10.
18. Cezari EJ. Plantas medicinais: atividade antitumoral do extrato bruto de sete plantas do cerrado e o uso por povos tradicionais. Dissertação (Mestrado) – Universidade Federal do Tocantins, Programa de Pós-Graduação em Ciências do Ambiente. Palmas: UFT; 2010.
19. Pasa MC. Saber local e medicina popular: a etnobotânica em Cuiabá, Mato Grosso, Brasil. Universidade Federal de Mato Grosso: 2011.
20. Lima CP, Santos MG, Calabrese KS, Silva ALA, Almeida P. Evaluation of the leishmanicidal activity of plant species of the Brazilian savanna. Rev Patol Trop 2015;44(1):45-55. doi: 10.5216/rpt.v44i1.34800
21. Rizzi ES, Pereira KCL, Abreu CAA, Silva BCFL, Fernandes RM, Oliveira AKM, et al. Allelopathic potential and phytochemistry of camburarazinho (Vochysia haenkeana (Spreng.) Mart.) leaves in the germination and development of lettuce and tomato. Bioso Ci 2015;32(1):98-107.
22. Colaço RCO, Cogo JC, Rocha T, Oshima-Franco Y, Randazzo-Moura P. Protection by Mikania laevigata (guaco) extract against the toxicity of Philodryas olfersii snake venom. Toxicon 2012;60(4):614-22. doi: 10.1016/j.toxicon.2012.05.014
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23. Costa KN, Gomes RM, Farrapo NM, Oshima-Franco Y. Validação farmacológica de fitoquímicos comerciais sob o parâmetro da junção neuromuscular (JNM) e do perfil cromatográfico. In: Oliveira-Junior JM, et al, editors. *Os Múltiplos Olhares na Área da Pesquisa – da Observação ao Conhecimento*. Brazil: Eduniso; 2011. PP. 11-29.

24. Bülbring E. Observation on the isolated phrenic nerve diaphragm preparation of the rat. *Br J Pharmacol* 1946;1:38-61.

25. Tribuiani N, Silva AM, Ferraz MC, Silva MG, Bentes APG, Graziano TS, et al. *Vellozia flavicans* Mart. ex Schult. hydroalcoholic extract inhibits the neuromuscular blockade induced by *Bothrops jararacussu* venom. *BMC Complement Altern Med* 2014;14:48. doi: 10.1186/1472-6882-14-48

26. Ambikabothy J, Ibrahim H, Ambu S, Chakravarthi S, Awang K, Vejayan J. Efficacy evaluations of *Mimosa pudica* tannin isolate (MPT) for its anti-ophidian properties. *J Ethnopharmacol* 2011;137(1):257-62. doi: 10.1016/j.jep.2011.05.013

27. Félix-Silva J, Souza T, Menezes YA, Cabral B, Câmara RB, Silva-Junior AA, et al. Aqueous leaf extract of *Jatropha gossypifolia* L. (Euphorbiaceae) inhibits enzymatic and biological actions of *Bothrops jararaca* snake venom. *PLoS One* 2014;9(8):e104952. doi: 10.1371/journal.pone.0104952

28. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193(1):265-75.

29. Ferraz MC, Parrilha LAC, Moraes MSD, Filho JA, Cogo JC, Santos MG, et al. The effect of lupane triterpenoids (*Dipteryx alata* Vogel) in the *in vitro* neuromuscular blockade and myotoxicity of two snake venoms. *Curr Org Chem* 2012;16(22):2717-23. doi: 10.2174/138527212804004481

30. Melo RS, Farrapo NM, Rocha-Junior DS, Silva MG, Cogo JC, Dal Belo CA, et al. Antiophidian mechanisms of medicinal plants. In: Keller RB, editor. *Flavonoids: Biosynthesis, Biological Effects and Dietary Sources*. New York: Nova Science; 2009. PP. 249-62.

31. Oshima-Franco Y, Dal Belo CA. Recognizing antiophidian plants using the neuromuscular junction apparatus. *Int J Complement Alt Med* 2017;5(5):00165.

32. Ministério da Saúde: Textos Básicos de Saúde (Cadernos de Atenção Básica; n. 22). Brasil: Health surveillance: zoonoses; 2009.

33. Oshima-Franco Y, Rosa LJR, Silva GAA, Amaral Filho J, Silva MG, Lopes PS, et al. Antithropic action of *Camellia sinensis* extract against the neuromuscular blockade by *Bothrops jararacussu* snake venom and its main toxin, bothropstoxin-I. Croatia: Intech; 2012.

34. Mors WB, Nascimento MC, Pereira BM, Pereira NA. Plant natural products active against snake bite-the molecular approach. *Phytochemistry* 2000;55(6):627-42.