NEUTRALIZATION OF THE NEUROMUSCULAR ACTIVITY OF BOTHROPSTOXIN-I, A MYOTOXIN FROM *Bothrops jararacussu* SNAKE VENOM, BY A HYDROALCOHOLIC EXTRACT OF *Casearia sylvestris* Sw. (GUAÇATONGA)

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ABSTRACT: Numerous plants are used as snakebite antidotes in Brazilian folk medicine, including *Casearia sylvestris* Swartz, popularly known as guaçatonga. In this study, we examined the action of a hydroalcoholic extract from *C. sylvestris* on the neuromuscular blockade caused by bothropstoxin-I (BthTX-I), a myotoxin from *Bothrops jararacussu* venom, in mouse isolated phrenic nerve-diaphragm (PND) preparations. Aqueous (8 and 12 mg/ml, n=4 and 5, respectively) and hydroalcoholic (12 mg/ml, n=12) extracts of the leaves of *C. sylvestris* caused facilitation in PND preparations followed by partial neuromuscular blockade. BthTX-I (20 μg/ml, n=4) caused 50% paralysis after 65±15 min (mean ± S.E.M). Preincubation (30 min at 37°C) of BthTX-I (20 μg/ml, n=4) with a concentration of the hydroalcoholic extract (4 mg/ml) that had no neuromuscular activity, such as the control (n=5), prevented the neuromuscular blockade caused by the toxin. This protection may be mediated by compounds such as flavonoids and phenols identified by thin-layer chromatography and colorimetric assays.

KEY WORDS: alternative medicine, phytotherapy, plant extracts, snakebite.

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INTRODUCTION

Currently, the standard treatment for snakebites in humans is the administration of antivenom (5). However, numerous species of plants have also been used to treat snakebites in animals and humans. Thus, in Trinidad, the standard treatment for snakebites and scorpion stings in dogs involves the use of steroids, antibiotics, and the enzyme ananase (from the pineapple *Ananas comosus*). This enzyme, the administration of which must occur within 2 hours of the bite (15), reduces the inflammatory response and helps to degrade necrotic tissue.

Casearia sylvestris Sw. (Flacourtiaceae), popularly known as guaçatonga, grows wild throughout the tropics, where it easily adapts to forests and plains (28). This plant is widely used in folk medicine as an antiseptic, wound healer, topical anesthetic, and antitumor, anti-ulcer and anti-snakebite agent (1, 2, 3, 14). In this study, we examined the neuromuscular activity of aqueous and hydroalcoholic extracts of *C. sylvestris*, and assessed the activity of the hydroalcoholic extract to neutralize the neuromuscular blockade caused by BthTX-I, a myotoxin from *Bothrops jararacussu* venom, that causes muscle damage (12, 24) and blockade of directly and indirectly evoked contractions in mouse phrenic nerve-diaphragm preparations (11).

MATERIALS AND METHODS

1. Plant material and extraction

The leaves of *C. sylvestris* were collected in February 2003 from adult plants growing in an orchard at the University of Sorocaba (UNISO). A voucher specimen was deposited in the UNISO herbarium after identification by the Botanical Institute (Instituto de Botânica) in São Paulo, Brazil. The leaves were dried and powdered, and aqueous and hydroalcoholic (70%) extracts were obtained by maceration, concentration, and lyophilization. The lyophilized material was stored at room temperature and protected from light and humidity until assayed. Partial physicochemical characterization of the extracts was done as described in the sections 2 and 3 below.

2. Thin-layer chromatography

Aliquots of the hydroalcoholic (HA) extract were spotted onto thin-layer (0.3 mm thick) silica gel plates (Merck[®]), along with appropriate standards. All solvents were purchased from Merck[®] (Darmstadt, Germany). The solvent system for running the hydroalcoholic extract consisted of ethyl acetate : formic acid : glacial acetic acid : water (100:11:11:26, v/v). The following phytochemical groups were used as standards (all from Sigma-Aldrich[®], St. Louis, MO, USA): apigenin, quercetin, rutin, caffeic acid, and chlorogenic acid. The separated spots were visualized with diphenylboric acid 2-aminoethyl ester (NP) and polyethylene glycol 4000 (PEG) and observed with ultraviolet light at 365 nm. The retention factor (Rf) of each spot was compared to the standards indicated above.

