RESEARCH REPORT

The inhibitory effect of *Camellia sinensis* extracts against the neuromuscular blockade of *Crotalus durissus terrificus* venom

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ABSTRACT

In geographically isolated populations where intensive medical care or serum therapy is not easily accessible snake envenomation is a major cause for concern. The aim of the present study was to test *Camellia sinensis* extracts, theaflavin and epigallocatechin (two of the main *C. sinensis* components) against the irreversible neuromuscular blockade induced by *Crotalus durissus terrificus* venom in mouse phrenic-nerve diaphragm preparations. A quantitative histological study was also performed. The venom (20µg/ml) completely decreased twitch tension after 70min and 5µg/ml venom abolished 50% of twitch amplitude after 60min. *C. sinensis* extract induced intense facilitatory effect in the preparation activity at 0.2mg/ml and slightly facilitatory effect at 0.05mg/ml. Both 0.05mg/ml *C. sinensis* extract and 0.05µg/ml commercial theaflavin maintained partial muscular activity in presence of 5µg/ml venom. The histological data confirms that Cs is able to protect the muscle from the myotoxic activity of the venom. Commercial epigallocatechin gallate did not show pre-synaptic nor post-synaptic activities. *C. sinensis* extract was able to protect the mouse phrenic-nerve diaphragm against the irreversible neuromuscular blockade induced by *C. durissus terrificus* venom.

KEYWORDS: Anticrotalic action, catechins, green tea, snake venom, theaflavins

INTRODUCTION

Snake venoms that have neurotoxins with phospholipase A_2 (PLA_2) activity, are able to inhibit the neuromuscular transmission and cause irreversible paralysis (Rigoni et al, 2008). *Crotalus durissus terrificus* (Cdt), the South American rattlesnake, is responsible for the majority of deaths caused by snakebites in Brazil (Brazil, 2001; Boldrini-França et al, 2009) and many other countries. The main toxin of Cdt venom is crotoxin, a specific complex of toxic proteins, that can be separated into a PLA_2 and crotapotin fragment. Crotapotin, consisting of three different amino acid chains, potentiates the enzyme and is markedly neurotoxic (MeSH, 2010). Many medicinal plants have anti-PLA_2 properties and compounds that are able to inhibit PLA_2 activity could be useful as therapeutic agents to treat lesions induced by PLA_2 activity, such as Cdt venom (Borges et al, 2005; Nirmal et al, 2008).

The infusion of *Camellia sinensis* L. (formerly *Thea sinensis*) leafs, which is an evergreen Asiatic bush of the Theaceae family, contains catechins and theaflavins that are responsible for the protecting effect attributed to the tea. *C. sinensis* is known as black (infusion of oxidized leaves) or green (infusion of dried leaves) tea. The oxidation process consists of drying, pulverizing and exposing the leaves to the air, in order to release the polyphenoloxydase enzyme inside the cell vacuoles (Matsubara and Rodriguez-Amaya, 2006). Many properties of *C. sinensis*, such as nutraceutical (Grove and Lambert, 2010), anti-inflammatory and immunostimulatory

(Kim et al, 2008), enhancing insulin activity (Ueda et al, 2008), chemoprevention (Clement, 2009), antimicrobial activity (Shah et al, 2008), and antihypertensive activity (Kurita et al, 2010), have been studied. In addition, black tea extract has been shown to prevent the neuromuscular blockade induced by botulinum neurotoxin (Satoh, 2005).

In the present study, a hydroalcoholic extract from dried/pulverized leaves of *C. sinensis* and two of the main chemical components (theaflavin and epigallocatechin gallate) were tested to determine if they would interfere with the neuromuscular blocking activity of venom of *Crotalus durissus terrificus*.

MATERIALS AND METHODS

Hydroalcoholic extract from leaves of *Camellia sinensis*

The leaves of *C. sinensis* were harvested from plants growing in an orchard at the University of Sorocaba - UNISO (Sorocaba, SP, Brazil). A voucher specimen was deposited in the Instituto Agronômico de Campinas (IAC, number 50.469) herbarium (http://herbario.iac.sp.gov.br) after identification by L.C. Bernacci.

Sixty-four grams of leaves powder were macerated along with 150ml of 70° GL ethanol, over 3 days. After this period, the resulting suspension was placed into a percolator with 50ml of 70° GL ethanol, and left for a further 3 days. The macerated drug was percolated and a 20% hydroalcoholic extract obtained. The solvent was evaporated until dryness and the dried extract was then protected from light and humidity at room temperature until the assays.

