RESEARCH REPORT

Cordia salicifolia and *Lafoensia pacari* plant extracts against the local effects of *Bothrops jararacussu* and *Philodryas olfersii* snake venoms

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ABSTRACT

Philodryas olfersii produces similar local effects to *Bothrops jararacussu* snakebite, which can induce misidentification and bothropic antivenom administration. Antivenom therapy is effective, but has its limitations regarding local damage. Since plants are used in folk medicine to treat snakebite victims, we evaluated the protective properties of *Cordia salicifolia* and *Lafoensia pacari* extracts against *Philodryas olfersii* and *Bothrops jararacussu* venoms. Preparations pretreated with both extracts inhibited > 90% the *B. jararacussu* venom-induced neuromuscular blockade, and 52% to 81% the *P. olfersii* venom-induced blockade. *C. salicifolia* inhibited the myonecrosis promoted by both venoms; however, *L. pacari* prevented only the myofilaments hypercontraction. Regarding haemorrhagic activity, *C. salicifolia* was more effective against *B. jararacussu* venom, while *L. pacari* was more effective against *P. olfersii* venom. On the other hand, for oedema-forming activity the results were the opposite. Considering that both extracts prevented (to different levels) the main manifestations of both snakebites (local symptoms), we endorse further studies involving these plants as coadjuvant in snakebite therapeutics.

KEYWORDS: Neuromuscular junction, myotoxicity, haemorrhage, oedema, protective effect, antiophidic

INTRODUCTION

Snakebite is a major public health issue and a neglected tropical disease. Brazil has the highest snakebite incidence within South America: 144,251 cases were reported between 2007 and 2015 (Mise et al, 2018), *Bothrops* genus being mainly responsible.

Despite different venom compositions, the symptomology following envenomation by *Bothrops jararacussu* is similar to other *Bothrops* sp. Local effects largely include pain, oedema, haemorrhage and myonecrosis. Severe local envenomation can induce permanent tissue loss. Rare systemic manifestations lead to acute renal failure, systemic haemorrhage and clotting disorders (Milani Júnior et al, 1997). *Philodryas olfersii*, even being opisthoglyphous, induces similar symptoms to those described for *Bothrops* sp. (Ribeiro et al, 1999).

Cordia salicifolia, widely used in Brazilian traditional medicine, has caffeine and tannin among its components (Viana et al, 2013), and has demonstrated several physiological

effects, such as cardiotonic activity (Matsunaga et al, 1997). *Lafoensia pacari*, also widely used in folk medicine, exhibits anti-inflammatory, sedative and antitumoral activities (Solon et al, 2000; Matos et al, 2008), among others. Considering the increasing importance of plants in treating local snakebite effects (as adopted by rural and tribal populations), which are not effectively treated by antivenom therapy, the lack of information regarding *C. salicifolia* and *L. pacari* antiophidic properties and the *B. jararacussu* and *P. olfersii* snakebite symptomatology, we evaluated protection by these plants against neuromuscular blockade, myotoxic, oedema-forming and haemorrhagic activities, induced by both snake venoms.

MATERIAL AND METHODS

Reagents, venoms and dried leaves

Halothane was purchased from Cristalia (Brazil) and other reagents from JT Baker Chemicals/Mallinckrodt (Mexico) and Merck (Brazil). Dr José Carlos Cogo provided the lyophilized pools of *B. jararacussu* and *P. olfersii* venoms. *C. salicifolia* dried powdered leaves were purchased from Vital Specialties Pharmaceutical Laboratory (lot 14.0010-1101; Brazil). Dr Cháriston André Dal-Belo donated the *L. pacari* dried extract. This work is registered at the National System for the Management of Genetic Resources and Associated Traditional Knowledge (no. AE3758A).

Preparation of plants extracts

Dried powdered leaves were macerated with ethanol (70°GL; room temperature) for seven days, being stirred for 5min/day. The suspensions were filtered and the precipitates were macerated again. This process was repeated 5 times. The resulting suspensions were evaporated under reduced pressure and stored (-4°C).

Animals

Male Swiss mice (18-25gm) and Wistar rats (200-300gm) were from Pontifical Catholic University of São Paulo (Brazil). Animal housing and protocols (approved by the Committee for Ethics in Animal Use, CER/UNIVAP, A025/CEP/2009) were according to ethical guidelines of the Brazilian Society for Laboratory Animal Science, National Council for Animal Experimentation and Federal Law No. 11.794 (October 08, 2008).