3. Determination of the flavonoid and polyphenol content

3.1. Flavonoids content

The content of flavonoids was determined as described by other authors (9, 17), based on the UV absorption of $Al-Cl_3$-flavonoid complexes, and was expressed as the total content of quercetin. Briefly, 2 g of the extract were extracted under reflux for 30 min with 50 ml of 50% methanol and 5 ml of this extract was then completed to 50 ml with 80% methanol. Aliquots (2 ml) of this solution (n=4) were mixed with 2 ml of methanol containing 5% anhydrous aluminum chloride ($AlCl_3$; complexing agent) and completed to 10 ml with 80% methanol. After 30 min, the absorbances were read at 420 nm against a blank containing 80% methanol (8 ml) and 5% $AlCl_3$ (2 ml). The percentage of flavonoids (%FL) was calculated from a standard curve of quercetin (Sigma-Aldrich[®]) prepared in methanol (0, 4, 8, 12, and 16 μg/ml).

3.2. Polyphenol content

The content of polyphenols was determined as described by other authors (13, 22), using an adaptation by Reicher *et al.* (22). Briefly, 2 g of the extract were extracted under reflux with 50 ml of 50% ethanol, and 5-ml aliquots of this solution (n=3) were completed to 50 ml with 50% ethanol. From this solution, 5-ml aliquots were withdrawn and completed to 25 ml using distilled water. Five hundred microliters of phosphomolybdotungstic reagent was added to 1 ml of this solution and the volume was completed to 10 ml with 15% sodium carbonate solution. After 30 min, the

absorbance at 720 nm was read against a blank of 15% sodium carbonate solution. The percentage of polyphenols (%PP) was determined from a standard curve (5, 10, 15, 20, 25, 30, 35, and 40 μg/ml) of pyrogallol (Sigma-Aldrich[®]).

4. Animals

Male Swiss white mice (26-32 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The animals were housed at 25 ± 3°C on a 12-h light/dark cycle and had access to food and water *ad libitum*. This project (protocol number 644-1) was approved by the institutional Committee for Ethics in Animal Experimentation (CEEA-IB, UNICAMP), and the experiments were done within the guidelines of the Brazilian College for Animal Experimentation (COBEA).

4.1. Mouse phrenic nerve-diaphragm muscle (PND) preparation

The phrenic nerve and diaphragm (6) were obtained from mice anesthetized with chloral hydrate (300 mg/kg, intraperitoneally) and sacrificed by exsanguination. The diaphragm was removed and mounted under a tension of 5 g in a 5 ml organ bath containing aerated Tyrode solution (control) of the following composition (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. After equilibration with 95% O_2/5% CO_2, the pH of this solution was 7.0. The preparations were stimulated indirectly with supramaximal stimuli (4 x threshold, 0.1 Hz, 0.2 ms) delivered from a Grass S48 stimulator to the nerve through bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (Load Cell BG-10 GM) coupled to a physiograph (Gould, Model RS 3400) via a Gould universal amplifier. The preparations were allowed to stabilize for at least 20 min before the addition of the following: Tyrode solution alone (control), lyophilized aqueous or hydroalcoholic extracts of *C. sylvestris* diluted in Tyrode solution (2, 4, 8, and 12 mg/ml), BthTX-I (20 μg/ml), or BthTX-I (20 μg/ml) + hydroalcoholic extract (4 mg/ml) of *C. sylvestris*.

5. Statistical analysis

Each experimental protocol was repeated at least three times. The results were expressed as the mean $\pm$ S.E.M., as appropriate. Student's *t*-test was used for statistical comparison of the data. A value of $p < 0.05$ indicated significance.

RESULTS

1. Thin-layer chromatography

Figure 1 shows the chromatographic profile of the hydroalcoholic extract of *C. sylvestris* leaves. Comparison with standards of quercetin, rutin, caffeic acid, and chlorogenic acid run, simultaneously, showed that the phenolic constituents of the extract had Rf values that were most similar to those of rutin and chlorogenic acid.

2. Content of flavonoids and polyphenolic compounds

Figure 2A shows the standard curve for flavonoids (quercetin). Using the equation $Y = 0.0563X - 0.041$ (r = 0.99939) for this curve, the concentration of quercetin in the hydroalcoholic extract of *C. sylvestris* was calculated to be 5.12 μg/ml and the content of flavonoids (%FL) was 0.640 g%. The standard curve for polyphenols (pyrogallol) (Figure 2B) resulted in the equation $Y = 0.135X + 0.0356$ (r = 0.99749). From this, the concentration of pyrogallol in the extract was calculated to be 1.77 μg/ml and the content of polyphenols (%PP) was 2.22 g%.

