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Bothrops jararacussu venom-induced neuromuscular blockade inhibited by *Casearia gossypiosperma* Briquet hydroalcoholic extract

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Abstract: The hydroalcoholic extract of *Casearia gossypiosperma* Briquet (Flacourtiaceae) was standardized for the first time through quality control procedures including pharmacognostic methods, fingerprint chromatograms, defined amounts of marker substances and physicochemical characteristics. The pharmacological activity of *C. gossypiosperma* (Cg) hydroalcoholic extract was assayed by a traditional *in vitro* test, which involved irreversible neuromuscular blockade induced by *Bothrops jararacussu* (Bjssu) venom (60 µg/mL) in mouse phrenic nerve-diaphragm preparations. Bjssu venom blocked muscle activity for 26 (± 2.0) minutes (n = 6). Cg extract (0.1 mg/mL) induced changes on the baseline muscle activity without impairing the muscle function and inhibited 87.6% (± 1.8) (n = 6) of the Bjssu venom-induced blockade. Both flavonoids (0.624 g%) and polyphenols (4.63 g%) from the extract were spectrophotometrically quantified. Therefore, the present study confirms the antibothropic activity of Cg extract, supporting the ethnomedical use of *Casearia* sp. in the treatment of snakebite victims.

Key words: antibothropic extract, *Bothrops jararacussu*, *Casearia gossypiosperma* Briquet, snake venoms.

INTRODUCTION

The genus *Casearia* is one of the 86 members of the family Flacourtiaceae. It includes around 180 species that occur throughout the tropics and are widespread in the Americas, Africa, Asia, Malaysia, Australia and islands of the Pacific Ocean (1). At least 16 *Casearia* species have shown biological activity such as cytotoxic [*C. arborea; C. grayi; C. grewiifolia; C. lucida; C. multinervosa; C. nigrescens; C. obliqua* (2-8)]; antifungal [*C. decandra* (9)]; antidiabetic [*C. esculenta* (10)]; immunomodulatory [*C. guianensis* (11)]; antifertility [*C. ilicifolia* (12)]; antiophidian [*C. mariquitensis* (13)]; antitumor [*C. membranacea* (14)]; genotoxic [*C. tomentosa* (15)]; and antioxidant actions [*C. velutina* (16)]. The most studied among them is *Casearia sylvestris* Sw. that has shown several biological activities, including antitumor, antiulcer, analgesic, anti-inflammatory, cytotoxic, antifungal, anticancer, anti-snake and bee venom PLA_2, genotoxic, trypanocidal, enzyme inhibitory, antihyperlipidemic and antibothropic properties (17-27). The latter have been used as antivenom in folk medicine by isolated Brazilian communities (22).

Geographical isolation helps reinforce the need of traditional and local medical practices, selecting natural resources for the treatment of many diseases, including snakebites (28). In addition, recognizing the potential of a medicinal plant could also contribute to preserve it.

In this study, *Casearia gossypiosperma* Briquet, a native tree from Brazil and popularly known as "pau-de-espeto", was pharmacognostically and

pharmacologically assessed for the first time. *In vivo* myotoxic effects and *in vitro* irreversible neuromuscular blockade effects of crude venom from the snake *Bothrops jararacussu* are well known pharmacological methods used to study drugs showing antivenom properties (29, 30). Thus, the aim of the present study was to verify the capability of *Casearia gossypiosperma* hydroalcoholic extract to neutralize the neuromuscular blockade induced by *Bothrops jararacussu* venom.

MATERIALS AND METHODS

Plant Material and Extraction

Leaves from an adult *C. gossypiosperma* Briquet (Cg) tree were collected from the herbarium at the Higher School of Agriculture Luiz de Queiroz (ESALQ) of the University of São Paulo, Piracicaba, São Paulo, Brazil, in January 2006; the species was previously identified in 1988 (protocol IAC 38115). Cg leaves were dried at 35°C to 40°C for 24 hours. The dried leaves were then powdered, ground in a mill, macerated (200 g, during five days) in 2 L of 70% ethanol and the suspension was percolated (under protection against light) at 20 drops/minute, resulting in a 10% (m/v) hydroalcoholic extract.