Myographical assays

Mice were euthanized (overdose of halothane followed by exsanguination). Hemidiaphragms and corresponding phrenic nerves were removed and mounted (5g-tension) in a 5ml organ baths containing Tyrode solution [(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] at 37°C, gassed with 95% (v/v) O_2 and 5% (v/v) CO_2. The muscles were indirectly stimulated (0.1Hz, 0.2ms, supramaximal voltage; Grass S48 stimulator) and their contractions were recorded (Gould RS 3200 physiograph). The preparations were allowed to stabilize (20min) before the following treatments: i) Tyrode solution alone (control); ii) venoms [*B. jararacussu* (100µg/ml) or *P. olfersii* (50µg/ml)]; iii) extracts [*C. salicifolia* (750µg/ml) or *L. pacari* (400µg/ml)]; iv) 30min-preincubation with each extract (concentration as in (iii) before each venom addition (as in ii).

Haemorrhagic activity

Rats were anesthetized (sodium thiopental, 30mg/kg, i.p.) and their dorsal region was shaved. The haemorrhagic activity was measured in response to intradermal injection (100µl) of Tyrode solution (control), and *B. jararacussu* (100µg) or *P. olfersii* (50µg) venoms alone or after preincubation (30min) with *C. salicifolia* (750 or 1500µg) or *L. pacari* (400 or 800µg). After 24hr, the rats were euthanized (overdose of halothane followed by cervical dislocation), the dorsal skins were removed and the hemorrhagic halos on the inner surface were measured.

Oedema-forming activity

Rats were anesthetized (sodium thiopental, 30mg/kg, i.p.), the dorsal region was shaved, and 2.5% Evan's blue dye (300µl) was injected via penile vein. The plasma protein extravasation was measured in response to intradermal injection (100µl) of Tyrode solution (control), and *B. jararacussu* (10µg) or *P. olfersii* (10µg) venoms alone or after preincubation (30min) with *C. salicifolia* (75 or 150µg with *B. jararacussu*; 150 or 300µg with *P. olfersii*) or *L. pacari* (40 or 80µg with *B. jararacussu*; 80 or 160µg with *P. olfersii*). After 30min, the rats were euthanized (overdose of halothane followed by cervical dislocation), the dorsal skins were removed and the halos measured.

Histological analysis

Diaphragm muscles were maintained in 4% (v/v) paraformaldehyde solution (24hr), washed in PBS (3x), dehydrated in increasing ethanol concentrations (70%, 90%, 100% 3x) and clarified in xylol for paraffin inclusion. Sections (5µm thick) were obtained using a Leica RM 2035 microtome (Germany). The slides [deparaffinized in xylol, hydrated in descending series of ethanol (100%, 90%, 80%, 70% (v/v)) and water, stained with Hematoxylin (5min) and Eosin (3min), dehydrated in xylol in increasing series of ethanol] were mounted with Permount for analysis (Eclipse E200 - Nikon microscopy) and images capture (Image Pro Plus 6.0; USA). Myonecrosis was characterized by hypercontracted, vacuolated, oedematous and ghost fibers. Normal fibres were considered those with both polygonal profile and myofilaments homogeneously distributed.

Statistical analysis

Results were expressed as mean ± SEM. Statistical comparisons were done using analysis of variance (ANOVA) followed by the Tukey-Kramer test with $p<0.05$ indicating significance. All data analyses were done using the software Origin (USA).

RESULTS AND DISCUSSION

Several studies involving the use of plants extracts against *B. jararacussu* venom hemorrhagic and oedema-forming activities have been reported previously; however, this is the first study involving the use of plant extracts for the protection of hemorrhagic and oedematogenic activities induced by *Philodryas* sp. venom.

Plants are composed by a complex mixture of molecules able to interact with snake venom proteins. Tannins are among the main components of *C. salicifolia* and *L. pacari* extracts (Viana et al, 2013; Solon et al, 2000) and are

known for their anti-inflammatory effects and for precipitating proteins and metal – an important mechanisms which can chelate venom toxins and reduce their local effects (Ambreen and Mirza, 2020). Since several studies have demonstrated the antiophidic potential of condensed and hydrolysable tannins (de Moura et al, 2016), we suggest that tannins are the potential responsible for *C. salicifolia* and *L. pacari* protection against the local effects of *B. jararacussu* and *P. olfersii* venoms observed in this study.