3. Pharmacological assays in mouse PND preparations

3.1. Aqueous and hydroalcoholic extracts

Figure 3 shows the effects of aqueous (A) and hydroalcoholic (B) extracts of *C. sylvestris* leaves on mouse PND preparations at concentrations of 2, 4, 8, and 12 mg/ml (n=3-6 and 3-12 for the aqueous and hydroalcoholic extracts, respectively). Both extracts caused significant facilitation (p<0.05, compared to the Tyrode control) of an "all or nothing" type, starting with 8 mg/ml for the aqueous extract and 12 mg/ml for the hydroalcoholic extract. High concentrations of the extracts caused neuromuscular blockade. The facilitation and neuromuscular blockade were more intense with the hydroalcoholic extract. For this reason, this extract was chosen for the neutralization experiments with BthTX-I. A concentration of 4 mg/ml was considered ideal because it produced no facilitation or significant neuromuscular blockade.

3.2. Neutralization assays

Figure 4A shows the neuromuscular blockade caused by BthTX-I (20 μg/ml, n=4, p<0.05) compared to the Tyrode control (n=5) and the response to a mixture of

BthTX-I (20 μg/ml) + hydroalcoholic extract of *C. sylvestris* (4 mg/ml) preincubated for 30 min at 37°C prior to testing (n=4). These experiments were performed in parallel using hemidiaphragm preparations. The hydroalcoholic extract prevented the blockade by BthTX-I. Figures 4B and 4C show representative records of the responses to BthTX-I (20 μg/ml) and a mixture of BthTX-I + hydroalcoholic extract.
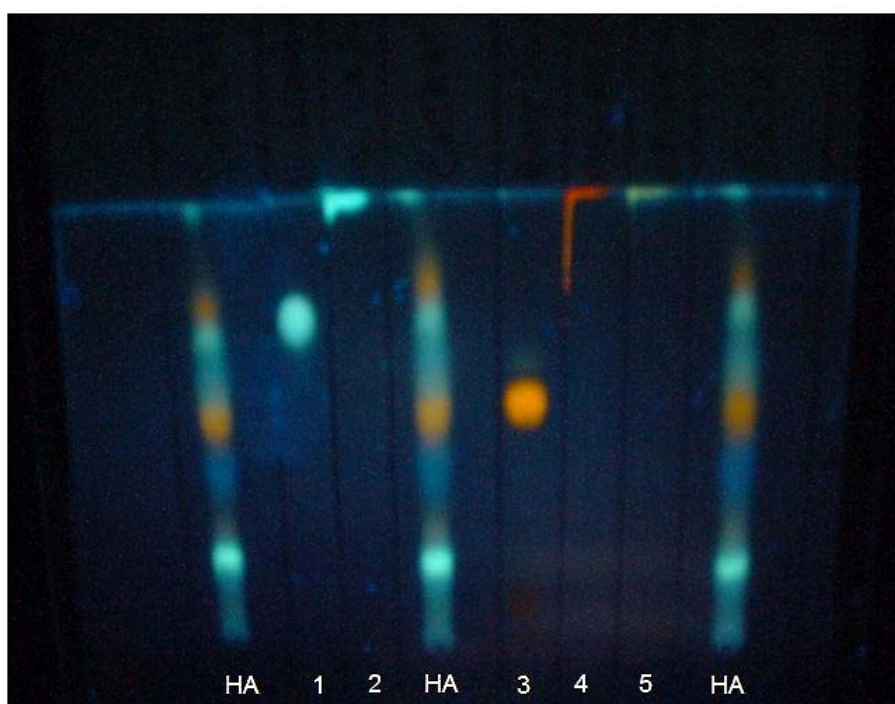

Figure 1: Thin-layer chromatography. Hydroalcoholic (HA) extract of leaves from *Casearia sylvestris* Sw. Solvent system: ethyl acetate : formic acid : glacial acetic acid : water (100:11:11:26, v/v). Spot detection: NP (diphenylboric acid 2-aminoethyl ester) and PEG (polyethylene glycol 4000). Phytochemical standards: 1 – chlorogenic acid, 2 –caffeic acid, 3 – rutin, 4 – quercetin, 5 – apigenin. Note the similarity between the Rf values of the constituents present in the extract and those of rutin (3, orange) and chlorogenic acid (5, blue).

