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Neuromuscular block induced by *Bothrops moojeni* snake venom and the effect of *Jatropha elliptica* starch

This research evaluated the effect of *Bothrops moojeni* venom on neuromuscular activity and verified the antiophidian potential of the *J. elliptica* starch (JeP) in this model. The venom was obtained from snakes collected in the state of Tocantins, the protein profile evaluated in electrophoresis (SDS-PAGE), and the activity on the phrenic-diaphragm nerve of mice determined in 50 µg/mL of venom. The JeP, obtained by hand, was sieved in a sieve for granulometric analysis, and concentrations of 100 and 1000 µg/mL were tested to verify its antiophidian potential on the action of the poison. The following models were used: pre-venom - addition of venom and after 15 min addition of JeP; post-poison - addition of JeP and after 15 min the addition of poison; and pre-incubation - for 30 min, of poison with JeP or commercial antivenom. The poison had proteins from 10 to 60 kDa, which induced 50% of neuromuscular activity block in 71.5 ± 8.9 min (T50). The pre-venom and post-venom models with JeP 100 µg/mL prolonged phrenic nerve activity time (100.9 ± 7.6 min and 97 ± 6.1 min, respectively). The results obtained for 1000 µg/mL and in the model of pre-incubation of the venom with JeP 100 (78.2 ± 9.2); 1000 µg/mL (86.5 ± 8.9) and with commercial bothropic antivenom (80.2 ± 14.1), did not interfere with the activity of the venom on the phrenic nerve. *Bothrops moojeni* venom induces neuromuscular block and the lower concentration of the starch caused a protective effect on the junction, before and after the administration of the venom.

Keywords: *Bothrops moojeni*; Neuromuscular junction; *Jatropha elliptica*; starch.

Bloqueio neuromuscular induzido pelo veneno da serpente Bothrops moojeni e o efeito do polvilho de Jatropha elliptica

Esta pesquisa avaliou o efeito do veneno de *Bothrops moojeni* sobre a atividade neuromuscular e verificou o potencial antiofídico do polvilho de *J. elliptica* (JeP), nesse modelo. O veneno foi obtido de serpentes coletadas no estado do Tocantins, o perfil proteico avaliado em eletroforese (SDS-PAGE), e a atividade sobre o nervo frênico-diafragma de camundongos determinada em 50 µg/mL de veneno. O JeP, obtido de modo artesanal, foi tamisado em peneira para análise granulométrica e concentrações de 100 e 1000 µg/mL foram testadas para verificar seu potencial antiofídico sobre ação do veneno. Foram utilizado os modelos: pré-veneno - adição do veneno e após 15 min adição do JeP; pós-veneno - adição do JeP e após 15 min a adição do veneno; e pré-incubação – por 30 min, de veneno com o JeP ou com antiveneno comercial. O veneno apresentou proteínas de 10 a 60 kDa, o qual induziu 50% de bloqueio da atividade neuromuscular em 71,5±8,9 min (T50). Os modelos pré-veneno e pós-veneno com JeP 100 µg/mL prolongaram o tempo de atividade do nervo frênico (100,9 ± 7,6 min e 97±6,1 min, respectivamente). Os resultados obtidos para 1000 µg/mL e no modelo de pré-incubação do veneno com JeP 100 (78,2±9,2); 1000 µg/mL (86,5±8,9) e com antiveneno botrópico comercial (80,2±14,1), não interferiram na atividade do veneno sobre o nervo frênico. O veneno de *Bothrops moojeni* induz bloqueio neuromuscular e a menor concentração do polvilho causou efeito protetor sobre a junção, antes e após a administração do veneno.

Palavras-chave: *Bothrops moojeni*; Junção neuromuscular; *Jatropha elliptica*; polvilho.

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INTRODUCTION

In Brazil, among the snakes of medical interest or the *Bothrops* (Viperidae) genus, accident incidence rates are considered responsible for 90% of the changes in the Notification Recordings Information System (SINAN, 2020). The severity of the bothropic accident is marked by intense reactions triggered by various components present in the venom, such as phospholipases, metalloproteases, and serine proteases. The main changes are systemic and are observed in hemostasis and the local ones trigger pain, inflammation, and tissue destruction in the injured limb, which can lead to amputation or death (GUTIÉRREZ et al., 1989; RIBEIRO et al., 1998; BRAUD et al., 2000; PINHO et al., 2001). The treatment for snake venom poisoning has been the administration of antivenom, which improves the immune response, reduces mortality, and neutralizes the clinical symptoms triggered by the poisoning (ALMEIDA et al., 2012).