Antivenom therapy, the main treatment for snakebites, is effective for systemic symptoms, but not for local manifestations: the patient's death can be prevented, but the affected limb may face deformities or even amputation. Several therapeutic options, complementary to antivenom therapy, are being investigated, including the plants used in traditional medicine as antiophidics (Gutiérrez, 2002). In this work, we evaluated *C. salicifolia* and *L. pacari* protection against neuromuscular blockade, myotoxic, oedema-forming and haemorrhagic activities, induced by *B. jararacussu* and *P. olfersii* snake venoms.

The neuromuscular activity and myotoxicity were accessed in mouse phrenic nerve-diaphragm preparations (PND). The adopted extract concentrations, 750μg/ml of *C. salicifolia* and 400μg/ml of *L. pacari*, were the highest concentrations unable to produce neuromuscular blockade; *C. salicifolia* promoted concentration-dependent contractility facilitation (data not shown), which can be attributed to the extract's caffeine content (Viana et al, 2013), since it promotes endoplasmic reticulum Ca^{2+} mobilization, increasing twitch-tensions amplitudes *in vitro* (Tarnopolsky, 2008). The venom concentrations used in this work (100μg/ml of *B. jararacussu* and 50μg/ml of *P. olfersii*) were based on previous studies and our preliminary experiments (Collaço et al, 2012; Melaré et al, 2016).

B. jararacussu venom caused total irreversible neuromuscular blockade, attributed to its major myotoxin, the $Lys49\text{-}PLA_2$ Bothropstoxin-I (Homsi-Brandeburgo et al, 1988; Ferraz et al, 2014). On the other hand, *P. olfersii* venom caused only partial irreversible blockade, also probably due to postsynaptic mechanism as myotoxic activity (Prado-Franceschi et al, 1996; 1998; Collaço et al, 2012); however, the responsible constituents remain largely unknown (Figure 1). The PND pre-treatment with *C. salicifolia* or *L. pacari* extract inhibited >90% neuromuscular blockade induced by *B. jararacussu* venom (Figure 1A), which could be due to a Bothropstoxin-I inhibition, similar to those produced by *Vochysia haenkeana* (Harder et al, 2017). Pre-treatments with *C. salicifolia* and *L. pacari* extracts reduced neuromuscular blockade by *P. olfersii* venom by 52% and 81%, respectively (Figure 1B). Since *C. salicifolia* provides better protection against myotoxicity (as discussed later) and *L. pacari* a better protection against the neuromuscular blockade, we suggest that the blockade induced by *P. olfersii* venom is mainly, but not exclusively, myotoxins-related.

Diaphragm muscles treated with Tyrode solution alone or with both extracts presented normal fibres with polygonal profile, with myofibrils well distributed (Figures 2A, D, G).
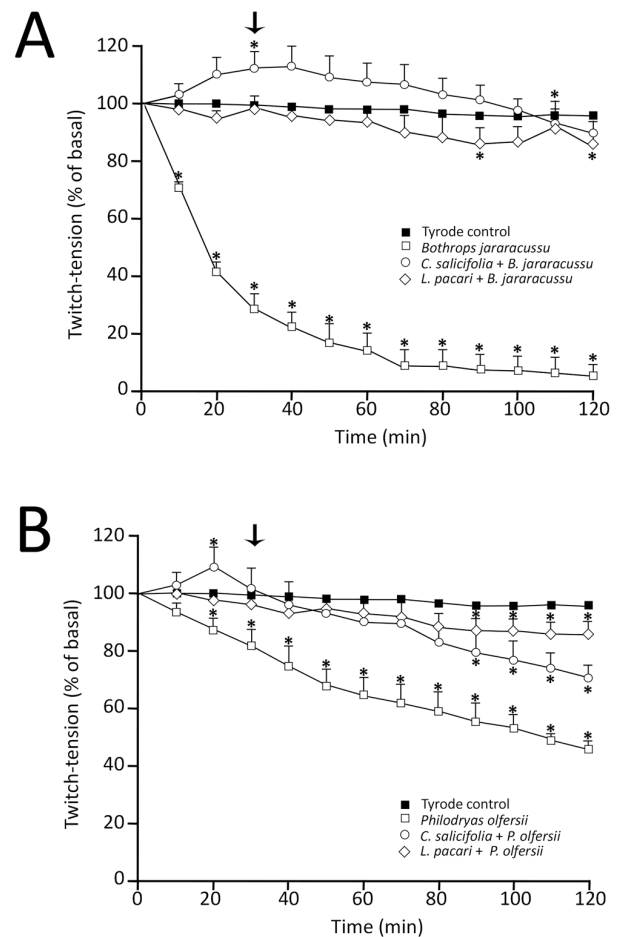