Quality control assays of the vegetal drugs – the ash and humidity tests

In order to observe their elementary physical and chemical characteristics, the powder obtained from the *C. sinensis* leaves was submitted to the ash and humidity tests (Brazilian Pharmacopeia, 1988). Briefly, 100gm of each specimen powder were placed in six calibrated melting pots, which were warmed until total powder carbonization. The melting pots were kept at 650°C and the ashes were weighed. Results were presented in grams of ashes/100gm of the sample.

The humidity test was performed by placing 1gm of each specimen powder in six calibrated porcelain capsules, which were warmed at 105°C for 4hr and then weighed.

Determination of flavonoids and polyphenols

Flavonoid content

The content of flavonoids was determined in the plant hydroalcoholic extract as described elsewhere (Harborne, 1998). The method is based on the UV absorption of Al-Cl_3-flavonoid complexes and is expressed as total content of quercetin. Briefly, 80% (v/v) methanol (50ml) was added to 10ml of extract and 5ml of solution were transferred to volumetric flasks and diluted again with 80% (v/v) methanol (50ml). Four aliquots (2ml) of solution were mixed with 2ml of 5% (w/v) anhydrous aluminum chloride solution ($AlCl_3$; complexing agent) and adjusted to 10ml with 80% (v/v) methanol. After 15 min, the absorbance of sample was read at 420nm, considering a blank sample containing 80% (v/v) methanol (8ml) and 5% (w/v) $AlCl_3$ (2ml). The percentage of flavonoids (%) was calculated from a standard curve of quercetin (0, 4, 8, 12, and 16µg/ml) prepared in methanol.

Polyphenol content

The contents of polyphenols in the plant hydroalcoholic extract were determined as previously described (Reicher et al, 1981). Briefly, 5ml of extract was poured into a volumetric flask and distilled water was added to 250ml, after which a 1ml aliquot was transferred to another volumetric flask and distilled water added to 25ml (final solution). Aliquots (1ml) of the final solution received 1ml of phosphomolybdotungstic reagent and the final volume (10ml) was adjusted with 15% (w/v) sodium carbonate solution. After 30min, the absorbance of sample was read at 720nm, considering a blank sample containing 15% (w/v) sodium carbonate solution. The percentage of polyphenols (%) was determined from a standard curve (5, 10, 15, 20, 25, 30, 35, and 40µg/ml) of pyrogallol (Sigma Chemical Co, St Louis, MO, USA).

Thin layer chromatography (TLC)

Aliquots of *C. sinensis* hydroalcoholic extract were spotted onto 0.2mm thickness silica gel $60F_{254}$ on aluminum plates, 20.10 cm, (Merck, Germany) and developed with ethyl acetate:methanol:water (100:13.5:10, v/v) in a pre-saturated chromatographic chamber along with appropriate phytochemical standards (Simões et al, 2004). These standards (theaflavin and epigallocatechin gallate, Sigma-Aldrich®, USA) were solubilized in methanol (1mg/ml). The separated spots were visualized (under UV light at 360nm) with NP/PEG as follows: 5% (v/v) ethanolic NP (diphenylboric acid 2-aminoethyl ester, Sigma Chemical Co, St Louis, MO, USA) followed by 5% (v/v) ethanolic PEG 4000 (polyethylene glycol 4000, Synth Chemical Co, Sao Paulo, SP, Brazil). The retention factor (Rf) of each standard was compared with spots exhibited by *C. sinensis* extracts.

Pharmacological study

Animals

Male Swiss white mice (26-32 g) were supplied by the Anilab - Animais de Laboratório (Paulínia, São Paulo, Brazil). The animals were housed at 25±3°C on a 12hr light/dark cycle with access to food and water *ad libitum*. This study was approved (protocol number A077/CEP2007) by the Committee for Ethics in Research from the University of Vale do Paraiba (UNIVAP) and all experiments were performed according to the guidelines of the Brazilian College for Animal Experimentation.

Crotalus *venom*

The crude venom was obtained from adult *Crotalus durissus terrificus* snakes (Serpentário do Centro de Estudos da Natureza) and certified by Professor Dr Jose Carlos Cogo, Universidade do Vale do Paraiba (Univap), Sáo Jose dos Campos, SP, Brazil.