The state of Tocantins maintains the national epidemiological standard for snakebite accidents, with accidents predominating for rural workers, with the lower limbs being the most affected (FEITOSA et al., 2015; LEOBAS et al., 2016). These accidents are aggravated by the increase in time between the accident and the start of treatment, which is more frequent in rural accidents, due to the distance and difficulty of commuting to the treatment units (GUTIÉRREZ et al., 2011; FEITOSA et al., 2015; LEOBAS et al., 2016; CÂMARA et al., 2020). In the state of Tocantins, patients treated in the first hour after the accident represented 24% of the notifications (1640 bothropic accidents), a percentage lower than that observed in Brazil (27% of bothropic accidents).

Thus, the search for alternatives against the effects of snake poisoning becomes a necessity (FONSECA et al., 2004), mainly for populations that live far from hospital units. They often make use of medicinal plants as an alternative to minimize the deficiencies of primary health care, which includes ophidism (CASTRO et al., 2004; SOARES et al., 2005; COSTA et al., 2011).

Due to the relevance of the effects caused by bothropic poisoning, some species of plants are being studied to prove the popular use of antiophidian activity (ASSAFIM et al., 2011; FERRAZ et al., 2014; TRIBUIANI et al., 2014), including *Jatropha elliptica* (Pohl.) Müll. Arg. This plant is characteristic of the Brazilian cerrado (PAULA et al., 2010; CORDEIRO et al., 2015; FERREIRA-RODRIGUES et al., 2016) whose loss of biodiversity has been aggravated as a result of various activities, such as agricultural activity (CALDEIRA et al., 2020). It is used in the treatment of various diseases, inflammations and indicated for snakebites (VAN DER BERG et al., 1988; LIMA et al., 2006; SILVA et al., 2010; GOMES et al., 2016; YAZBEK et al., 2016).

So far, research on *Jatropha elliptica* extracts or secondary metabolites ratifies the medicinal potential of this plant and validated its activity on some of the symptoms of ophidian poisoning (BRUM et al., 2006; LIMA et al., 2006; PESSOA et al., 1999; 2007; FERREIRA-RODRIGUES et al., 2016). However, an ethnobotanical study also describes the use of powder (starch), obtained from the rhizomes of the plant, administered orally and in small amounts as a treatment for this type of poisoning (SANTOS et al., 2006), which still needs to be investigated.

In the Tocantins, the most incident snake of medical interest is *Bothrops moojeni* (SILVA et al., 2017), and there are no studies that evaluate the toxicity of its poison, nor about the neuromuscular junction. Therefore, to fill these gaps, the present study aimed to evaluate the effect of *Bothrops moojeni* venom on neuromuscular activity and to verify the antiophidian potential of *J. elliptica* (JeP) starch in this model.

MATERIALS AND MÉTHODS

Obtaining plant rhizomes

The collection of rhizomes of *Jatropha elliptica* (Location: S 10º 19.207’ and W 047º 47.954’) was carried out in 2019, as described by Ferreira-Rodrigues et al. (2016), in the municipality of Santa Tereza (TO). The plant was identified according to the International Code of Botanical Nomenclature and compared to the exsiccates deposited in the herbarium of the Federal University of Tocantins (UFT), Porto Nacional Campus (Record n. 10.681).

Obtaining the *Jatropha elliptica* (JeP) starch

The starch was obtained by a small-scale process. The rhizomes (1,815kg) of the plant were washed and grated in a conventional drain, adding water, then sifted and set for decanting. After decanting, the supernatant was removed and the remainder dried at room temperature and later was sifted in a Granutest (Bertel Metalúgica Ltda.) sieve for granulometric analysis, Tyler 80 (0.180 mm). The starch yield was 124.7g. The JeP did not solubilize in dimethyl sulfoxide or polyethylene glycol 400 (PEG 400), recommended for use in neuromuscular preparation (CINTRA-FRANCISCHINELLI et al., 2008), which is why dilution in Tyrode and ultrasonication have opted, for 10 minutes, before the addition to the bath.