Quality Control Assays of the Medicinal Plants

Ash and humidity tests

To observe elementary physical and chemical characteristics, Cg powder was subjected to ash and humidity tests (31). Briefly, 100 g of the specimen powder were placed in six calibrated melting pots, which were warmed until total carbonization of the powders. The melting pots were kept at 650°C and the ashes were then weighed. Results are expressed as grams of ashes/100 g of sample. The humidity test was performed by placing 1 g of specimen powder in six calibrated porcelain capsules, which were heated at 105°C during four hours and then weighed.

Thin Layer Chromatography (TLC)

Aliquots of the hydroalcoholic extract were spotted on thin-layer silica gel plates (0.3-mm thick, Merck, Germany) with appropriate standards (32). The solvent system consisted of solvent A (ethyl acetate:formic acid:water, 90:10:10, v/v) and solvent B (acetone:chloroform:formic acid, 10:75:8, v/v). The phytochemical groups used as standards (1% methanol m/v, PA solution, Sigma Chemical Co., USA) were apigenin, quercetin and rutin. Methanol extract from *Casearia sylvestris* Sw. was compared to Cg hydroalcoholic extract (27). The separated spots were visualized (under UV light at 360 nm) with NP/PEG, as follows: 5% (v/v) ethanolic NP (diphenylboric acid 2-aminoethyl ester, Sigma Chemical Co., USA) followed by 5% (v/v) ethanolic PEG 4000 (polyethylene glycol 4000, Synth Chemical Co., Brazil). The retention factor (Rf) of each standard was compared with the spots exhibited by the tested extracts.

Determination of Flavonoids and Polyphenols

Flavonoid content

The flavonoid content was determined in Cg hydroalcoholic extract as described elsewhere (33). The method is based on the UV absorption of $AlCl_3$-flavonoid complexes, expressed as total quercetin content. Briefly, 80% methanol (50 mL) was added to 10 mL extract, and 5 mL solution were transferred to volumetric flasks and diluted again with 80% methanol (50 mL). Four aliquots (2 mL) of solution were mixed with 2 mL of 5% anhydrous aluminum chloride solution ($AlCl_3$; complexing agent) and adjusted to 10 mL with 80% methanol. After 15 minutes, the absorbance of samples was read at 420 nm, considering a blank sample containing 80% methanol (8 mL) and 5% $AlCl_3$ (2 mL). The percentage of flavonoids (%) was calculated from a standard curve of quercetin (0, 4, 8, 12, and 16 µg/mL) prepared with methanol.

Polyphenol content

The polyphenol content in Cg hydroalcoholic extract was determined as previously described (34). Briefly, 5 mL extract was poured into a volumetric flask and distilled water was added to 250 mL; then, a 1 mL aliquot was transferred to another volumetric flask and distilled water added to 25 mL (final solution). Aliquots (1 mL) of the final solution received 1 mL of phosphomolybdotungstic reagent and the final volume (10 mL) was adjusted with 15% sodium carbonate solution. After 30 minutes, the absorbance of samples was read at 720 nm, considering a blank sample containing 15%

sodium carbonate solution. The percentage of polyphenols (%) was determined from a standard curve (5, 10, 15, 20, 25, 30, 35, and 40 µg/mL) of pyrogallol (Sigma Chemical Co., USA).

Pharmacological Tests

Animals

Male Swiss white mice (26-32 g) were supplied by Anilab Animais de Laboratório (Brazil). Animals were housed at 25 ± 3°C under a 12-hour light-dark cycle with access to food and water *ad libitum*. This study was approved (protocol number A009/2006 CEP) by the Research Ethics Committee of the Vale do Paraiba University (UNIVAP) and all experiments were performed according to the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL/CEUA).

Bothrops venom

Crude venom was obtained from adult *Bothrops jararacussu* (Bjssu) snakes (Serpentarium of the Nature Study Center, UNIVAP) and certified by Prof. Dr. Jose Carlos Cogo, Vale do Paraiba University (UNIVAP), São José dos Campos, SP, Brazil.