Standard curve of quercetin

$y = 0.0563x - 0.041$
$r = 0.99939$

A

Standard curve of pyrogallol
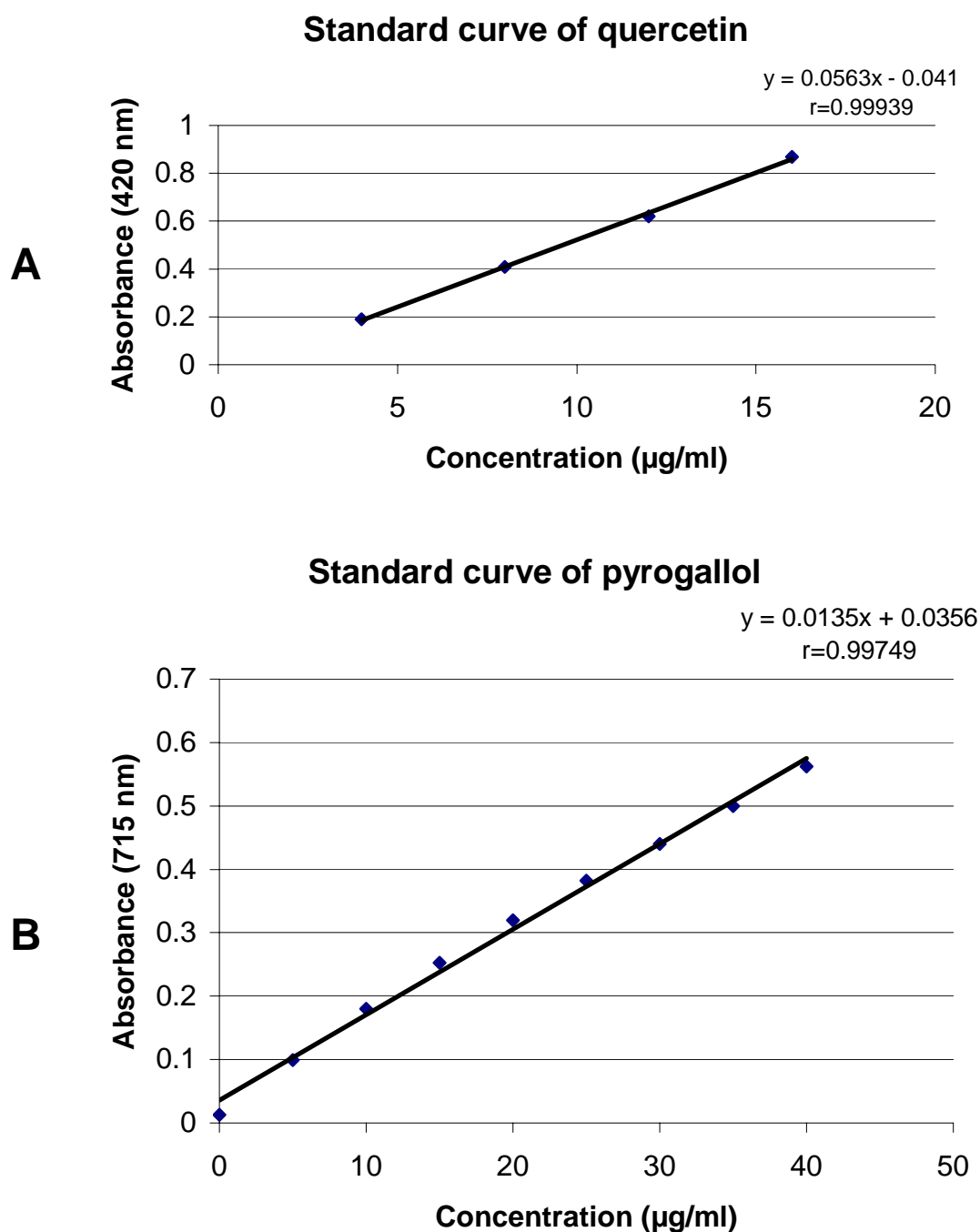
$y = 0.0135x + 0.0356$
$r = 0.99749$