Mouse phrenic-nerve diaphragm muscle (PND) preparation

The PND was obtained from mice anesthetized with halotane and sacrificed by exsanguination. The diaphragm was removed (Bülbring, 1946) and mounted under a tension of 5gm in a 5ml organ bath containing continuous-aerated Tyrode solution (control) with the following composition: 137mM NaCl,

2.7mM KCl, 1.8mM $CaCl_2$, 0.49mM $MgCl_2$, 0.42mM NaH_2PO_4, 11.9mM $NaHCO_3$, and 11.1mM glucose. After stabilization with 95% (v/v) O_2/5% (v/v) CO_2, the pH was 7.0. The PND myographic recording was performed according to Melo et al (2009). Briefly, preparations were stimulated indirectly with supramaximal stimuli (4x threshold, 0.06Hz, 0.2ms) delivered from a stimulator (model ESF-15D, Ribeirão Preto, Brazil) to the nerve through bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (cat# 7003, Ugo Basile), coupled to a 2-Channel Recorder Gemini physiograph (cat# 7070, Ugo Basile) via a Basic Preamplifier (cat# 7080, Ugo Basile). PND were allowed to stabilize for at least 20min before addition of the following substances: *C. sinensis* extracts 0.05mg/ml (n=7), 0.1mg/ml (n=7) and 0.2 mg/mL (n=7); Cdt alone at 5µg/ml (n=10), 10µg/ml (n=10) and 20µg/ml (n=6); 5µg/ml Cdt + 0.05mg/ml *C. sinensis* extract (n=5); 5µg/ml Cdt + 0.05mg/mL epigallocatechin gallate (n=4, Sigma-Aldrich, SP, Brazil); 5µg/ml Cdt + 0.05mg/ml theaflavin (n=4); and Tyrode solution (control, n=7).

Quantitative histological study

Preparations resulting from pharmacological assays were analyzed by quantitative morphometry. At the end of the experiments, the preparations (120 min period) used in Tyrode (control), *C. sinensis* hydroalcoholic extract (0.05mg/ml), Cdt venom (5µg/ml), and *C. sinensis* (0.05mg/ml) + Cdt venom (5µg/ml) groups (n=3) were fixed in Bouin solution and processed by routinely morphological techniques. Cross-sections (2µm thick) of diaphragm muscle were stained with 0.5% (w/v) toluidine blue for microscopy examination. Tissue damage was expressed in percentage (number of damaged muscle cells divided by the total number of cells in three non-overlapping, non-adjacent areas of each preparation) according to Cintra-Francischinelli et al (2008).

Statistical analysis

Each pharmacological protocol was repeated at least three times. Results were expressed as the mean ± standard deviation (SD). The Student's *t*-test or repeated measures ANOVA were used for statistical comparison of the data. The significance level was set at 5%.

RESULTS

Ash and humidity (mean ± SD) were 5.65 ± 0.06 g/100g and 11.3 ± 0.4 g/100g, respectively. Concentration of flavonoids (calibration curve R=0.998) and polyphenols (calibration curve, R=0.995) were 1.4 ± 0.04 % and 14.5 ± 0.07%, respectively. Figure 1 shows the thin layer chromatographic profile obtained by using a polar eluent system of the followings substances (retention factor = Rf): theaflavin (1, Rf=0.5), epigallocatechin gallate (3, Rf=0.72) and *Camellia sinensis* extract (Cs, 2, 4) . Cs spots are suggestive of several flavonoids (orange color) and phenolics constituents (blue color), including theaflavin and epigallocatechin gallate, respectively.

We then investigated the PND blockade activity of Cdt crude venom (Figure 2). However, we did not observe statistically significant differences were between 5 and 10µg/ml Cdt concentrations. There was no contraction recovery of PND after washing of preparations.

The pharmacological profile of 0.05mg/ml (n=7), 0.1mg/ml (n=7) and 0.2mg/ml (n=7) of *C. sinensis* is shown in Figure 3. An intense facilitatory effect was observed when 0.2mg/ml *C. sinensis* extract was added to the bath containing the neuromuscular preparation. The minor concentration (0.05mg/ml) of Cs was chosen for further Cdt-neutralization assays since it induced minor changes in basal response of PND. The same concentration was used for the comparative study using commercial phytochemicals, theaflavin and epigallocatechin gallate.