Obtaining *Bothrops moojeni* poison

Samples from the first extraction of crude venom from *Bothrops moojeni* were obtained from six snakes. The animals were collected in rural areas of the municipalities of Araguaína (two females), Palmas (one male and two females), and Santa Rosa (one female), in the state of Tocantins, in the years 2016 and 2017. The capture of the animals received the scientific authorization nº 52416-1 of the Biodiversity Authorization and Information System (SISBIO). The procedure followed Normative Instruction 03/2014 and the biological material was registered in the National System for the Management of Genetic Heritage and Associated Traditional knowledge (SisGen nº A2C170C). The venom (Pool / TO) was collected before the first feeding in captivity, lyophilized, and stored at -20ºC. The procedures were performed at the Herpetology Laboratory-IB of the Butantan Institute-SP, certified by Dr. Sávio Stefanini.

SDS-PAGE Electrophoresis

The protein profile of the venom was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) (LAEMMLI, 1970) using a concentration of 4% in the application gel and 10% in the running gel (200v; 40mA and 20W). The venom samples (10 µg) were applied in the presence of β-mercaptoethanol and in parallel, a 10 to 250 kDa molecular mass standard, Dual Color Precision Plus (Bio-Rad) was used. The electrophoresis gel was stained with Coomassie-Bio Rad.

Venom neutralization tests

Commercial Bothropic Antivenom (CBA)

Bothropic antivenom (CBA) was donated by the Butantan Institute and the experiments were carried out following the doses recommended in the CBA description leaflet (batch: 180187, production date: 03/2018, expiration date: 06/2021), namely: each mL neutralizes 5 mg of venom of *B. jararaca* reference.

Animals used and experimental conditions

Male *Mus musculus* Swiss mice (25-30g) were acquired from Anilab (Laboratory Animals, Paulínia, SP, Brazil), kept in chambers with exhausts and appropriate ventilation (environmental micro ventilation system, Smaflex®), with controlled temperature (25 ± 3 ° C) in 12-hour light-dark cycles, controlled by a timer, where they received feed and water *ad libitum* in the bioterium of the University of Sorocaba/SP, following the COBEA Animal Welfare Rules, with a project approved by the Ethics of Animal Use of the same institution (CEUA Report 163/2019).

Phrenic nerve-diaphragm preparation (PND) of mice

The phrenic nerve-diaphragm preparation of mice was isolated after anesthetizing the animals with halothane (Cristália, Brazil) and killing them by exsanguination. After recording in control conditions for 15 minutes of preparation stabilization, pharmacological protocols and myographic recording were performed according to Farrapo et al. (2011).

A starch concentration-response curve was performed using 100 µg/mL (n = 6), 200 µg/mL (n = 5) and 1000 µg/mL (n = 10). Tyrode's nutrient solution represented the control condition. The 100 and 1000 µg/mL concentrations of JeP were subsequently analyzed with 50 µg/mL of *B. moojeni* venom (n = 9), using three models (Table 1).

Table 1: Protocols and the number of experiments.

Models	JeP 100 µg/mL	JeP 1000 µg/mL
PreV: JeP* added 15 min before the venom	n=9	n=13
postV: JeP added 15 min after the venom	n=8	n=6
Pre-Incubation (30 min, before adding to the organ bath): *Pool*/TO + JeP or *Pool*/TO + CBA*	n=7	n=7

*JeP, starch obtained from rhizomes of *Jatropha elliptica*
*CBA, Commercial Bothropic Antivenom

Análise Estatística

Statistical analysis

The results were expressed as mean ± standard error of the mean and significance was determined by the unpaired t-Student test. The level of significance was set at $p < 0.05$, using the Origin 8.0 software

(OriginLab Corporation, Northampton, MA, USA).

RESULTS AND DISCUSSION

The protein profile of the venom from *Bothrops moojeni* snakes (in SDS PAGE 10%), is shown in Figure 1. The proteins expressed in greatest intensity, in the gel, were those with a molecular mass around 10, 20, and 45 kDa. The importance of studying the composition of the venom is highlighted, since the constituents influence differently the effects of poisoning and, consequently, the necessary treatment (CASEWELL et al., 2014).