B

Figure 2: Standard curves for the flavonoid quercetin (A) and the phenolic compound pyrogallol (B). The content of these substances in the leaves of *C. sylvestris* was determined by interpolation.
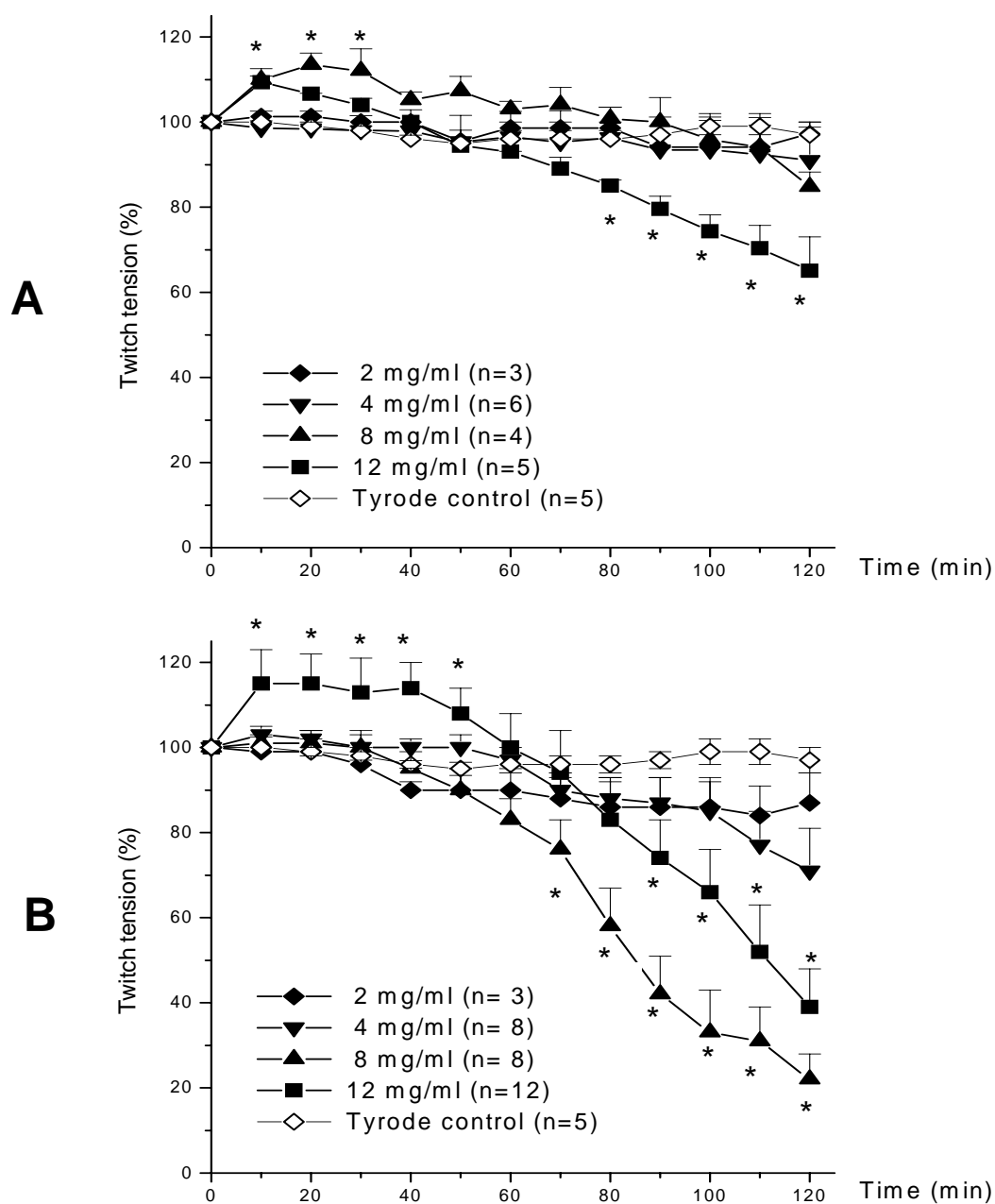
Figure 3: Neuromuscular activity of aqueous (A) and hydroalcoholic (B) extracts from the leaves of *C. sylvestris* in mouse isolated phrenic nerve-diaphragm preparations. Each point represents the mean ± S.E.M. of the number of experiments indicated in parentheses. *p<0.05 compared to the Tyrode control.