The study of the effects of 0.05mg/ml *C. sinensis* on twitch blockade induced by 5µg/ml Cdt venom is presented in Figure 4. The early effects of Cdt on twitch height (0.5µg/ml) remained unaltered by the addition of 0.05mg/ml of *C. sinensis*, however, the late effects of the venom were completely abolished, *i.e.*, after 2hr twitch height was 50% of the untreated control and the treated control was completely blocked (*p<0.05 compared to Tyrode control and to venom alone). In addition, following washing out of Cs-Cdt with Cs-Cdt free physiological salt solution twitch height was restored to 76 ± 7% of control in Cs treated preparations while in untreated preparations twitches did not recover. The commercial phytochemical from *C. sinensis* 0.05mg/ml epigallocatechin gallate in mixture with Cdt (n=4) did not protect the neuromuscular blockade induced by the venom. However, the commercial phytochemical from *C. sinensis* 0.05mg/ml theaflavin did protect the preparations from the

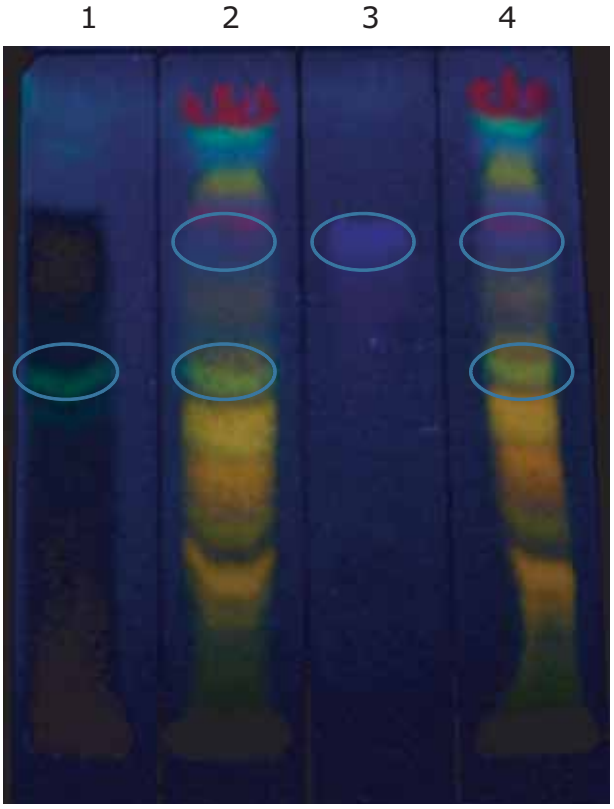

Figure 1. Thin Layer Chromatography performed by using ethyl acetate:methanol:water (100:13.5:10) solvent/Developer: NP/PEG. Phytochemical standards: 1 - Theaflavin; 3-Epigallocatechin gallate. 2, 4-*Camellia sinensis* extract [1; Theaflavin, 2; *Camellia sinensis* extract, 3; Epigallocatechin gallate, 4; *Camellia sinensis* extract]

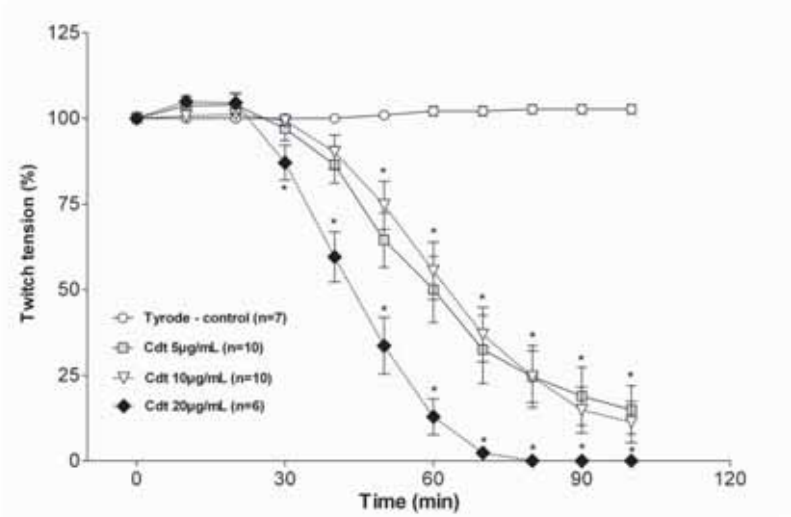

Figure 2. Isolated mouse phrenic nerve-diaphragm preparations under indirect stimuli. Concentration-response curve of *Crotalus durissus terrificus* (Cdt) venom. Each point represents the mean ± SEM. * = $p<0.05$ in comparison with Tyrode control.