Mouse phrenic nerve-diaphragm muscle (PND) preparation

PND was obtained from mice anesthetized with halothane and sacrificed by exsanguinations (35). The diaphragm was removed and mounted under a tension of 5 g in a 5 mL organ bath containing continuously aerated Tyrode solution (control) with the following composition: 137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, and 11.1 mM glucose. After stabilization with 95% O_2/5% CO_2, pH was 7.0. The preparations were indirectly stimulated with supramaximal stimuli (4x threshold, 0.06 Hz, 0.2 ms) delivered from a stimulator (model ESF-15®, Bonter, Brazil) to the nerve through bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (cat. 7003, Ugo Basile, Italy) coupled to a Gemini two-channel physiographic recorder (cat. 7070, Ugo Basile) via a basic preamplifier (cat. 7080, Ugo Basile, Italy).

Organ preparations (n = 6) were allowed to stabilize for at least 20 minutes before the addition of the following: Tyrode solution (control); 0.05, 0.1, 1 and 2 mg/mL Cg (i.e. 0.25, 0.5, 5 and 10 mg Cg, respectively); 60 µg/mL Bjssu venom (i.e., 300 mg or 0.3 mg); and 60 µg/mL Bjssu venom + 0.05, 0.1 and 1 mg/mL Cg. Two other experiments were carried out: organ preparations preincubated with 0.1 mg/mL Cg extract during 30 minutes, followed by addition of 60 µg/mL crude Bjssu venom; and organ preparations subjected to 60 µg/mL crude Bjssu venom during 15 minutes, followed by addition of 1 mg/mL Cg extract.

Statistical analysis

Each pharmacological protocol was repeated at least five times. Results were expressed as the mean ± standard deviation (SD). ANOVA and Tukey's (post hoc) tests were used for data comparison. The significance level was set at 5%.

RESULTS AND DISCUSSION

The chemical composition of plant extracts is very complex and variable as it depends on the climate and/or the soil composition (36). Thus, physicochemical control is indispensable for phytotherapics. Drug discovery techniques applied to the standardization of herbal medicines have revealed numerous analytical marker compounds. In the present study, quality control tests such as pharmacognostic methods, characteristic fingerprint chromatograms, defined amounts of marker substances and physicochemical characteristics were used for the extract standardization (37).

According to Brazilian Pharmacopeia (31), the usual values for extract ashes are up to 2% and for humidity, 14%. The results (mean ± SD, in grams/100 g) obtained in the ash and humidity tests for Cg were 3.67 ± 0.48 and 11.47 ± 1.02, respectively. The high ash content found in Cg powder indicates the presence of inorganic compounds due to the soil composition, although no correlation between elemental composition of medicinal plants and their curative properties have been established (38). The humidity level found for Cg powder confirms that no enzymatic reaction occurred during sample collection.

Preliminary phytochemical analysis of the plant crude extract, using thin layer chromatography (Figure 1), showed the presence of flavonoids (orange coloration) and other phenolic constituents (blue coloration), revealing a similar profile with *Casearia sylvestris* Sw., a well known *Casearia* species. The Rf values obtained

using solvent system A were 0.105, 0.158, 0.211, 0.303, 0.338, 0.434, 0.487, 0.526, 0.632, 0.737, 0.829 and 0.882 for *C. sylvestris* (Cs); 0.110, 0.178, 0.411, 0.480, 0.548, 0.616, 0.658, 0.712, 0.781 and 0.959 for *C. gossypiosperma* (Cg); and 1.0, 0.987 and 0.321 for the phytochemical standards apigenin, quercetin and rutin, respectively. The presence of substances with the same Rf for both *C. gossypiosperma* and *C. sylvestris* suggests common metabolites.

The Rf values obtained using solvent system B were 0.307, 0.387, 0.493, 0.547 and 0.880 for Cs; 0.181, 0.403 and 0.458 for Cg; and 0.663 and 0.400 for apigenin and quercetin, respectively, whereas rutin was not displaced by the solvent. In this solvent system, Cg spots are suggestive of rutin, quercetin and another flavonoid not identified (orange coloration).