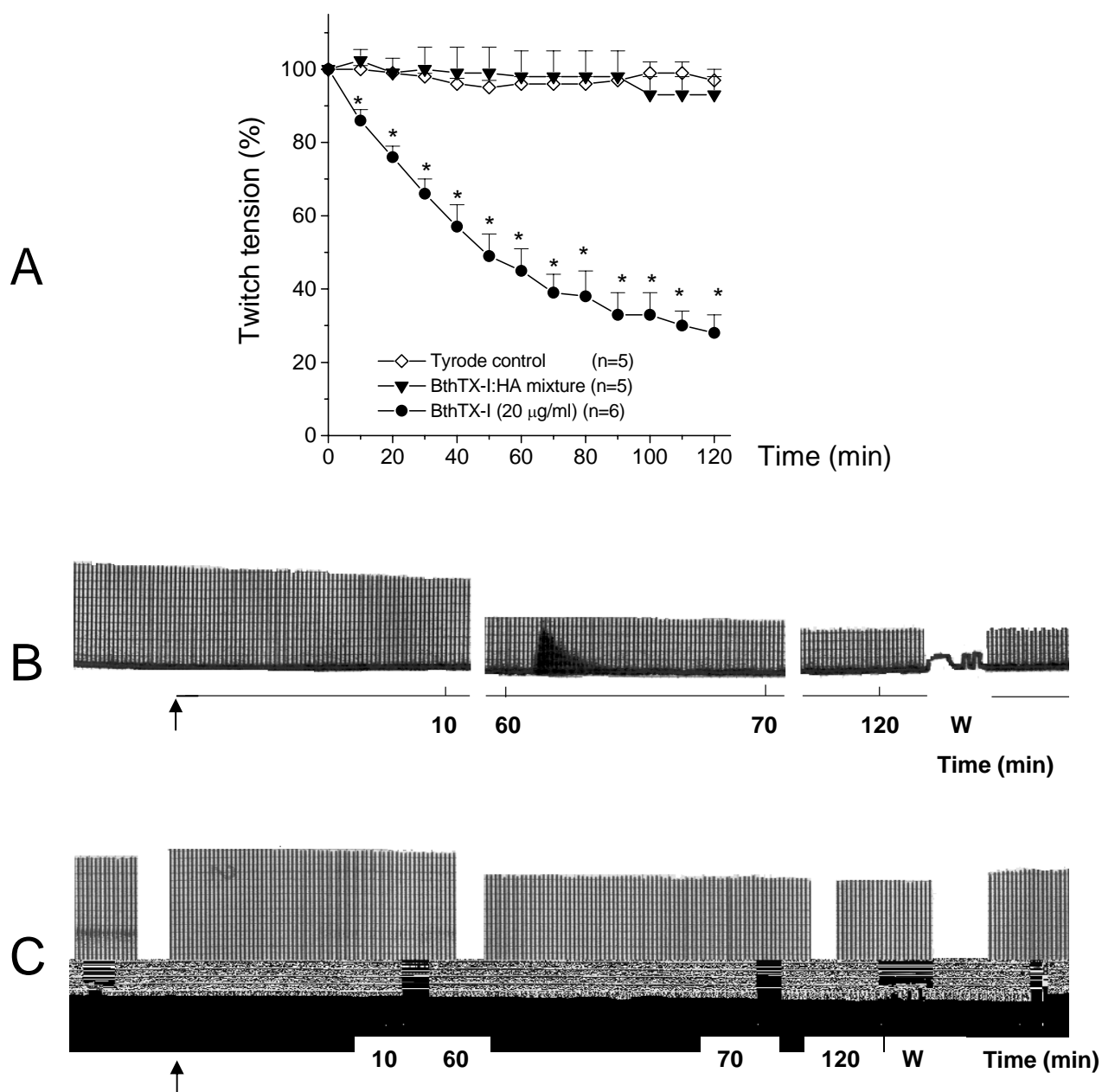
Figure 4: Neutralization of the neuromuscular blockade of BthTX-I by a hydroalcoholic extract of *C. sylvestris* leaves. In A, the points are the mean ± S.E.M. of the number of experiments indicated in parentheses. *p<0.05 compared to the Tyrode control. B and C show representative recordings of the responses to BthTX-I and a BthTX-I:HA mixture. HA: hydroalcoholic extract of *C. sylvestris*. Arrows: addition of BthTX-I (in B) or BthTX-I:HA mixture (in C).