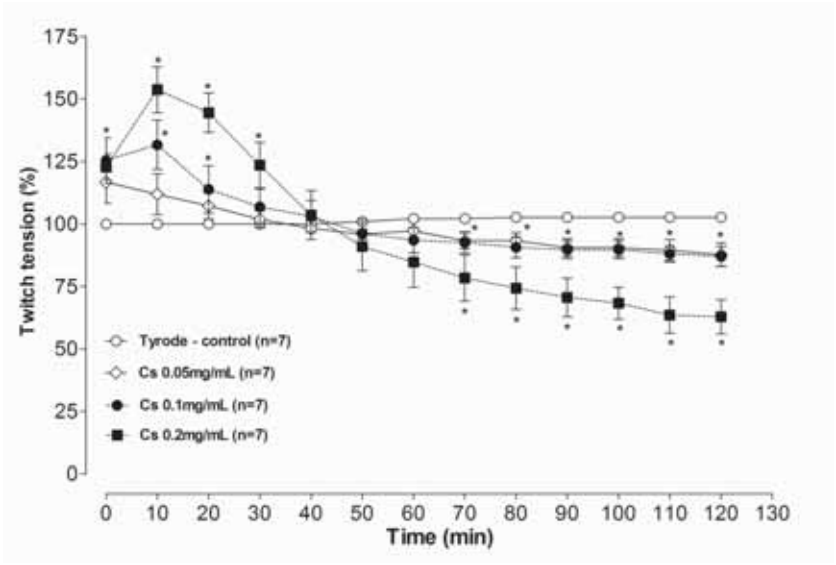

Figure 3. Isolated mouse phrenic nerve-diaphragm preparations under indirect stimuli. Concentration-response curve of *Camellia sinensis* (Cs) extract. Each point represents the mean ± SEM. * = $p<0.05$ in comparison with Tyrode control.

paralytic effect of the venom (n=4, no statistically differences with Cs extract alone).

In neuromuscular preparations exposed either to Tyrode or *C. sinensis* extract, the muscle fibers were well-preserved, showing changes not significantly different between one each other of 15,9 % ±0.8 or 25.3 % ±1.1 damaged fibers, respectively. These changes were related to loss of the typical cell cross-sectional polygonal profile (not shown). The venom alone clearly showed in transversal sections myonecrosis (m), edematous cells (e), loss of polygonal profile; sarcolemma disruption, delta lesion (arrow), "ghost" cells (g), and nuclei (n) dispersed in the tissue, whereas in longitudinal sections (not shown) these changes plus atrophy of the muscle fibers, hypercontraction, and condensation and/or lysis of the myofibrils also are seen. Figure 5 shows cross-sections of PND muscle fibers during 120 min of indirect electrical stimulation (n=3 each) of Cdt venom exposition (A, 5µg/ml, 59.5 ±1 % of lesioned fibers) and after *in vitro* neutralization by *C. sinensis* extract (B, Cdt + *C. sinensis*, 31.4 ±0.8 % of lesioned fibers, $p<0.05$). Note in B, a major cell density due to Cs extract protection.

DISCUSSION

In the present study, the efficacy of *Camellia sinensis* L. extract against the irreversible blockade of *Crotalus durissus terrificus* venom was investigated, using a consolidated myographic experimental model – the mouse phrenic nerve-diaphragm preparation. Plants are an interesting source of new compounds against many diseases, especially potential lethal accidents, such as snakebites. Access to intensive medical care or serum therapy depends on being close to modern medical resources where the accident occurs and the medicinal plants could be a viable alternative to treat snakebite envenomation (Soares et al, 2005).

C. sinensis is a well known herb being spread around the world. It has shown many pharmacological properties, even in its commercial form. Tea is one of the most popular

