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Chemical and functional analyses of *Rhinella icterica* (Spix, 1824) toad secretion screened on contractions of the heart and oviduct in *Locusta migratoria*

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ABSTRACT

Rhinella icterica is a Brazilian toad with a parotoid secretion that is toxic to insects. In this work, we examined the entomotoxicity of this secretion in locust (*Locusta migratoria*) semi-isolated heart and oviduct preparations *in vitro*. The parotoid secretion caused negative chronotropism in semi-isolated heart preparations (at the highest dose tested: 500 μg) and markedly enhanced the amplitude of spontaneous contractions and tonus of oviduct muscle (0.001–100 μg). In addition, the secretion enhanced neurally-evoked contractions of oviduct muscle, which was more sensitive to low concentrations of secretion than the semi-isolated heart. The highest dose of secretion (100 μg) caused neuromuscular blockade. In zero calcium-high magnesium saline, the secretion still enhanced muscle tonus, suggesting the release of intracellular calcium to stimulate contraction. Reverse-phase HPLC of the secretion yielded eight fractions, of which only fractions 4 and 5 affected oviduct muscle tonus and neurally-evoked contractions. No phospholipase A_2 activity was detected in the secretion or its chromatographic fractions. The analysis of fractions 4 and 5 by LC-DAD-MS/MS revealed the following chemical compounds: suberoyl arginine, hellebrigenin, hellebrigenin 3-suberoyl arginine ester, marinobufagin 3-pimeloyl arginine ester, telocinobufagin 3-suberoyl arginine ester, marinobufagin 3-suberoyl arginine ester, bufalin 3-adipoyl arginine, marinobufagin, bufotalinin, and bufalitoxin. These findings indicate that *R. icterica* parotoid secretion is active in both of the preparations examined, with the activity in oviduct possibly being mediated by bufadienolides.

1. Introduction

Natural toxins are valuable sources of biologically-active compounds, many of which have potential biotechnological applications. Poisonous animals, including toads, produce or acquire through their diet, compounds that are used for defense against predators and microorganisms (Cunha Filho et al., 2005; Saporito et al., 2012; Sciani et al., 2013; Heus et al., 2014).

Toads of the family Bufonidae (~612 species) have a worldwide distribution (except in polar and arid regions) and are characterized by

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having dry, leathery skin, short legs, and large warts covering the parotoid glands (Escoriza and Hassine, 2019; Frost, 2019). Toad cutaneous glands are widely distributed on the body surface and are classified as mucous and granular types (Toledo and Jared, 1995; Mendes et al., 2016; Qi et al., 2018). The mucus produced by mucous glands is involved in physiological functions such as cutaneous respiration, thermoregulation, reproduction, and defense against pathogens (Toledo and Jared, 1995). The toad granular glands secrete toxins, mainly steroid derivatives (including cholesterol, ergosterol, bufotoxins and bufadienolides), biogenic amines (epinephrine, norepinephrine, 5-hydroxytryptamine or serotonin and *N*-dimethyl-5-hydroxytryptamine or bufotenine), alkaloids (dehydrobufotenine, bufoviridine), proteins and peptides (baserpin, buforin I, buforin II) (Daly et al., 2005; Tempone et al., 2008; Cunha-Filho et al., 2010; Sciani et al., 2013; Siano et al., 2014; Rodríguez et al., 2017).

The neurotoxins in toad secretions exert a range of pharmacological activities, including calcium channel blockade, vaso- and bronchodilation, antitumor and antidiabetic actions and hepatoprotection, in addition to functioning as opioid peptides and neuropeptides (Kamboj et al., 2013; Baldo et al., 2017; Kowalski et al., 2018). The bufotenines and dehydrobufotenins cause hallucinogenic effects in mammals through a direct action on the central nervous system and increase neurotransmitter release at neuromuscular junctions (Rostelato-Ferreira et al., 2011; Kryukova et al., 2017). Toad parotoid secretions also cause positive inotropic and negative chronotropic responses in vertebrates mediated by bufadienolides and bufotoxins that inhibit Na^+/K^+-ATPase activity (Rohrer et al., 1982; Radford et al., 1986; Kwan et al., 1992; Chen et al., 2006; Cunha-Filho et al., 2010).