Orange and blue compounds in the plants, visualized by TLC, are suggestive of flavonoids and polyphenol compounds and here were quantitatively confirmed as 0.624 g% (quercetin calibration curve Y = 0.0632X + 0.0035, r = 0.998) and 4.63 g% (pyrogallol calibration curve Y = 0.1693X – 0.0004, r = 0.999) for Cg (32). The latter has very similar flavonoid content but twice more polyphenol concentration compared to *C. sylvestris* (0.640 g% and 2.22 g% for flavonoids and polyphenols, respectively); these parameters were measured by the methodology described elsewhere (39). This variation in plant compounds can change medicinal properties (40). Parameters such as ash production, humidity, flavonoids and phenols are important for phytochemical characterization and maintenance of a physicochemical standard of plant extracts. In the present study, the plant assays showed reproducibility, which could indicate that the extracts had good standardization.

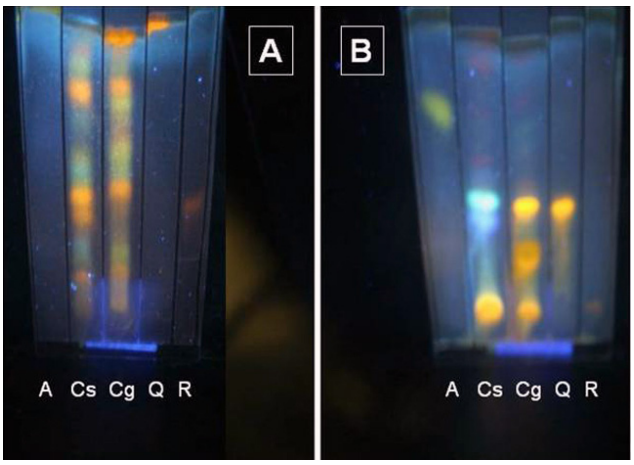

Figure 1. Comparative thin layer chromatography between *Casearia sylvestris* (Cs) and *Casearia gossypiosperma* (Cg). Chromatographical profile of extracts and phytochemical standards under solvents A (ether acetate:formic acid:water, 9:1:1, v/v) and B (acetone:chloroform:formic acid, 10:75:8, v/v). Note the similarity of compounds between (**A**) *Casearia* spp. and the presence of rutin and quercetin and another unidentified orange compound in (**B**) *C. gossypiosperma*. A: apigenin; Q: quercetin; R: rutin.

The major constituents (flavonoids and polyphenols) of Cg hydroalcoholic extracts were identified by TLC and spectrophotometrically quantified, and their capability to inhibit neuromuscular activities of bothropic venom was demonstrated *in vitro*. Crude Bjssu venom induces neurotoxicity *in vitro* and causes muscle necrosis *in vivo*, resulting in irreversible tissue loss and limb amputation in severe accidents (29, 30). Muscle necrosis is usually attributed to bothropstoxin-I, which is a myotoxic Lys49 PLA_2 homologue and the main toxic component of Bjssu venom (41). The myotoxic components of snake venoms can reduce the initiation of muscle contractures, muscle response after direct stimulation or elevated potassium exposure (42). In this work, the addition of Bjssu venom (60 µg/mL or 0.3 mg, n = 6) alone to the bath produced total muscle paralysis starting at 26 ± 2 minutes until the end of the experiment.

Figure 2 shows the bioassays on the muscle activity of mouse phrenic nerve-diaphragm preparations using 0.05, 0.1, 1 and 2 mg/mL (or 0.25, 0.5, 5 and 10 mg, respectively) Cg hydroalcoholic extract over 120 minutes. Except 0.05 mg/mL, all other Cg extract concentrations significantly decreased the muscle activity. However, in all assays, a total recovery of the muscle activity was verified after total removal of extracts by washing the preparations with fresh Tyrode solution. The concentrations of 0.05, 0.1 and 1 mg/mL Cg extract were assayed with crude Bjssu venom.