Figure 1. Nerve-evoked twitch-tension responses of mouse phrenic nerve-diaphragm preparations (PND). The PND were electrically stimulated (0.1Hz, 0.2ms, supramaximal voltage) and incubated with Tyrode solution in the absence (control) or in the presence of *B. jararacussu* (100μg/ml; A) or *P. olfersii* (50μg/ml; B) venoms at 37oC. Preparations were also pre-treated with *C. salicifolia* (750μg/ml) or *L. pacari* (400μg/ml) for 30min (0-30min of experiment) before the venom addition (↓). Each point is the mean ±SEM of 3-6 experiments, as follows: Tyrode control n=6, *B. jararacussu* and *P. olfersii* venoms, *C. salicifolia* + *B. jararacussu* venom and *L. pacari* + *P. olfersii* venom n=5; *L. pacari* + *B. jararacussu* venom and *C. salicifolia* + *P. olfersii* venom n=3. *p<0.05 compared to Tyrode control.

Myonecrosis (Figures 2B-C) was found in all muscles treated with *B. jararacussu* and *P. olfersii* venoms. Hypercontracted, oedematous and ghost fibres were observed among normal fibres. The pretreatment with *C. salicifolia* extract inhibited myonecrosis both induced by both venoms (Figures 2E-F) while *L. pacari* pretreatment prevented only myofilaments hypercontraction; neither hypercontracted nor ghost fibers were observed. However, some oedematous fibres were still present (Figures 2H-I).

P. olfersii venom-induced myonecrosis is a common finding *in vitro* (Prado-Franceschi et al, 1996; 1998; Collaço et al, 2012) and *in vivo* (Acosta et al, 2003; Oliveira et al, 2017), but unlike *B. jararacussu,* this venom lacks PLA_2 (Assakura et al, 1992; Rocha et al, 2006; Oliveira et al, 2017), suggesting that such effect is related to other classes of substances. A
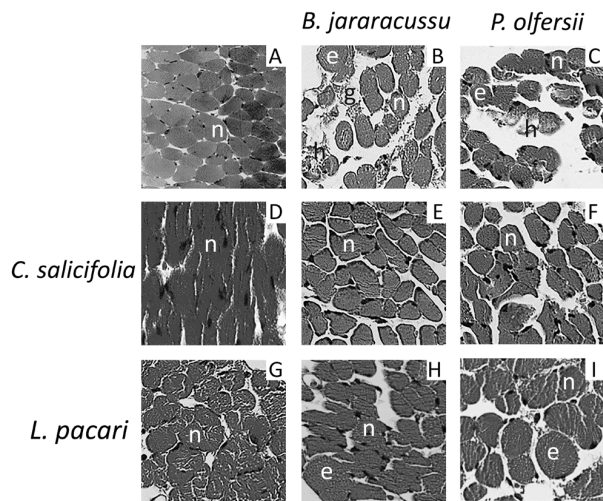
Figure 2. Histological analysis of diaphragm muscle. The tissues were incubated with: **A)** Tyrode; **B)** *B. jararacussu* venom (100µg/ml); **C)** *P. olfersii* venom (50µg/ml); **D)** *C. salicifolia* extract (750µg/ml); **(E)** *C. salicifolia* extract (750µg/ml) + *B. jararacussu* venom (100µg/ml); **(F)** *C. salicifolia* extract (750µg/ml) + *P. olfersii* venom (50µg/ml); **(G)** *L. pacari* extract (400µg/ml); **(H)** *L. pacari* (400µg/ml) + *B. jararacussu* venom (100µg/ml); **(I)** *L. pacari* extract (400µg/ml) + *P. olfersii* venom (50µg/ml). The myonecrosis is characterized by oedematous (e), hypercontracted (h) and ghost fibers (g). Note some normal (n) fibers. Bar: 50µm.