Rhinella icterica (Spix, 1824), the "Yellow Cururu Toad", is a South American anuran with a wide geographic distribution that includes central, southeastern and southern Brazil, northeastern Argentina and eastern Paraguay (Silvano et al., 2010; Frost, 2019). *Rhinella icterica* occurs in a large variety of habitats, from forests to open areas, and has been the subject of several ecological studies (Colombo et al., 2008; Forti, 2009; Pinhão et al., 2009; dos Santos et al., 2013; Moretti et al., 2018; Titon et al., 2018; Assis et al., 2019). The parotoid secretion of *Rhinella* spp. has been screened for antiproliferative, antiparasitic, antiviral, antimicrobial and insecticidal activities (Tempone et al., 2008; Pinto et al., 2009; Kamboj et al., 2013; Baldo et al., 2017; Rodríguez et al., 2017; Leal et al., 2018). Although bufadienolides, a class of C-24 steroids, have been identified in *R. icterica* parotoid secretion (RIPS), little is known of their chemical and pharmacological profiles (Sciani et al., 2013; Mailho-Fontana et al., 2018; Oliveira et al., 2018, 2020). We recently showed that the toxicity of RIPS on the cockroach nervous system is mediated mainly by the direct modulation of octopaminergic neurotransmission (Leal et al., 2018); the chemical constituents responsible for this activity were not identified at the time. In this work, we screen RIPS and its chromatographic fractions for activity on locust semi-isolated heart and oviduct muscle. We also used liquid chromatography coupled to mass spectrometry (LC-MS) to examine the composition of two active fractions (F4 and F5).

2. Material and methods

2.1. Reagents and poison

All chemicals and reagents used were of the highest purity and were obtained from Sigma-Aldrich, Merck or BioRad. The secretion was collected by manual compression of the large post-orbital parotoid glands of adult male and female toads captured at Derrubadas in the southern Brazilian state of Rio Grande do Sul under authorization provided by the System of Authorization and Information on Biodiversity (SISBIO; permit number no. SISBIO 24867-2). The extraction of toads' parotoid secretions were accomplished right after the animals were collected. After collection, the yellowish, viscous secretion was weighed (in liquid form), on a Shimadzu (Kyoto, Japan) high precision analytical balance. In all protocols of this work, a pool of the extract obtained from 18 animals was used. According to Rostelato-Ferreira et al. (2011), two grams of RIPS were pretreated with 50 mL of methanol for three days at room temperature and lyophilized (Liobras, Liotop K105, São Paulo, Brazil) resulting in ~ 100 mg of a powdered compound. The desired amount of extract was dispersed in ultrapure water (18.2 MΩ, Milli-Q water, Millipore, USA) before all biological assays. For LC–MS analysis, HPLC grade acetonitrile and formic acid (both from J.T. Baker) were used, as well as ultrapure water.

2.2. Experimental animals

The experiments were done using mated female *L. migratoria*. The insects were reared under crowded conditions on a 12 h light/dark cycle at 30 °C and were fed fresh wheat seedlings and bran daily (Lange et al., 1986).

2.3. RP-HPLC analysis of R. icterica secretion

The parotoid secretion was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a 20A Prominence chromatograph (Shimadzu Co., Japan). Aliquots (100 µL) of RIPS were loaded onto a C18 column (Luna 10 µm New Column C18, 100 Å, 250 mm × 10 mm) in a two-solvent system: solvent A – ultrapure H_2O and solvent B – acetonitrile (ACN). The column was eluted at a constant flow rate of 1 mL/min with a 0 to 100% gradient of solvent B (0 min = 0%, 10 min = 80%, 15 min = 100%, 15.01 min = 0%, 20 min = stop) over 20 min. The elution profile was monitored with a Shimadzu SPD-20A/20AV detector at 254 nm and 280 nm simultaneously. The fractions were collected with a model MRC-10A fraction collector (Shimadzu Co., Japan), lyophilized (Liobras, Liotop K105, São Paulo, Brazil) and screened for biological activities, as described below. Each subfraction was dissolved in Locust saline before aplication in biological assays.

2.4. Identification of fraction constituents by LC-DAD-MS

RIPS was analyzed by ultra-fast liquid chromatography (Prominence UFLC; Shimadzu) coupled to a diode array detector (DAD) and mass spectrometer (MicroTOF-Q III, Bruker Daltonics, Billerica, MA, USA) with electrospray ionization. A Kinetex C18 column (2.6 µm, 150 × 2.1 mm, Phenomenex) was used for the analyses, and the following conditions was applied: a flow rate of 0.3 mL/min, oven temperature of 50 °C, injection volume of 2 µL and a mobile phase composed of ultrapure water (solvent A) and acetonitrile (solvent B), both containing 0.1% formic acid (v/v). The gradient elution profile was as follows: 0–2 min: 3% B, 2–25 min: 3–25% B, 25–40 min: 25–80% B and 40–43 min: 80% B. The analyses were acquired in positive ion mode (m/z 120–3000) using nitrogen as the nebulizer gas (4 bar), collision gas and dry gas (9 L/min). All samples were prepared at a concentration of 1 mg/mL and filtered before injection (0.22 µm PTFE filters, Millex®).

Compounds were identified by using a combination of UV and mass spectrometric data (accurate mass and ion fragmentation pathway) and comparing this with information reported in the literature. The molecular formula of each compound was determined based on the mass errors to within ± 5 ppm and mSigma < 20.