Figure 3 shows the neutralization of Bjssu venom by Cg extract mixed with venom in the bath kept at 37°C for 120 min. Curiously, after decreasing muscle activity, 0.1 mg/mL Cg extract significantly ($p < 0.05$) reduced muscle paralysis by 87.6±1.8% (n = 6). The protective action

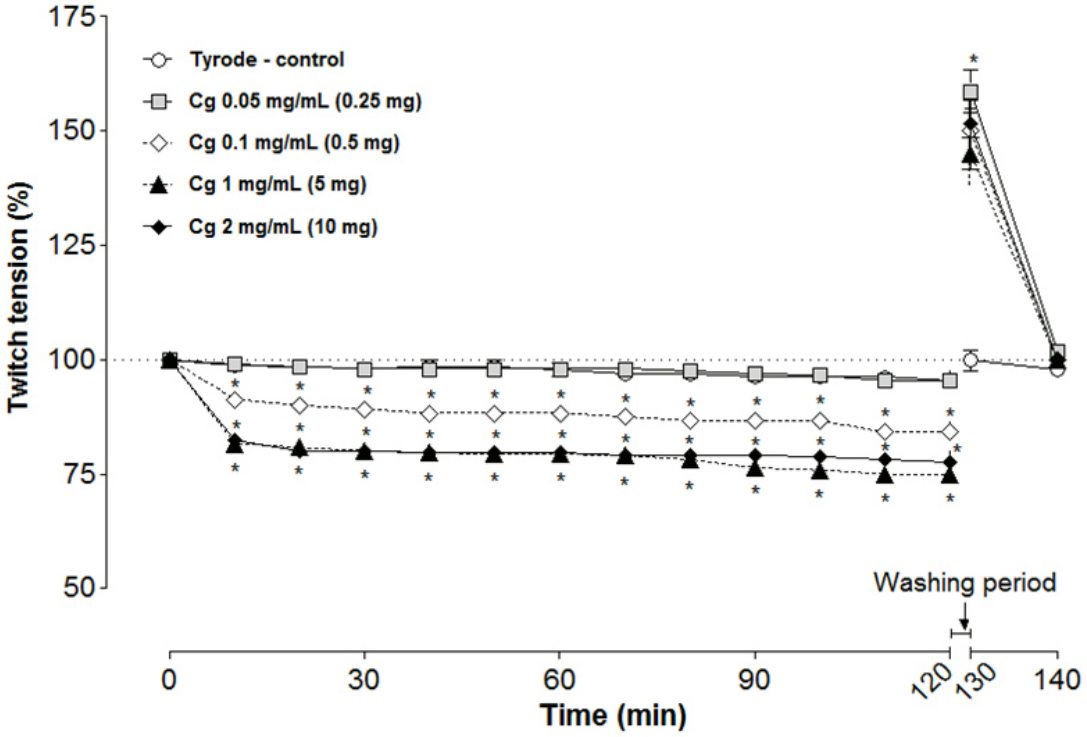

Figure 2. Twitch tension of mouse isolated phrenic nerve-diaphragm preparations under indirect stimuli, in percentage (each point represents the mean ± SEM, n = 6). Action of Cg hydroalcoholic extracts at concentrations of 0.05, 0.1, 1 and 2 mg/mL (or 0.25, 0.5, 5 and 10 mg, respectively). All extract concentration, except the first one, significantly differed from that of Tyrode solution (control) during 120 minutes (* $p < 0.05$).