myotoxin (~20 kDa, 182 amino acid residues; probably too large for a PLA_2), which accounts for ~25% of the venom's protein content, has been isolated (Prado-Franceschi et al; 1998). Also, snake venom metalloproteinases, which play an important role in *Philodryas* sp. snakebite (Assakura et al, 1994; Acosta et al, 2003; Rocha et al, 2006), could be responsible for myotoxicity as reported for *Bothrops* and other colubrids snakes (Mamede et al, 2016; Torres-Bonilla et al, 2016). In addition, a CRiSP from *P. patagoniensis* venom causes myonecrosis, but not edema or hemorrhage (Peichoto et al, 2009). Considering that the neuromuscular blockade promoted by these two venoms is mainly related to myotoxicity/postsynaptic activity, we believe that the extracts are, somehow, protecting the preparations from the muscle damage and consequently neuromuscular blockade.

P. olfersii venom (50µg) presented higher haemorrhagic activity (1.15 ± 0.20cm halo) than *B. jararacussu* venom (100µg; 0.85 ± 0.15cm halo), corroborating with studies in mice (Assakura et al, 1992; Alves et al, 2018). Such effect is attributed to venom-content metalloproteinases, which degrade endothelial membrane proteins leading to microvessel lesions (Gutiérrez et al, 2016). The pre-incubation of *C. salicifolia* (750 and 1500µg) with *B. jararacussu* venom reduced the venom-induced haemorrhagic activity by 60.0% and 93.0%, respectively, while for *L. pacari* pre-incubation (400 and 800µg) the reduction was 88.2% and 76.5%, respectively (Figure 3A). In turn, the pre-incubation of *P. olfersii* venom with *C. salicifolia* extract (750 and 1500µg) prevented the venom-induced hemorrhage by 52.2% and 69.6%, respectively, while *L. pacari* preincubation (400 and 800µg) lead to 43.5% and 91.3% reduction, respectively (Figure 3B). These results suggest that the extracts interact with metalloproteinases mechanism of action.
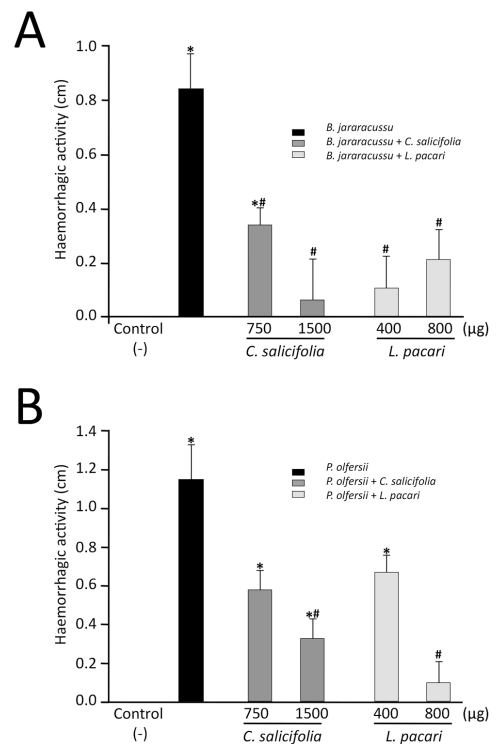

Figure 3. Haemorrhagic activity assessed in rat dorsal skin. The Tyrode solution (negative control), and *B. jararacussu* (100µg; **A**) or *P. olfersii* (50µg; **B**) venoms alone or pre-incubated with different concentrations of *C. salicifolia* and *L. pacari* were intradermally injected into rat dorsal skin 24hr before the hemorrhagic halo measurement. Each column represents the median ±EPM of 4 (**A**) or 3 (**B**) experiments. *p<0.05 compared to negative control; #p<0.05 compared to venom alone.

Since vascular damage induces plasma extravasation in a different mechanism to oedema formation, the chosen concentration for oedema-forming experiments (10µg) was based in preliminary experiments which assessed the highest venom concentration produced oedema without presence of haemorrhage (data not shown). After 30min injection, the oedematogenic halo produced by *B. jararacussu* and *P. olfersii* venoms measured 2.40 ± 0.1cm and 1.80 ± 0.1cm, respectively. *B. jararacussu* venom is known to produce local early oedema by increasing COX-2 expression, IL-1b production, and neutrophil chemotaxis mainly by PLA_2 (Wanderley et al, 2014). On the other hand, *P. olfersii* venom oedematogenic activity may be related to other components since PLA_2 are absent (Oliveira et al, 2017).