2.5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Samples (12 µg) of RIPS and RP-HPLC fractions diluted in sample buffer (0.01 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol) were loaded onto a BioRad Tris-Tricine Mini-protean TGX (4–20%) precast gel that was run at a constant voltage (100 V). After electrophoresis, the gels were stained with silver (BioRad silver stain kit) and documented.

2.6. Locust semi-isolated heart preparation

The cardiac activity of RIPS was assessed on semi-isolated heart preparations of *L. migratoria*. For this, a lateral incision was made along the thorax and abdomen of adult locusts of either sex to expose the dorsal vessel and heart. The dorsal cuticle was affixed to a Sylgard-coated dissection plate using micro-pins and the ventral cuticle (along with the digestive and reproductive systems) was removed and the cavity then filled with 500 μL of locust saline (composition, in mM: 150 NaCl, 10 KCl, 4 $CaCl_2$, 2 $MgCl_2$, 5 $NaHCO_3$, 90 sucrose, 5 trehalose and 5 Hepes, pH 7.2). The frequency of dorsal vessel contractions was recorded using an impedance converter (UFI model 2991, Morro Bay, CA, USA) equipped with electrodes and attached to a PicoScope oscilloscope (model 2202, Pico Technology Ltd., UK). The electrodes were inserted between the fifth and sixth abdominal segment on each side of the dorsal vessel. The preparations were stabilized for 10 min prior to starting each experiment. All treatments were added to the bath in a final volume of 500 μL and when changes in saline were required, the preparations were washed with locust saline. Heart rate was recorded for 10 min in the presence of RIPS (5 pg to 500 μg/500 μL of saline) or physiological saline alone and frequency determined from traces recorded using PicoLog software (Pico Technology Ltd., UK). Data were presented as a percentage of the control saline, before the aplication of treatements.

2.7. Locust oviduct preparation

The ability of RIPS to influence insect visceral muscle contraction was assessed on the locust oviduct. The common and lateral oviducts of adult female *L. migratoria* were dissected in locust saline. The lateral oviducts were fixed to a Sylgard coated plate with micropins and the common oviduct was attached to a Grass FT03 force transducer (Grass Medical Instrument Co., Quincy, MA, USA) via a silk thread to allow the recording of basal tension and the amplitude and frequency of oviduct muscle spontaneous contractions. Muscle contractile force was recorded using a PicoScope oscilloscope (model 2202) and Picolog software (Pico Technology Ltd.). Neurally-evoked contractions of the oviducts were obtained by electrically-stimulating the oviducal nerves via suction electrodes, as previously described (Noronha and Lange, 1997). All treatments were added in a final volume of 200 μL. Changes to the solution were achieved by washing the preparations with locust saline. The effects of modified saline on neurally-evoked contractions in the presence of RIPS were also examined. Calcium free, high-magnesium saline was prepared by removing $CaCl_2$ from the saline and increasing the concentration of $MgCl_2$ from 2 mM to 20 mM. The contractions in response to RIPS (1 pg to 100 μg/200 μL) were analyzed 100 s and 600 s after the addition of secretion.

2.8. Statistical analysis

The results are expressed as the mean ± S.E.M. Each experiment was repeated at least three times. Student's *t*-test for unpaired data was used for simple comparisons involving only two groups. When data from more than two experimental groups were analyzed, one-way ANOVA was used followed by Dunnett's test (the groups were compared with a positive control or saline) or two-way ANOVA followed by the Tukey multiple comparisons test, with $p < 0.05$ indicating significance. All statistical analyses were done using GraphPad Prism v.7.0 (Software Inc., San Diego, CA, USA).

3. Results

3.1. RP-HPLC of RIPS and identification of constituents by LC-DAD-MS

RP-HPLC of RIPS on a C18 column resulted in eight fractions (F1-F8). Electrophoretic analysis showed that RIPS and the RP-HPLC fractions contained proteins with molecular masses ranging from ~10 kDa to 250 kDa, with the main differences being in the region of 37–50 kDa and 75–100 kDa (Fig. 1). Fractions F1 and F2 showed a strongly staining band at 10–15 kDa, while the other fractions contained very faint bands at ~50 kDa (Fig. 1).

Screening of the fractions using locust oviduct preparations showed that only fractions F4 and F5 were more active than RIPS in their ability to increase the muscle tonus and amplitude of spontaneous contractions, respectively (see later). LC-DAD-MS/MS analysis of F4 and F5 identified 28 compounds (Fig. 2, Table 1). The compounds were identified by using a combination of UV, MS and MS/MS spectral data that were then compared with data reported in the literature (Schmeda-Hirschmann et al., 2017; Petroselli et al., 2018).