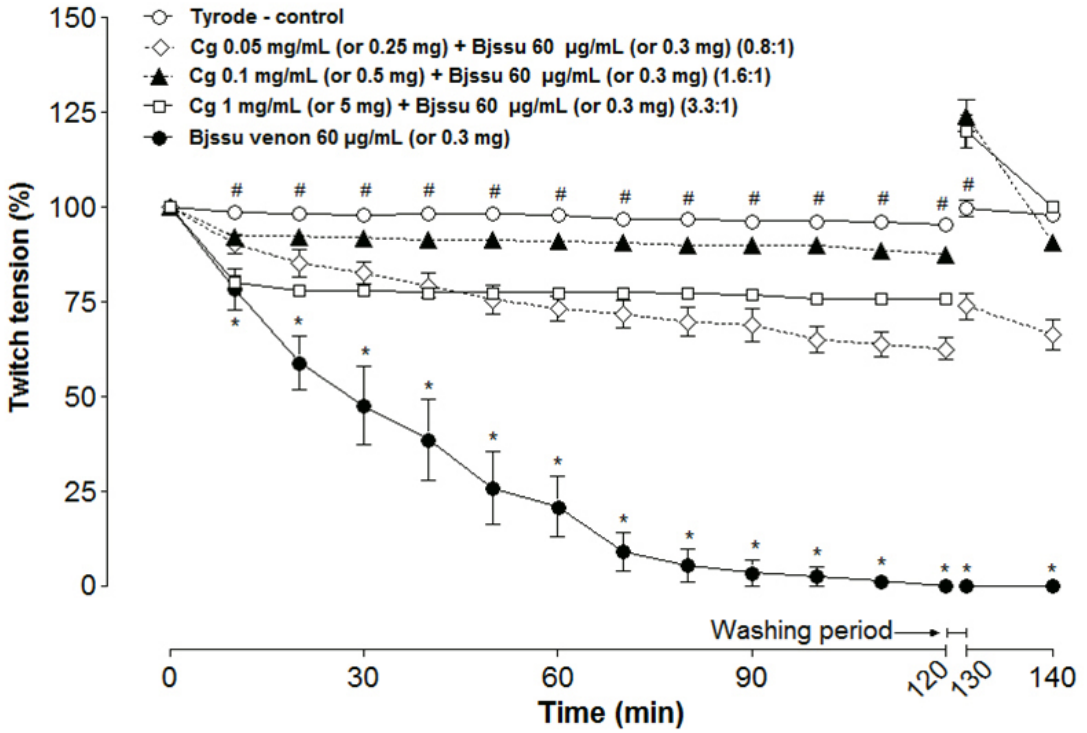

Figure 3. Twitch tension of mouse isolated phrenic nerve-diaphragm preparations under indirect stimuli, in percentage (each point represents the mean ± SEM, n = 6). Neuromuscular blockade by Bjssu venom (60 µg/mL or 300 mg or 0.3 mg) and assays of neutralization by Cg hydroalcoholic extracts (0.05, 0.1 and 1 mg/mL or 0.25, 0.5 and 5 mg, respectively), compared to Tyrode solution (control) during 120 minutes. * $p < 0.05$ compared to Tyrode control for all periods (except 0). # $p < 0.05$ compared to Tyrode control and Bjssu venom.