DISCUSSION

Snake venoms of the genus *Bothrops* induce local effects such as myonecrosis, edema, and hemorrhage (8, 25). *Bothrops jararacussu* venom produces these effects mainly through bothropstoxin-I (BthTX-I), the major myotoxin of this venom (12).

The use of plant extracts as antidotes for envenomings represents an alternative for communities that do not have access to antivenom therapy, and may be useful against the local effects of venoms (3, 16, 21, 23, 26). Plant products also have applications against snakebites and scorpion stings (15).

The efficacy of *C. sylvestris* against snake venom has been assessed in some studies (2, 3). As shown here, alcohol was a better solvent for extraction than water. The presence of active compounds such as flavonoids and phenols was initially demonstrated by thin-layer chromatography followed by direct quantification. Many of these plant substances structurally resemble biologically active compounds, and this similarity may be the basis of their physiological action (10).

In plants, flavonoids most likely serve to filter out UV light (adstringency), scavenge free radicals *in situ*, and exert an antipredatory effect. Flavonoids can also act as free radical scavengers *in vitro* and may modulate the redox properties and mechanisms of radical interactions (4). As shown here, the aqueous and hydroalcoholic extracts caused facilitation followed by partial inhibition of the twitch tension. Similar results have been reported for green tea (*Camellia sinensis*) extracts in rat diaphragm and were reproduced by a crude polyphenol preparation (7). The hydroalcoholic extract of guaçatonga contains flavonoids and phenolic compounds. The role of the phenolic compounds in the neuromuscular blockade caused by this extract is unclear.

In addition to the extracts tested here, other substances with a facilitatory action on neuromuscular transmission, such as heparin (20) and manganese (a neuromuscular blocker that shows spontaneous reversion and post-wash facilitation) (18, 19, 27), also protect against neuromuscular blockade.

The results reported here extend the findings of Borges *et al.* (2, 3) regarding the ability of extracts of *C. sylvestris* to protect against the hemorrhagic and phospholipase A_2 activities of snake venom. However, the mechanism by which the hydroalcoholic extract of *C. sylvestris* protected against the paralysis induced by BthTX-I remains unclear. Further purification and identification of the active fractions of the extract will contribute to our understanding of the pathways involved.

ACKNOWLEDGEMENTS

REFERENCES

1 BASILE AC., SERTIE JA., PANIZZA S., OSHIRO TT., AZZOLINI CA. Pharmacological assay of *Casearia sylvestris* – I: preventive anti-ulcer activity and toxicity of the leaf crude extract. *J. Ethnopharmacol.*, 1990, 30, 185-97.

2 BORGES MH., SOARES AM., RODRIGUES VM., ANDRIÃO-ESCARSO SH., DINIZ H., HAMAGUCHI A., QUINTERO A., LIZANO S., GUTIÉRREZ JM., GIGLIO JR., HOMSI-BRANDEBURGO MI. Effects of aqueous extract of *Casearia sylvestris* (Flacourtiaceae) on actions of snake and bee venoms and on activity of phospholipases A_2. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.*, 2000, 127, 21-30.

3 BORGES MH., SOARES AM., RODRIGUES VM., OLIVEIRA F., FRANSHESCHI AM., RUCAVADO A., GIGLIO JR., HOMSI-BRANDEBURGO MI. Neutralization of proteases from *Bothrops* snake venoms by the aqueous extract from *Casearia sylvestris* (Flacourtiaceae). *Toxicon*, 2001, 39, 1863-9.

4 BORS W., HELLER W., MICHEL C., STETTMAIER K. Polyphenols and flavonoids: chemistry and biology. In: CADENAS E., PACKER L. Eds. *Handbook of antioxidants*. 2.ed. New York: Marcel Dekker, 2001: 279-370.

5 BRASIL. Ministério da Saúde. Fundação Nacional da Saúde. *Manual de diagnóstico e tratamento de acidentes por animais peçonhentos*. Brasília, 1998. 131p.

6 BÜLBRING E. Observation on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmacol.*, 1946, 1, 38-61.

7 DAS M., VEDASIROMONI JR., CHAUHAN SP., GANGULY DK. Effect of green tea (*Camellia sinensis*) extract on the rat diaphragm. *J. Ethnopharmacol.*, 1997, 57, 197-201.

8 GUTIÉRREZ JM., ROMERO M., DÍAZ C., BORKOW G., OVADIA M. Isolation and characterization of a metalloproteinase with weak hemorrhagic activity from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon*, 1995, 33, 19-29.

9 HARBONE JB. *Phytochemical methods – a guide to modern techniques of plant analysis*. 3.ed. London: Chapman & Hall, 1998. 302p.

10 HAVSTEEN B. Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol.*, 1983, 32, 1141-8.

11 HELUANY NF., HOMSI-BRANDEBURGO MI., GIGLIO JR., PRADO-FRANCESCHI J., RODRIGUES-SIMIONI L. Effects induced by bothropstoxin, a component from *Bothrops jararacussu* snake venom, on mouse and chick muscle preparations. *Toxicon*, 1992, 30, 1203-10.

12 HOMSI-BRANDEBURGO MI., QUEIROZ LS., SANTO-NETO H., RODRIGUES-SIMIONI L., GIGLIO JR. Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. *Toxicon*, 1988, 26, 615-27.

13 INSTITUTO NACIONAL DA FÁRMACIA E DO MEDICAMENTO. *Farmacopéia portuguesa*. 7.ed. Lisboa: Infarmed, 2002. 2792p.

14 ITOKAWA H., TOTSUKA N., TAKEYA K., WATANABE K., OBATA E. Antitumor principles from *Casearia sylvestris* Sw. (Flacourtiaceae), structure elucidation of new clerodane diterpenes by 2 – D NMR spectroscopy. *Chem. Pharm. Bull. (Tokyo)*, 1988, 36, 1585-8.