Compound **1** was putatively identified as suberoyl arginine (*m/z* 331). Compounds **3**, **6**–**10**, **12**–**13**, **16**–**21**, **24** and **27** showed fragment ions at *m/z* 331 that were compatible with $C_{14}H_{27}N_4O_5^+$ and the presence of the substituent suberoyl arginine; thus, compounds **3, 6–8, 10, 16–17, 20–21, 23–28** showed the substituent suberoyl argininyl, but bufadienolide aglycones could not be identified. In addition, 3-(*N*-suberoylargininyl) hellebrigenin (**9**, *m/z* 729.4079 $[M + H]^+$, $C_{38}H_{56}N_4O_{10}$), 3-(*N*-suberoyl argininyl) telocinobufagin (**12**, *m/z* 715.4252 $[M + H]^+$, $C_{38}H_{58}N_4O_9$), 3-(*N*-suberoylargininyl) marinobufagin(**13**, *m/z* 713.4120 $[M + H]^+$, $C_{38}H_{56}N_4O_9$), 3-(*N*-adipoylargininyl) bufalin (**14**, *m/z* 671.4028 $[M + H]^+$, $C_{36}H_{54}N_4O_8$) and 3-(*N*-suberoylargininyl) bufalin (bufalitoxin) (**18**–**19**, *m/z* 699.4343/699.4338 $[M + H]^+$, $C_{38}H_{58}N_4O_8$) were also identified. For compound **11**, the fragment ion at *m/z* 317 suggested the substituent pimeloyl argininyl and was putatively identified as 3-(*N*-pimeloyl argininyl) marinobufagin. Bufadienolide aglycones were also detected, including hellebrigenin (**2**) and its isomers (**4** and **5**), marinobufagin (**15**) and bufotalinin (**22**).

3.2. Toxicity of RIPS in locust semi-isolated heart

The cardiotoxicity of RIPS (5 pg/500 μL to 500 μg/500 μL) was examined using locust semi-isolated heart preparations. Only the highest concentration tested (500 μg/500 μL) caused a decrease in heartbeat rate: an initial reduction of 21 ± 6% ($p < 0.05$, n = 6) 100 s (~1.7 min) after application, followed by a transient recovery and then a progressive decrease throughout the remainder of the experiment (Fig. 3). After a 10 min incubation, this same high concentration of RIPS reduced the heart rate by 47 ± 7% compared to the saline control ($p < 0.0001$, n = 6; Fig. 3A, B). This decrease in heart rate was reversible by washing. With an intermediate concentration (0.5 ng/500 μL), there was a transient increase (23 ± 8%) in heartbeat rate in the first 100 s ($p < 0.05$, n = 6, Fig. 3A). The other concentrations of RIPS did not alter the heart rate relative to the saline control (n = 6, Fig. 3A).

3.3. Effect of RIPS on L. migratoria oviduct muscle

RIPS (1 pg to 100 μg/200 μL) increased the amplitude of spontaneous contractions and tonus of *L. migratoria* oviduct muscle (Fig. 4), with the greatest increases in muscle contraction amplitude being caused by the two highest doses tested (1 μg and 100 μg). The highest dose (100 μg) increased the amplitude by 36 ± 14% ($p < 0.05$, n = 6, Fig. 4C) and the basal muscle tonus by 71 ± 11 mg ($p < 0.05$, n = 6, Fig. 4B).

The effect of RIPS on neurally-evoked contractions in locust oviduct preparations was also examined. In these experiments, low doses of RIPS enhanced neurally-evoked contractions, but the highest dose of RIPS (100 μg) caused neuromuscular blockade that was maximal after 10 min (85 ± 3% blockade, n = 6, $p < 0.0001$, Fig. 5A). This amount of RIPS also caused a maximal increase in the muscle basal tonus (Fig. 5B). In contrast to semi-isolated heart preparations, low doses of RIPS also enhanced the basal tonus in neurally-evoked preparations (Fig. 5B), indicating that the latter preparations were more sensitive to the action of RIPS.