B. jararacussu venom edema-forming activity was partially inhibited by *C. salicifolia* extract pre-incubation (reduction of 37.5% and 41.7% to 75 and 150µg of extract) and more effectively by *L. pacari* preincubation (40 and 80µg), which reduced the halo by 68.8% and 54.2%, respectively (Figure 4A). In turn, the *P. olfersii* venom preincubation with *C. salicifolia* extract (150 and 300µg) reduced the halo by 66.7% and 77.8% respectively, while *L. pacari* extract (80 and 160µg) demonstrated to be slightly less effective by inhibiting this activity by 58.3% and 65.5%, respectively (Figure 4B). The extracts may have reduced the venom-induced edema by venom components inhibition or by an anti-inflammatory activity.
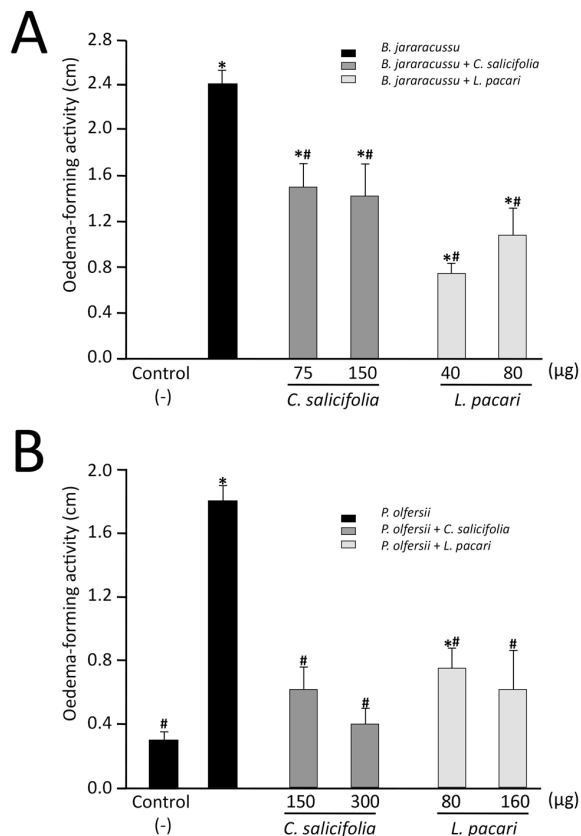
Figure 4. Oedema-forming activity assessed in rat dorsal skin. The Tyrode solution (negative control), and *B. jararacussu* (10µg; **A**) or *P. olfersii* 10µg; **B**) venoms alone or pre-incubated with different concentrations of *C. salicifolia* and *L. pacari* were intradermally injected into rat dorsal skin 30hr before the halo formed by the extravasation of Evan's blue stained plasma be measured. Each column represents the median ±EPM of 3 experiments. *p<0.05 compared to negative control; #p<0.05 compared to venom alone.

CONCLUSION

C. salicifolia and *L. pacari* extracts can, to different levels, ameliorate all tested activities induced by *B. jararacussu* and *P. olfersii* venoms. Considering that local effects, such as myotoxicity, haemorrhage and oedema, are the main symptoms of both snakebites, and that antivenom therapy is not effective in treating local effects, we suggest that both extracts may have some therapeutic potential for treating the local damages that follows *Bothrops* and *Philodryas* sp. snakebites, and endorse further studies involving these plants as coadjuvant in snakebite treatment.

ACKNOWLEDGEMENTS

We thank Gildo B Leite, Dr Mário LR Cesaretti and Mariana P Góes for technical assistance and Luiz PM Filho for English revision. This work was a part of PRM's postdoctoral work.

COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

B. jararacussu: *Bothrops jararacussu*
C. salicifolia: *Cordia salicifolia*
CRiSP: Cysteine-rich secretory protein
L. pacari: *Lafoensia pacari*
P. olfersii: *Philodryas olfersii*
PND: Phrenic nerve-diaphragm preparation

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