The molecular standard values allowed us to infer that the ~14 kDa protein belongs to the phospholipase class (CALGAROTTO et al., 2008) and the one with ~20 kDa to the metalloproteinases (MESH, 2020). It is also possible to observe the presence of proteins between 20 and 50 kDa, almost undetectable in the gel, which shows little expressiveness of them in this material, such as serine proteases (~32 kDa) (BHAT et al., 2016; OLIVEIRA et al., 2016). Proteins around 50 kDa, on the other hand, belong to the hemorrhagic factor class, with intense proteolytic activity (CRISTINA et al., 2020), and have not yet been described for the *B. moojeni* venom. Pool/TO did not present proteins with molecular weight above 50 kDa.

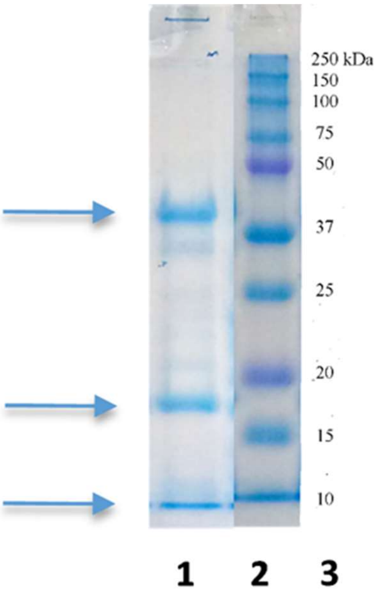

Figure 1: SDS-Page Electrophoresis. (1) Pool/TO (10µg). (2) Dual Color Precision Plus (Bio-Rad) standards. (3) molecular markers (kDa). The arrows indicate differential bands expressed in Pool/TO. The proteins were stained with Coomassie-Bio Rad according to the manufacturer's instructions. *Pool*/TO, *pool* of venom collected from 6 *Bothrops moojeni* snakes.

There are no studies with the *B. moojeni* venom using the experimental model on the neuromuscular junction, that is, the neuromuscular preparation of mammals. The chosen venom concentration of 50 µg/mL was based on studies with other bothropic venoms, performed on the same model (GALBIATTI, 2008; PUEBLA et al., 2010; CARDOSO, 2011; FERRAZ et al., 2014; TRIBUIANI et al., 2014; FERREIRA-RODRIGUES et al., 2016).

Therefore, at this concentration, the *B. moojeni* venom caused 50% of neuromuscular block in a time of T50 = 71.5 ± 8.9 min (n = 9). Figure 2 illustrates a myographic recording representing the number of experiments carried out with *B. moojeni* venom exposed to neuromuscular preparation for 120 minutes.

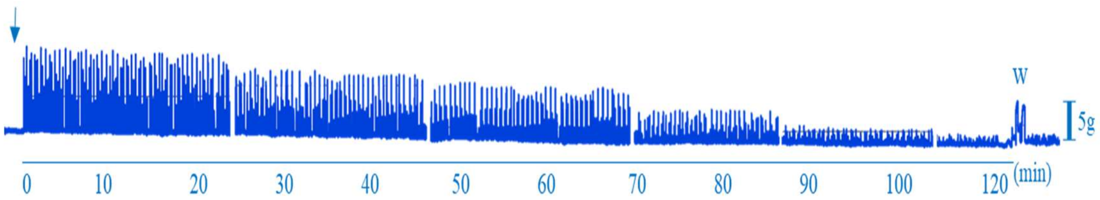

Figure 2: Myographic recording representative of the neuromuscular block induced by Pool/TO (50 µg/mL). The arrow represents the time of addition of the poison and the T50 of 71.5 ± 8.9 min. W, washing of the preparation. *Pool*/TO, a *pool* of venoms collected from *B. moojeni* snakes from the state of Tocantins

Snake venoms are sometimes more active, sometimes less active, with variation in the blocking time at the neuromuscular junction (OLIVEIRA et al., 2019), a parameter that also depends on the species, such as, for example, the *B. jararacussu* venom, which at a concentration of 40 µg/mL led to a 50% block, in a time of 56.2 ± 8.3 min (T50; n = 4) (FERREIRA-RODRIGUES, 2016). On the other hand, it is known that differences in the proportion and composition of venoms influence their toxicity, as revealed by proteomic studies (CHIPPAUX et al., 1991; BRAUD et al., 2000; CASTRO et al., 2004; BRAHMA et al., 2015; YIN et al., 2016; AMORIM et al., 2017).