The eight RIPS fractions obtained by RP-HPLC were screened for

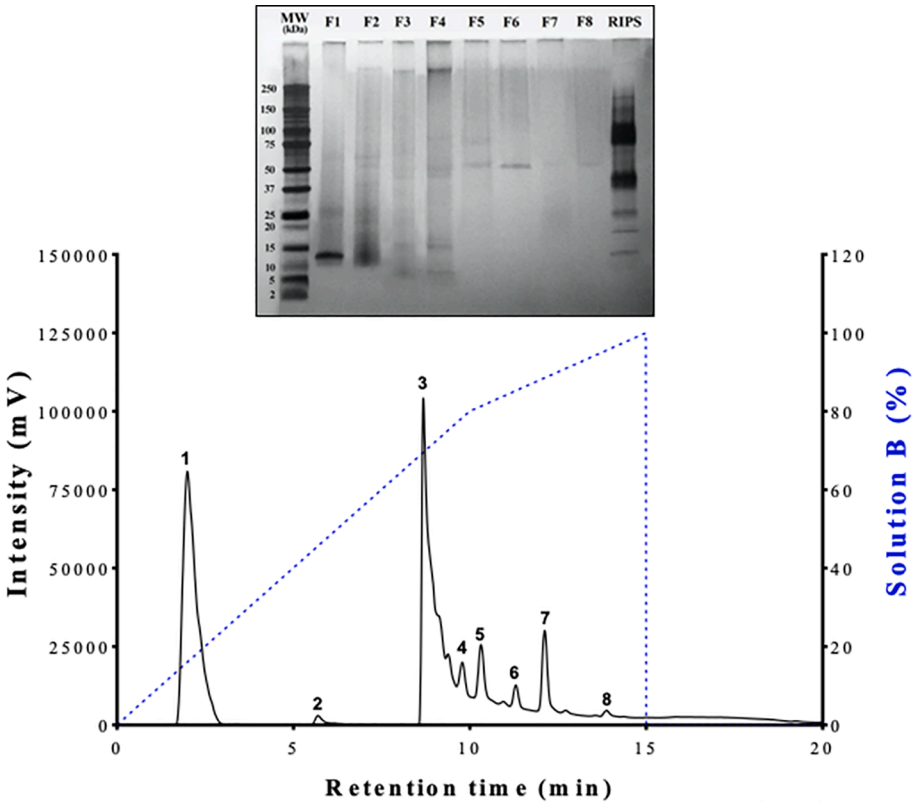

Fig. 1. RP-HPLC elution profile of RIPS (100 μL of a 1 mg/mL solution in 0.1% TFA; lower panel). The secretion was applied to a C18 column (Luna 10 μm, 250 × 10 mm, 100 Å, 5 mm particles) and then eluted with a stepwise gradient (0–80% for 10 min; 80–100% for 5 min) of solution B (80% acetonitrile in 0.1% TFA), at a flow rate of 1 mL/min at 25 °C, using a Shimadzu HPLC system. The elution profile was monitored at 254 nm. Upper panel: SDS-PAGE profile of RIPS and its eight RP-HPLC fractions (F1 to F8). RIPS –*R. icterica* parotoid secretion. The lanes were loaded with 12 μg of protein and the gel was silver-stained (BioRad) at the end of the electrophoretic run.

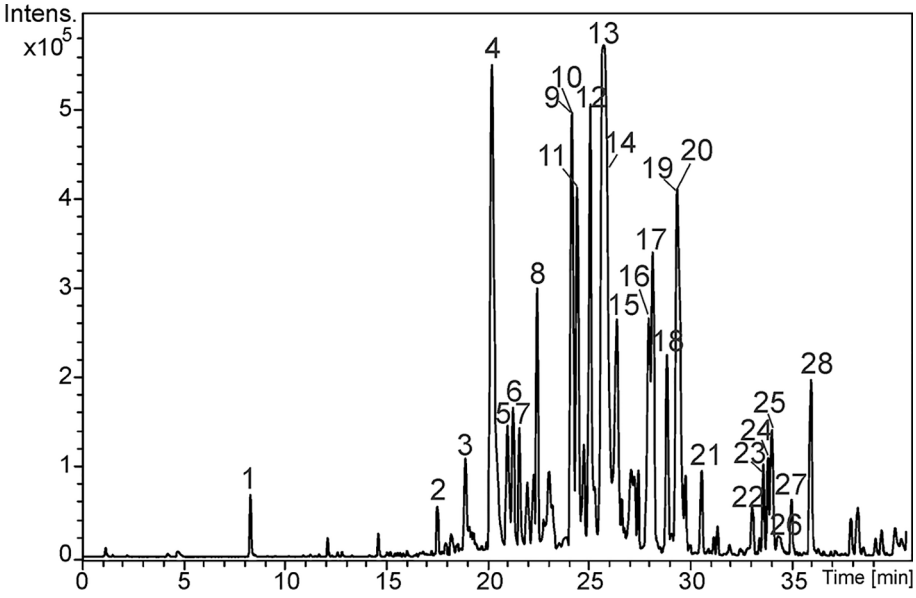

Fig. 2. A chromatogram generated during LC-DAD-MS/MS analysis (positive ion mode) of the components of fractions F4 and F5 from RIPS. For this analysis, a pool of fractions F4 and F5 was used. The peak numbers correspond to the compounds identified as indicated in the main text and in Table 1.

activity on the locust oviduct (Fig. 6). Only fractions F4 and F5 mimicked the pharmacological profile of RIPS. The addition of F4 and F5 (100 μg/200 μL) to the preparations markedly increased the amplitude of spontaneous contractions (maximal increases of 811 ± 391% and 744 ± 340%, respectively; n = 3, $p < 0.01$, Fig. 6A) and basal tonus (maximal increases of 32 ± 0.5 mg and 44 ± 3 mg, respectively; n = 3, $p < 0.0001$, Fig. 6B). The other fractions increased basal tonus but did not statistically increase the amplitude of spontaneous contractions (Fig. 6).