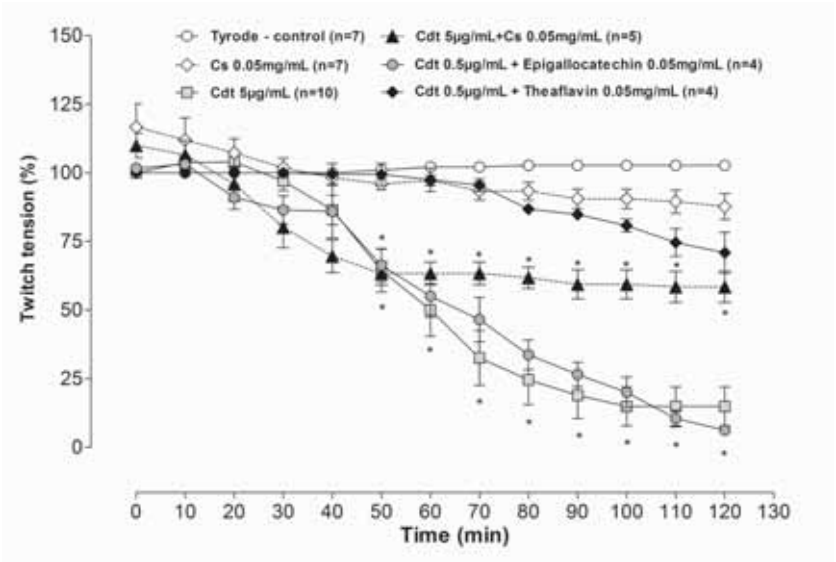

Figure 4. (A) Isolated mouse phrenic nerve-diaphragm preparations under indirect stimuli. Each point represents the mean ± SEM. * = $p<0.05$ in comparison with Cdt venom.

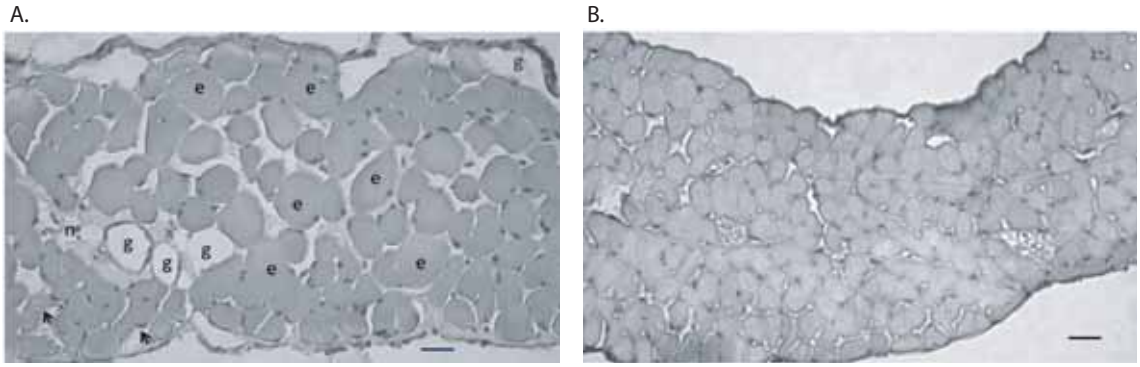

Figure 5. Diaphragm muscles (cross-sections of 2µm thick). **A.** Muscle incubated with 5µg/ml Cdt venom shows 59.5 ±1% of fibers exhibiting myonecrotic states characterized by edema (e), delta lesion (arrow), sarcolemmal disruption with nuclei (n) dispersion, and "ghost" cells (g) visualized by spaces optically empty. Area with extensive myonecrosis assumes a hyaline aspect. **B.** Muscle incubated with 5µg/ml Cdt venom plus hydroalcoholic extract shows fibers maintaining its characteristic polygonal profile, major quantity of cells due minor edema resulting in a slower percentage of damage (31.4 ±0.8 %) compared to venom alone ($p<0.05$). Bar = 50µm.

beverages in the world, perhaps second only to water (for example, see Chen et al, 2008) and a major source of dietary flavonoids (Hollman et al, 2010).

Crotoxin is the main toxic component of South American *C. d. terrificus* rattlesnake venom. Two different subunits (Hendon and Fraenkel-Conrat, 1971; Rubsamen et al, 1971) were identified in crotoxin: CA, crotapotin, which is non-toxic; and CB, a basic subunit, which is a phospholipase A_2 with high enzymatic activity. The abundance of PLA explains the invasive mode of the Cdt venom that disrupt the cell membrane integrity via phospholipids hydrolysis (Oliveira et al, 2009). Besides its neurotoxic action with digestive function, a wide range of pharmacological activities, such as myotoxic, edema-inducing, hypotensive, platelet-aggregating, cardiotoxic, and anticoagulant effects have been attributed to venom containing PLA (Oliveira et al, 2009).

The main toxicity of Cdt venom is attributed to crotoxin, since this substance is highly concentrated (more than 50%) in this venom (Faure et al, 1994). Crotoxin affects primarily presynaptic membrane of neuromuscular junctions by impairing neurotransmitter release (Hawgood and Bon, 1990).

In the present study, the neutralizing assays showed that the *C. sinensis* extract was unable to completely protect the preparations from the effects of the venom and a progressive neuromuscular blockade was observed, possibly induced by crotoxin. However, the total muscle paralysis was prevented by the *C. sinensis* extract. The exact mechanism of this blockade partial-reversion is unclear and demands further investigation. It is possible that the plant was also able to protect the muscle fibers against the well known myotoxic effect of *C. d. terrificus* venom (Oshima-Franco et al, 1999), since the most part of twitches was recovery after washing PND. A strong confirmation in behalf of the muscle protection was showed by quantitative morphological analysis.