It is important to note that *Pool*/TO expressed a small number of serine proteases (Figure 1), which may explain the greater T50% block in this *pool*. Another possibility is related to the intense local myotoxic effect caused by bothropic venom (NISHIJIMA et al., 2015), also observed in neuromuscular preparation (FERREIRA-RODRIGUES et al., 2016). Possivelmente a ação local mascare, reduza ou mesmo limite a ação neurotóxica do veneno botrópico.

The venoms have toxins that cause neuromuscular paralysis in vitro, and this has been explained by the presence of components that can act both presynaptically, blocking the release of acetylcholine (ACh), as well as postsynaptically, blocking its receptors, or even destroying the skeletal muscle architecture, causing myonecrosis (RODRIGUES et al., 2004). Therefore, our study demonstrated that *B. moojeni* venom also causes an important block in isolated mouse preparations, similar to the venom of other snake species (GALBIATTI, 2008; PUEBLA et al., 2010; CARDOSO, 2011; FERRAZ et al., 2014; TRIBUIANI et al., 2014; FERREIRA-RODRIGUES et al., 2016). However, further studies are needed to elucidate the mechanisms involved. Popularly, some species of plants are used to minimize or neutralize the effects of poisoning by snakes due to the severity of accidents, and also due to the time of access to treatment, especially in rural areas, which are far from healthcare (MOURA et al., 2015).

Included in this context is the *Jatropha elliptica* plant, with occurrences in the Brazilian cerrado, known as “purga-de-lagarto”, “erva-de-teiú”, “batata-de-tiú”, among others. It is found in the states of Alagoas, Pernambuco, Mato Grosso, Goiás, Bahia and Tocantins and can be observed during the transition from the dry to the rainy season, having a seasonal characteristic (GOULART et al., 1993; SILVA et al., 1998; CORDEIRO et al., 2015).

Concerning medicinal use, the ethnobotanical and pharmacological information reveals that in the regions where it occurs, its root is widely used in popular medicine as a bactericide, depurative, against venereal diseases, itches and for ophidian treatment (VAN DEN BERG et al., 1988; LIMA et al., 2006; SANTOS et al., 2006; YAZBEK et al., 2016).

To minimize the effects of snake poisoning, the traditional use of *J. elliptica* includes the root powder (starch) for the treatment (SANTOS et al., 2006), in the *cerrado* region of Tocantins, and is done by oral administration, in small amounts. This important information guided the present study and used the experimental model of the neuromuscular junction, by the myographic technique, as a tool for the scientific validation of ethnobotanical extracts of various plants against venoms and toxins (OSHIMA-FRANCO and DAL BELO, 2017). For this, hand-made starch was obtained, and since there were no previous studies on the starch response, a concentration-response curve was performed with 100, 200, and 1000 µg/mL of starch, in this experimental model (Figure 3). Figure 3 illustrates JeP concentrations 100 µg/mL (n = 6); 200 µg / ml (n = 5); and 1000 µg / mL (n = 10), which were added to the vat containing the neuromuscular preparation.