To investigate the possible mechanisms involved in the action of RIPS, locust oviduct was incubated with RIPS in calcium free-high magnesium saline and the basal tonus and neurally-evoked contractions were monitored. The incubation of oviduct muscle with calcium-free saline containing 20 mM magnesium chloride in the absence of RIPS resulted in a slight initial increase in the baseline tension followed by a progressive decrease in basal tonus and complete inhibition of the neurally-evoked contractions after 10 min (Fig. 7, left-hand side). However, in these conditions, RIPS still caused a marked increase in basal tonus followed by complete relaxation 10 min later. The effects of

Table 1
Identification of the constituents of fraction F4-5 of *Rhinella icterica* parotoid secretion by LC–MS/MS.

Peak	RT (min)	Compound	MF	UV	EM (*m/z*) $[M + H]^+$	EM/EM (*m/z*)
1	8.3	Suberoyl arginine	$C_{14}H_{26}N_4O_5$	–	331.1974	272, 250, 232, 208, 175
2	17.5	Hellebrigenin	$C_{24}H_{32}O_6$	296	417.2293	399, 159
3	19.0	NI	$C_{48}H_{69}N_5O_{13}$	–	462.7544*	453, 331
4	20.2	Hellebrigenin (isomer)	$C_{24}H_{32}O_6$	298	417.2268	399, 371, 335, 201, 159
5	21.1	Hellebrigenin (isomer)	$C_{24}H_{32}O_6$	300	417.2275	399, 335, 159
6	21.3	NI	$C_{38}H_{56}N_4O_{12}$	–	761.3982	743, 717, 331
7	21.6	NI	$C_{37}H_{56}N_4O_{11}$	–	733.4017	715, 367, 331
8	22.5	NI	$C_{37}H_{56}N_4O_{10}$	–	717.4080	699, 331, 278
9	24.2	3-(*N*-suberoyl argininyl) hellebrigenin	$C_{38}H_{56}N_4O_{10}$	300	729.4079	399, 331, 314, 296, 278
10	24.2	NI	$C_{38}H_{58}N_4O_{10}$	300	731.4248	713, 687, 331, 278
11	24.5	3-(*N*-pimeloyl argininyl) marinobufagin	$C_{37}H_{54}N_4O_9$	300	699.3967	681, 365, 317, 300, 264
12	25.2	3-(*N*-suberoyl argininyl) telocinobufagin	$C_{38}H_{58}N_4O_9$	300	715.4252	697, 331, 278
13	25.8	3-(*N*-suberoyl argininyl) marinobufagin	$C_{38}H_{56}N_4O_9$	298	713.4120	695, 331, 314, 278, 260
14	26.0	3-(*N*-adipoyl argininyl) bufalin	$C_{36}H_{54}N_4O_8$	300	671.4028	653, 351, 303, 286, 268
15	26.4	Marinobufagin	$C_{24}H_{32}O_5$	298	401.2328	365, 270, 253, 211
16	28.0	NI	$C_{37}H_{60}N_4O_8$	275	689.4498	671, 653, 331, 278
17	28.2	NI	$C_{42}H_{58}N_2O_6$	–	687.4351	669, 331, 278
18	28.9	3-(*N*-suberoyl argininyl) bufalin (bufalitoxin)-isomer	$C_{38}H_{58}N_4O_8$	296	699.4343	681, 331
19	29.4	3-(*N*-suberoyl argininyl) bufalin (bufalitoxin)-isomer	$C_{38}H_{58}N_4O_8$	280	699.4338	681, 331, 278
20	29.5	NI	$C_{37}H_{58}N_4O_8$	–	687.4338	669, 331, 278
21	30.7	NI	$C_{37}H_{58}N_4O_8$	–	687.4349	669, 331, 278
22	33.2	Bufotalinin	$C_{24}H_{30}O_6$	–	415.2124	–
23	33.7	NI	$C_{18}H_{39}NO_2$	–	302.3064	288
24	33.9	NI	$C_{37}H_{56}N_4O_8$	–	685.4187	657, 331, 278
25	34.0	NI	$C_{20}H_{43}NO_3$	–	346.3319	328, 284
26	34.1	NI	$C_{18}H_{39}NO_3$	–	318.3009	270
27	35.1	NI	$C_{37}H_{58}N_4O_7$	–	671.4377	653, 331, 278
28	36.1	NI	$C_{23}H_{32}O_4$	255	373.2369	355, 337, 319, 309, 296, 291, 281, 263

MF: molecular formula; NI: not identified; RT: retention time; *$[M + 2H]^{+2}$. Note that several compounds (indicated by '–') showed little or no UV absorption, making their spectra difficult to accurately detect. All the MF were determined from errors and mSigma values < 8 ppm and < 20, respectively.

calcium free-high magnesium saline were reversible since replacement with normal saline restored the neurally-evoked contractions and the contractile activity of RIPS (Fig. 7, right-hand side).