15 LANS C., HARPER T., GEORGES K., BRIDGEWATER E. Medicinal and ethnoveterinary remedies of hunters in Trinidad. BMC Complementary and Alternative Medicine, 2001, 1, 10 [cited 2003 Dec 24]. Available from: http://www.biomedcentral.com/1472-6882/1/10.

16 MARTZ W. Plants with a reputation against snakebite. *Toxicon*, 1992, 30, 1131-42.

17 MORI ALPM. *Própolis – identificação de flavonóides e ácidos aromáticos em tintura. Estimativa de FPS de extrato mole em base cosmética*. São Paulo: Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, 1997. 114p. [Masters Dissertation]

18 OSHIMA-FRANCO Y., LEITE GB., BELO CA., HYSLOP S., PRADO-FRANCESCHI J., CINTRA AC., GIGLIO JR., DA CRUZ-HOFLING MA., RODRIGUES-SIMIONI L. The presynaptic activity of bothropstoxin – I, a myotoxin from *Bothrops jararacussu* snake venom. *Basic Clin. Pharmacol. Toxicol.*, 2004, 95, 175-82.

19 OSHIMA-FRANCO Y., LEITE GB., PRADO-FRANCESCHI J., RODRIGUES-SIMIONI L. Neuromuscular transmission: influence of manganese ions on the study of pharmacology of the BthTX-I (*Bothrops jararacussu*). In: *PAN AMERICAN CONGRESS INTERNATIONAL SOCIETY ON TOXINOLOGY*, 6, Margarita Island, 1998. Abstracts... Margarita Island, 1998. p.36.

20 OSHIMA-FRANCO Y., LEITE GB., SILVA GH., CARDOSO DF., HYSLOP S., GIGLIO JR., DA CRUZ-HOFLING MA., RODRIGUES-SIMIONI L. Neutralization of the pharmacological effects of bothropstoxin – I from *Bothrops jararacussu* (jararacuçu) venom by crotoxin antiserum and heparin. *Toxicon*, 2001, 39, 1477-85.

21 PEREIRA BMR., GONÇALVES LC., PEREIRA NA. Abordagem farmacológica de plantas recomendadas pela medicina folclórica como antiofídicas III – atividade antiedematogênica. *Rev. Bras. Farmacol.*, 1992, 73, 85-6.

22 REICHER F., SIERAKOWSKI MR., CORRÊA JBC. Determinação espectrofotométrica de taninos pelo reativo fosfotúngstico – fosfomolíbdico. *Arq. Biol. Tecnol.*, 1981, 24, 407-11.

23 RIZZINI CT., MORS WB., PEREIRA NA. Plantas brasileiras tidas como ativas contra peçonhas animais, especialmente venenos de cobras. *Rev. Bras. Farmacol.*, 1988, 69, 82-6.

24 RODRIGUES-SIMIONI L., BORGESE N., CECCARELLI B. The effects of *Bothrops jararacussu* venom and its components on frog nerve-muscle preparation. *Neuroscience*, 1983, 10, 475-89.

25 ROSENFELD G. Symptomatology, pathology and treatment of snakebites in South America. In: BUCHERL W., BUCKLEY EE. Eds. *Venomous animals and their venoms*. New York: Academic Press, 1971: 345-62.

26 RUPPELT BM., PEREIRA EFR., GONÇALVES LC., PEREIRA NA. Abordagem farmacológica de plantas recomendadas pela medicina folclórica como antiofídicas I – atividades analgésica e antiinflamatória. *Rev. Bras. Farmacol.*, 1990, 71, 54-6.

27 SOARES AM., OSHIMA-FRANCO Y., VIEIRA CA., LEITE GB., FLETCHER JE., JIANG MS., CINTRA ACO., GIGLIO JR., RODRIGUES-SIMIONI L. Mn^{2+} ions reduce the enzymatic and pharmacological activities of bothropstoxin – I, a myotoxic Lys49 phospholipase A$_2$ homologue from *Bothrops jararacussu* snake venom. *Int. J. Biochem. Cell Biol.*, 2002, 34, 668-77.

28 TAYLOR L. Herbal secrets of the rainforest. 2.ed. États-Unis: Sage Press, Inc., 2002 [cited 2002 Oct 20]. Available from: http://www.rain-tree.com/guacatonga.htm