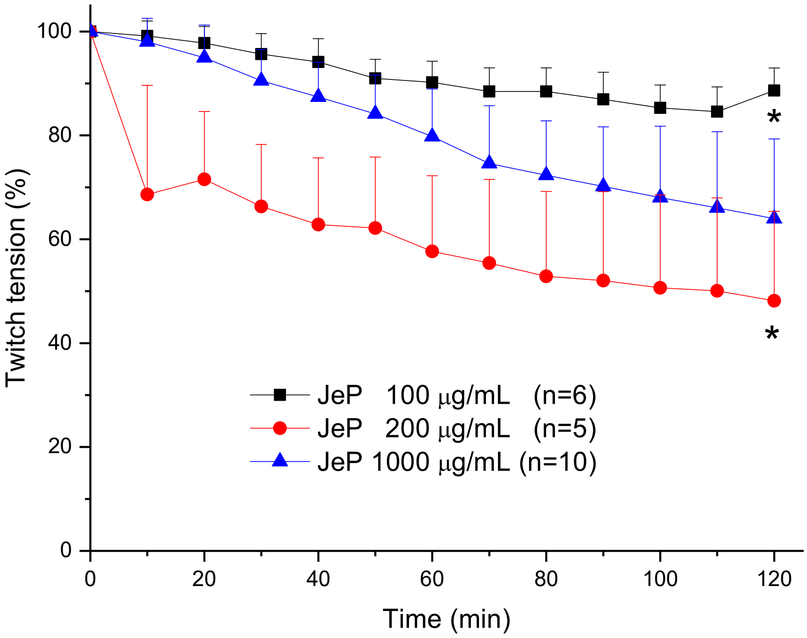

Figure 3: Phrenic nerve-diaphragm preparation of mice, indirect stimulus. Concentration-response curve of the starch (JeP 100, 200 and 1000 µg/mL). The number of experiments is shown in the labels. Each point represents M ± EPM. * p <0.05 compared to the concentration that caused the lowest baseline change.

From these results, two concentrations were selected for the subsequent neutralization tests, 100 µg/mL, because it caused the lowest baseline change in the contractile response, and 1000 µg/mL (p <0.05, compared to lowest concentration), as it is 10 times more concentrated. Thus, the amount of JeP to counteract the action of the venom can be tested.

Interestingly, the concentration of 200 µg/mL of JeP reduced neuromuscular activity more sharply than the concentration of 1000 µg/mL, which may be related to the technical procedure, as in this set of experiments the powder was not sonicated, which could have influenced the results.

The results obtained with the JeP starch against the neuromuscular block induced by Pool/TO are shown in Figure 4 concerning the time to block 50% of the contractile response. The pre-venom and post-venom treatments with JeP 100 µg/mL were statistically significant, increasing the activity time to 100.9 ±

7.6 (n = 9) and 97 ± 6.1 (n = 8), respectively. It was also observed that the increase in JeP concentration to 1000 µg/mL (10 times) was insignificant to improve phrenic nerve activity time, not differing significantly from the blocking time triggered by the venom (Pool/TO µg/mL) T50 on all experimental models. The JeP pre-incubation model showed an effect similar to that produced by the commercial antivenom (p> 0.05), with both concentrations of the starch, however, without a statistically significant difference when compared to the venom. Differences in the protective ability of antivenoms against certain effects of poisons have been reported in the literature (SEGURA et al., 2016; ESTEVÃO et al., 2016; GUTIÉRREZ, 2017).

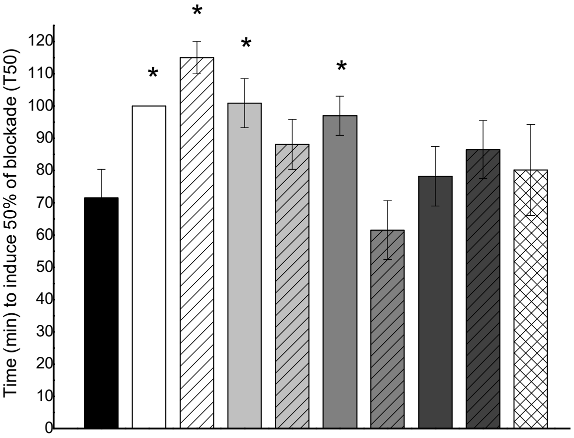

Figure 4: Phrenic nerve-diaphragm preparation of mice, indirect stimulus. Time to block 50% (T50) of each experimental model against 100 or 1000 µg/mL of JeP, as following: ■ *B. moojeni* 50 µg/mL (n=9); □ JeP 100 µg/mL (n=6); ▨ JeP 1000 µg/mL (n=10); ■ JeP 100 µg/mL 15 min, *B. moojeni* 50 µg/mL (n=9); ▨ JeP 1000 µg/mL 15 min, *B. moojeni* 50 µg/mL (n=13); ■ *B. moojeni* 50 µg/mL 15 min, JeP 100 µg/mL (n=8); ▨ *B. moojeni* 50 µg/mL 15 min, JeP 1000 µg/mL (n=6); ■ Pre-incubations *B. moojeni* 50 µg/mL + JeP 100 µg/mL (n=7); ▨ Pre-incubations *B. moojeni* 50 µg/mL + JeP 1000 µg/mL (n=7); ▩ Pre-incubations *B. moojeni* 50 µg/mL + antivenom (n=7). Note that the statistically significant differences (* p <0.05) were demonstrated. Each column represents M ± EPM. *B. moojeni* represents Pool/TO, pool of venoms collected from *B. moojeni* snakes from the state of Tocantins, JeP, starch obtained from rhizomes of *J. elliptica*.