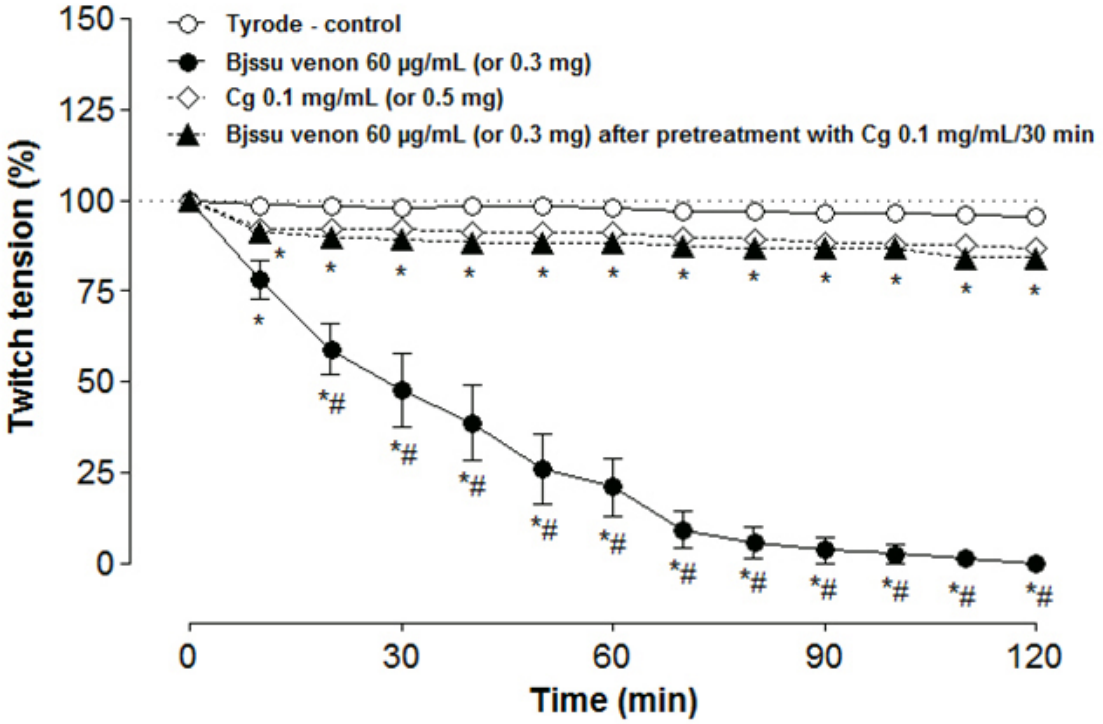

Figure 4. Twitch tension of mouse isolated phrenic nerve-diaphragm preparations under indirect stimuli, in percentage (each point represents the mean ± SEM, n = 6). Pretreatment of preparations with 0.1 mg/mL (or 0.5 mg) Cg hydroalcoholic extract for 30 minutes, followed by 60 µg/mL (or 300 mg or 0.3 mg) Bjssu venom addition during 120 minutes. * $p < 0.05$ compared to Tyrode control for all periods (except 0). # $p < 0.05$ compared to Cg extract alone or to pretreatment with Cg extract.