The *C. sinensis* extract used in the present study showed chromatographic patterns indicating many substances as candidates. The main Cs compounds, which are used to measure the tea quality, are catechins (poliphenol group also including epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate) and theaflavins (Matsubara and Rodriguez-Amaya, 2006; Saito et al, 2006). Epigallocatechin gallate is one of the most abundant catechins (Matsubara and Rodriguez-Amaya, 2006), but the protective action

of *C. sinensis* against the *C. d. terrificus* venom paralysis could not be only attributed to this phytochemical, as demonstrated in this study. Thus, theaflavins present in Cs could be the main chemical component that is responsible for the neuromuscular protection against Cdt venom as demonstrated here. The findings of Das et al (1997) and Basu et al (2005), describing on the most notable pharmacological action of *C. sinensis* extract, could corroborate with the results of the present study. The facilitatory effect (increased twitches amplitude) observed could be attributed to theaflavin (Basu et al, 2005). The ability of Cs against botulinum (Satoh, 2005) and tetanus (Satoh et al, 2002) neurotoxins was already described. The present study showed another and new ability of *C. sinensis* against the neuromuscular blockade induced by *C. d. terrificus* venom. Further studies are necessary for clearing the mechanism of action by which this protection occurs.

CONCLUSIONS

Camellia sinensis extract possess inhibitory effect against the neuromuscular blockade induced by *Crotalus durissus terrificus* venom, by an unclear mechanism of action, by which theaflavins have a strong participation.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

Cdt: *Crotalus durissus terrificus*
C. d.: *terrificus* - *Crotalus durissus terrificus*
Cs: *Casearia sylvestris*
C. sylvestris: *Casearia sylvestris*
PLA_2: Phospholipase A_2
PND: Phrenic nerve-diaphragm
Rf: Retention factor
TLC: Thin layer chromatography

REFERENCES

Basu S, Chaudhuri T, Chauhan SPS, Das Gupta AK, Chaudhury L and Vedasiromoni JR. 2005. The theaflavin fraction is responsible for the facilitatory effect of black tea at the skeletal myoneural junction. Life Sci, 76, 3081-3088.

Boldrini-França J, Rodrigues RS, Fonseca FP et al. 2009. *Crotalus durissus collilineatus* venom gland transcriptome: analysis of gene expression profile. Biochimie, 91, 586-595.

Borges MH, Alves DL, Raslan DS et al. 2005. Neutralizing properties of *Musa paradisiaca* L. (Musaceae) juice on phospholipase A2, myotoxic, hemorrhagic and lethal activities of crotalidae venoms. J Ethnopharmacol, 98, 21-29.

Brazil, Ministério da Saúde. 2001. *Manual de diagnóstico e tratamento de acidentes por animais peçonhentos*, Fundação Nacional da Saúde, Brasília, Brazil, 2nd Edition.

Brazilian Pharmacopeia. 1988. Atheneu São Paulo, São Paulo, Brazil, 4th Edition.

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

Chen D, Milacic V, Chen MS et al. 2008. Tea polyphenols, their biological effects and potential molecular targets. Histol Histopathol, 23, 487-496.

Cintra-Francischinelli M, Silva MG, Andréo-Filho N et al. 2008. Antibothropic action of *Casearia sylvestris* Sw. (Flacourtiaceae) extracts. Phytother Res, 22, 784-790.

Clement Y. 2009. Can green tea do that? A literature review of the clinical evidence. Prev Med, 49, 83-87.

Das M, Vedasiromoni JR, Chauhan SP and Ganguly DK. 1997. Effect of green tea (*Camellia sinensis*) extract on the rat diaphragm. J Ethnopharmacol, 57, 197-201.

Faure G, Choumet V, Bouchier C et al. 1994. The origin of the diversity of crotoxin isoforms in the venom of *Crotalus durissus terrificus*. Eur J Biochem, 223, 161-164.

Grove KA and Lambert JD. 2010. Laboratory, epidemiological, and human intervention studies show that tea (*Camellia sinensis*) may be useful in the prevention of obesity. J Nutr, 140, 446-453.

Harborne B. 1998. *Phytochemical Methods: A Guide to Modern Techniques of Plants Analysis*, Chapman & Hall, London, England, 3rd Edition.