Several species of plants have been studied from the perspective that, if some anti-ophidian activity is proven, they become possible adjuvants to the commercial antivenom, an effective treatment officially recommended for ophidian poisoning (WHO, 2018; GOMES et al., 2016). As examples, there are studies with the plant *Hypericum laxiusculum* (Hypericaceae) against the local effects of *Bothrops jararaca* venom (ASSAFIM et al., 2011) and *Jatropha molissima* against local effects induced by *Bothrops erythromelas* and *Bothrops jararaca* venom (GOMES et al., 2016).

Therefore, the validation of traditional knowledge is increasingly important in the scientific community, using protocols that contribute to the indication of medicine that can be used as an adjunct to conventional serum therapy, not only for the *cerrado* region, where the studied species occurred. Thus, the present study represents an advance in the determination of the antiophidian property of *J. elliptica* on the action of the venom of *B. moojeni*, concerning the use of the starch, confirming popular information.

However, other ways of extracting the components of the rhizome of this plant were used to investigate the action of snake venoms. The aqueous extract was effective against the action of the *Lachesis muta* venom about hemolysis, hemorrhage, coagulation, and proteolytic activity (PAULA et al., 2010).

The crude ethanolic extract also showed significant anti-inflammatory activity on the classic models of paw edema and neutrophil migration induced by *Bothrops jararacussu* venom. At the neuromuscular junction, the same extract prevented the evolution of paralysis, and histological analysis confirmed the protective effect by reducing the myotoxic index (IM) ($p < 0.05$) by up to three times, that is, it reduced the damage caused by the venom, namely, myonecrosis, edema, rupture of the membrane and presence of phantom cells. This protective effect was attributed to phenolic compounds, alkaloids (FERREIRA-RODRIGUES et al., 2016). Milad et al. (2014) affirm the need to establish a correlation between the presence of secondary metabolites, their effect, and popular usage. This highlights the relevance of further studies on the biological activity of these plants.

Obtaining the starch by hand persuades us to think that, during processing by decantation, portions of the secondary metabolites remain impregnated, causing their protective effect, and/or that the carbohydrate provides energy for the cell to respond more effectively against the actions of the poison. The studies by Bento et al. (2020) confirmed the presence of phenolic compounds in "batata-de-teiú" flour (12.67 mg eq gallic acid 100 g^{-1}), with antioxidant potential and nutraceutical resources. These results contribute to our hypothesis; however, new studies need to be carried out to elucidate the protective mechanism of the starch.

According to Moura et al. (2015), the search for a plant antidote against snake poisoning has been long. The rationale for indicating a plant with antivenom potential is complex (VILAR et al., 2005; MOURA et al., 2015), because there is no bioactive compound capable of interacting with the venom antigen as the antibody does. However, any plant that attenuates or blocks the progression of a poisoning event should be valued, for further prospection studies, mainly those from traditional knowledge, due to the known difficulties in accessing serum therapy, or even where its availability is non-existent (SOARES et al., 2005).

CONCLUSIONS

Our study showed that the venom of *B. moojeni*, from specimens collected in the *cerrado* of Tocantins, caused an important neuromuscular block in isolated preparations of phrenic nerve-diaphragm from mice. It also proved experimentally, for the first time, the protective effect of *Jatropha elliptica* (JeP) starch, when used in a lower dose, immediately before or after the addition of *Bothrops moojeni* venom.

The results presented here ratify the neuromuscular junction as an important tool for the study of bioactive substances, also taking into account the high incidence of bothropic accidents in the Tocantins, as well as the high diversity of species of antiophidian plants used by populations living in the *cerrado*, that still lack scientific validation.

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