4. Discussion

The results of this investigation show that RIPS is active on locust semi-isolated heart and oviduct muscle. RIPS exerted a marked negative chronotropic effect on *L. migratoria* cardiac activity at the highest dose tested (100 μg) that was similar to the response previously observed in *Nauphoeta cinerea* cockroaches (Leal et al., 2018). At doses < 100 μg, the only change observed was a transient positive chronotropism with 0.5 ng of RIPS after 100 s, an effect not seen in *N. cinerea* (Leal et al., 2018); this difference may indicate species selectivity in the action of low amounts of RIPS (Predel et al., 2001; Hertel and Pass, 2002). The control of rhythmic contractions in insect hearts is complex and involves both myogenic and neural activities, being influenced mainly by cholinergic (Schaefer and Miles, 1970), octopaminergic (Roeder, 1999) and proctolinergic (Orchard et al., 1989) neurotransmission. Proctolin, a short neuropeptide (RYLPT) initially isolated and characterized from the American cockroach *Periplaneta americana* (Starratt and Brown, 1975) is well known for its cardiostimulatory effects in various insects (Orchard et al., 1989; Chowański et al., 2016).

In contrast to the inhibitory effect of RIPS on cardiac contractility, in locust oviduct the secretion exerted a stimulatory or excitatory effect that was greatest with the highest dose tested (100 μg). *Locusta migratoria* oviduct consists of striated muscle that is under myogenic and neurogenic control (Lange et al., 1987). This muscle can be isolated from the insect while preserving its functional characteristics, including spontaneous and rhythmic contractions when kept in saline (Lange et al., 1987). The oviduct muscle contracts in response to electrical stimulation of the N2B nerve (Kiss et al., 1984) and these contractions control the movement of mature eggs and sperm within the oviducts and

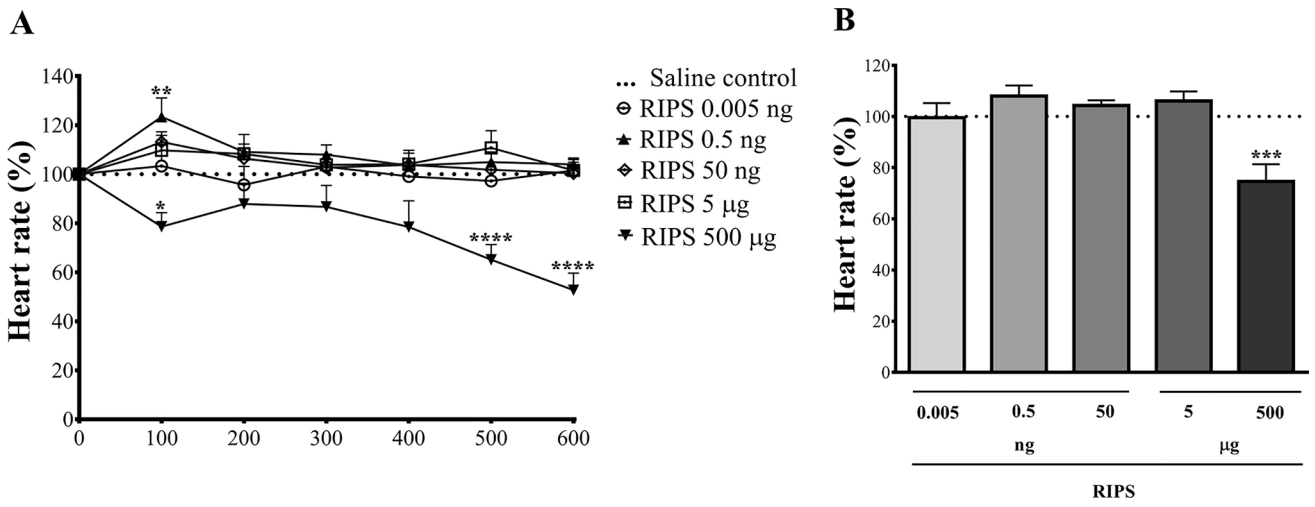

Fig. 3. Effect of RIPS on the heart rate of *L. migratoria* semi-isolated heart. (**A**) Time-course of changes in heart rate during 10 min after RIPS application. (**B**) The maximum change in heartbeat rate at the end of the 10 min observation period. RIPS was applied in a fixed volume of 500 µL. The points in (**A**) and columns in (**B**) represent the mean ± SEM (n = 6). *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ compared to the saline control.