Hawgood B and Bon C. 1991. Handbook of natural toxins, reptile and amphibian venoms. In: Tu AT (Ed) *Reptile venoms and toxins*, Marcel Dekker, New York, 5th Edition, pp 3-52.

Hendon RA and Fraenkel-Conrat H. 1971. Biological roles of the two components of crotoxin. Proc Natl Acad Sci USA, 68, 1560-1563.

Hollman PC, Geelen A and Kromhout D. 2010. Dietary flavonol intake may lower stroke risk in men and women. J Nutr, 140, 600-604.

Kim HR, Rajaiah R, Wu Q-L et al. 2008. Green tea affords protection against autoimmune arthritis in rats by modulating the disease-related immune events. J Nutr, 138, 2111-2116.

Kurita I, Maeda-Yamamoto M, Tachibana H and Kamei M. 2010. Antihypertensive effect of Benifuuki tea containing O-methylated EGCG. J Agric Food Chem, 58, 1903-1908.

Matsubara S and Rodriguez-Amaya DB. 2006. Teores de catequinas e teaflavinas em chás comercializados no Brasil. Ciênc Tecnol Aliment, 26, 401-407.

MeSH, Medical Subject Headings. *Crotoxin*. Available in: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=mesh>, Pubmed, 12 June 2010.

Nirmal N, Praba GO and Velmurugan D. 2008. Modeling studies on phospholipase A2-inhibitor complexes. Indian J Biochem Biophys, 45, 256-262.

Oliveira JG, Soares SG, Soares AM, Giglio JR, Teixeira JE and Barbosa JE. 2009. Expression of human recombinant antibody fragments capable of partially inhibiting the phopholypase activity of *Crotalus durissus terrificus* venom. Basic Clin Pharmacol Toxicol, 105, 84-91.

Oshima-Franco Y, Hyslop S, Prado-Franceschi J, Cruz-Höfling MA and Rodrigues-Simioni L. 1999. Neutralizing capacity of antisera raised in horses and rabbits against *Crotalus durissus terrificus* (South American rattlesnake) venom and its major toxin, crotoxin. Toxicon, 37, 1341-1357.

Reicher F, Sierakowski MR and Corrêa JBC. 1981. Determinação espectrofotométrica de taninos pelo reativo fosfotungstico-fosfomolíbdico. Braz Arch Biol Technol, 24, 407-411.

Rigoni M, Paoli M, Milanesi E et al. 2008. Snake phospholipase A2 neurotoxins enter neurons, bind specifically to mitochondria, and open their transition pores. J Biol Chem, 283, 34013-34020.

Rubsamen K, Breithaupt H and Habermann E. 1971. Biochemistry and pharmacology of the crotoxin complex. I. Subfractionation and recombination of the crotoxin complex. Naunyn Schmiedebergs Arch Pharmakol, 270, 274-288.

Saito ST, Welzel A, Suyenaga ES and Bueno F. 2006. A method for fast determination of epigallocatechin gallate (EGCG), epicatechin (EC), catechin (C) and caffeine (CAF) in green tea using HPLC. Ciênc Tecnol Aliment, 26, 394-400.

Satoh E. 2005. Ethyl acetate extract from black tea prevents neuromuscular blockade by botulinum neurotoxin type A *in vitro*. Int J Food Sci Nutr, 56, 543-550.

Satoh E, Ishii T, Shimizu Y, Sawamura S and Nishimura M. 2002. The mechanism underlying the protective effect of the thearubigin fraction of black tea (*Camellia sinensis*) extract against the neuromuscular blocking action of botulinum neurotoxins. Pharmacol Toxicol, 90, 199-202.

Shah S, Stapleton PD and Taylor PW. 2008. The polyphenol (-)-epicatechin gallate disrupts the secretion of virulence-related proteins by *Staphylococcus aureus*. Lett Appl Microbiol, 46, 181-185.

Simões CMO, Schenkel EP, Gosmann G, Mello JCP, Mentz LA and Petrovick PR. 2004. Farmacognosia: da Planta ao Medicamento, UFRGS/UFSC, Porto Alegre/Florianópolis, Brazil, 5th Edition.

Soares AM, Ticli FK, Marcussi S et al. 2005. Medicinal plants with inhibitory properties against snake venoms. Curr Med Chem, 12, 2625-2641.

Ueda M, Nishiumi S, Nagayasu H, Fukuda I, Yoshida K and Ashida H. 2008. Epigallocatechin gallate promotes GLUT4 translocation in skeletal muscle. Biochem Biophys Res Commun, 377, 286-290.