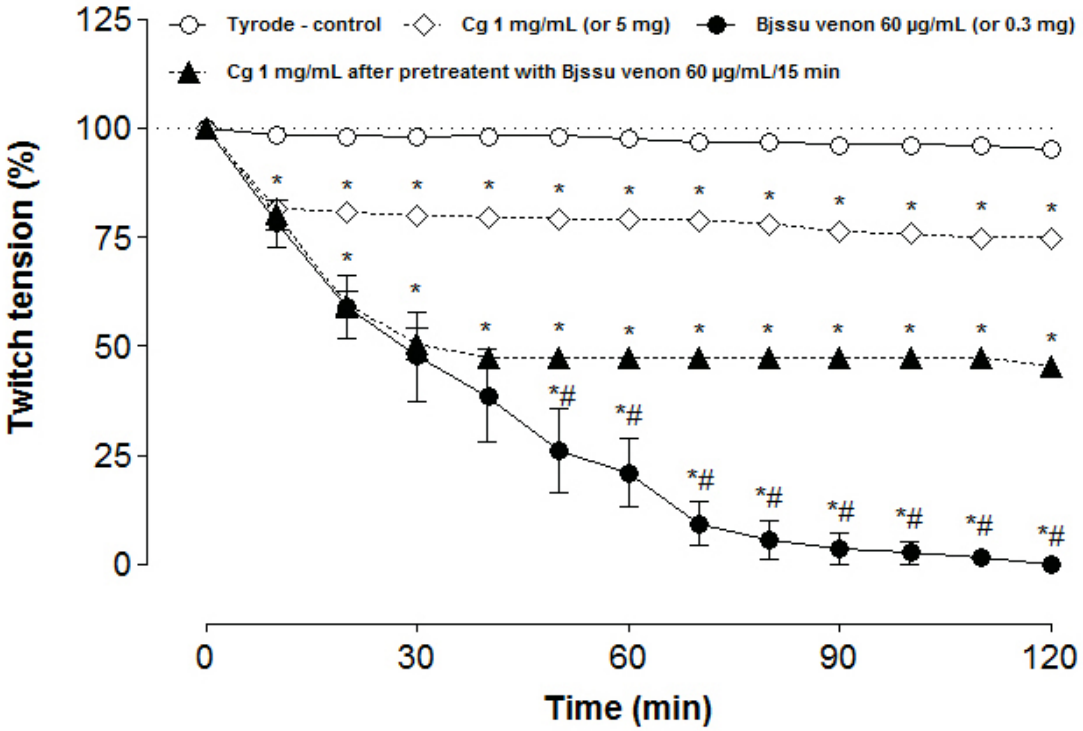

Figure 5. Twitch tension of mouse isolated phrenic nerve-diaphragm preparations under indirect stimuli, in percentage (each point represents the mean ± SEM, n = 6). Post-treatment of preparations with 1 mg/mL (or 5 mg) Cg hydroalcoholic extract after 15 minutes, followed by 60 µg/mL (or 300 mg or 0.3 mg) Bjssu venom addition. * $p < 0.05$ compared to Tyrode control for all periods (except 0). # $p < 0.05$ compared to treatment with Bjssu during 15 minutes, followed by Cg extract addition.

against crude venom was confirmed by a total recovery after washing (W).

Using the same experimental model, two organ preparations were observed, pretreatment: preincubation with Cg extract followed by addition of crude Bjssu venom (Figure 4, n = 6); and post-treatment: addition of Cg extract after 15 minutes of Bjssu venom exposure (Figure 5, n = 6). Preincubation with Cg extract protected the muscle against the blockade induced by Bjssu venom, similarly to the results shown in Figure 3. Post-treatment with 0.1 mg/mL Cg extract did not change the blockade profile (not shown), but 1 mg/mL Cg extract partially and significantly ($p < 0.05$) protected against the total blockade induced by Bjssu venom. This result could indicate that 1 mg/mL Cg extract could prevent the venom activity propagation. Since only higher Cg concentrations showed pharmacological effect, the bioactive compounds could be concentrated in the extract, which is interesting for a future *C. gossypiosperma* bio-prospection.

The inhibition of neurotoxic and myotoxic activities of snake venoms in similar experimental models was also previously observed for crude extracts of *Tabernaemontana catharinensis*, *Casearia sylvestris*, *Pentaclethra macroloba*, *Baccharis trimera* and *Mandevilla velutina* (27, 39, 43-47).

Our experience with *C. sylvestris* (27, 39) using the same experimental model and all attempts to identify the bioactive compounds (mainly rutin) showed that this plant protected the muscle against the toxic effects of Bjssu venom due to phytocomplex formation. Different molecules in the plant constitution inhibit Bjssu venom activity in different ways, for example, coumarin from *Mikania laevigata* and tannin from *Plathymenia reticulata*. In commercial phytochemical preparations, these molecules (coumarin and tannic acid) confirmed the capability to revert Bjssu venom-induced muscle blockade by pharmacological (*M. laevigata*) or protein precipitation (*P. reticulata*) (48). We also observed that the capability to protect against the neuromuscular blockade induced by Bjssu venom does mean protection against the paralysis caused by *Crotalus durissus terrificus* venom (data not published), which indicates that the mechanism of action of the venoms must be considered and the plant and venom mixing is not enough to neutralize the toxic effects.

In the present study, the neuromuscular blockade induced by Bjssu venom was inhibited by Cg hydroalcoholic extract, suggesting the presence of compounds with the ability to reduce neurotoxicity and effectively inhibit the myotoxic action of the venom. Based on the similarity of the constituents found in *C. gossypiosperma* to those seen in *C. sylvestris*, we suggest the same mechanism of action for both, i.e., phytocomplex formation between venom and antioxidants (flavonoids and polyphenols). This is the first attempt to show the antivenom activity of *C. gossypiosperma* and prove its ethnobotanical use. Further studies are needed to identify the bioactive compound(s) responsible for the antineurotoxic and antimyotoxic effects of Cg.

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CONFLICTS OF INTEREST

There is no conflict.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Research Ethics Committee of Vale do Paraíba University, UNIVAP, São José dos Campos, SP, Brazil (protocol number A009/2006 CEP). In addition, all animal experiments were performed according to the guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL/CEUA).

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