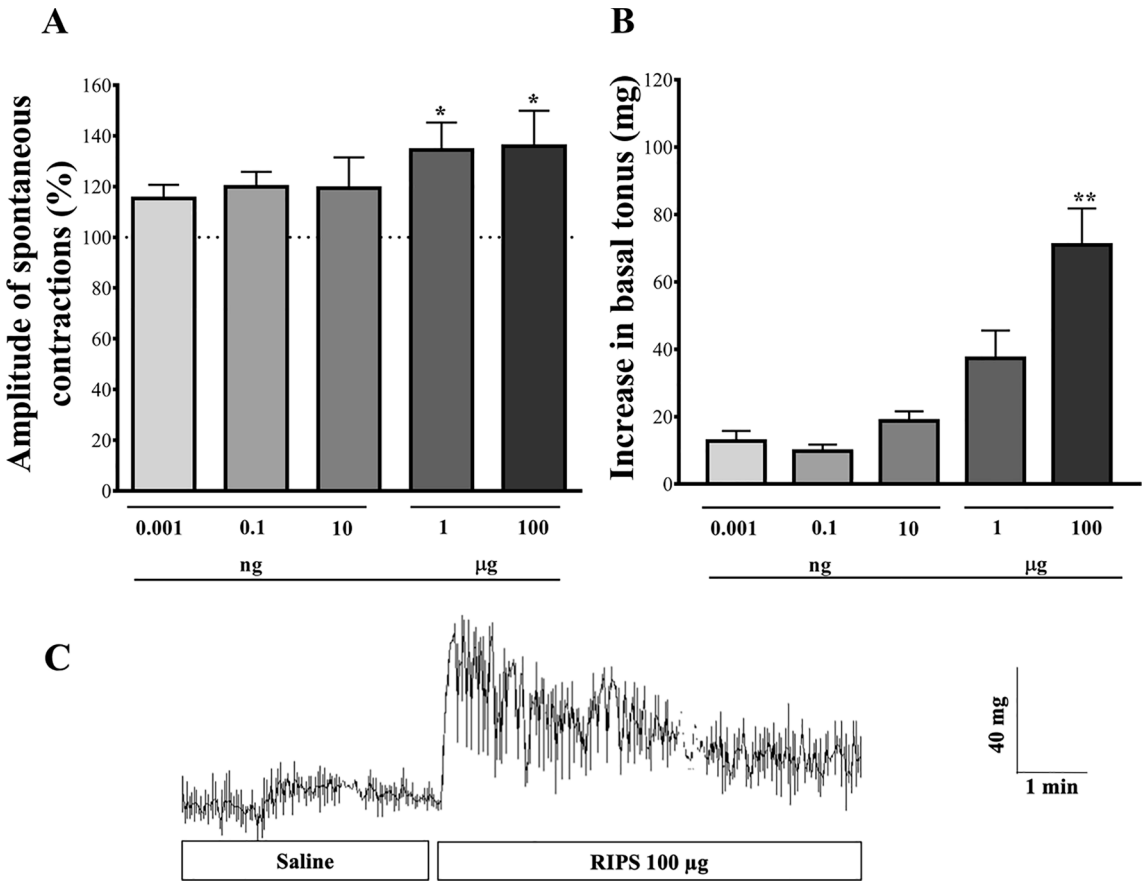

Fig. 4. RIPS influences muscle contraction in *L. migratoria* oviduct. (**A**) Representative trace of the muscle response to RIPS (100 µg). (**B**) Increase in muscle basal tonus (in mg) relative to the tonus before application of RIPS. (**C**) Increase in amplitude of spontaneous contractions, expressed as a percentage (%) of the amplitude in saline-treated preparations (control, dotted line). The quantities of RIPS indicated were applied in a fixed volume of 200 µL of saline. Contractile responses were monitored for 100 sec. The columns represent the mean ± S.E.M. (n = 6). **$p < 0.01$ compared to the responses with smaller amounts of RIPS in panel **B** and *$p <$ 0.05 compared to the saline control in panel (**C**).

assist in hemolymph circulation in the insect (Davey, 1958; Noronha and Lange, 1997; Lange, 2009). Locust oviducts are innervated by a small number of neurons located in the 7th abdominal ganglion (Lange, 2009). Among these neurons are two dorsal unpaired medial neurons containing octopamine/tyramine and three pairs of motor neurons containing proctolin (Lange and Orchard, 1986; Lange et al., 1986; Noronha and Lange, 1997; Donini and Lange, 2004).

Insect striated visceral muscle has properties similar to vertebrate smooth muscles, including contractions coordinated by peristaltic waves (Wilcox and Lange, 1995). The *L. migratoria* oviduct provides a suitable

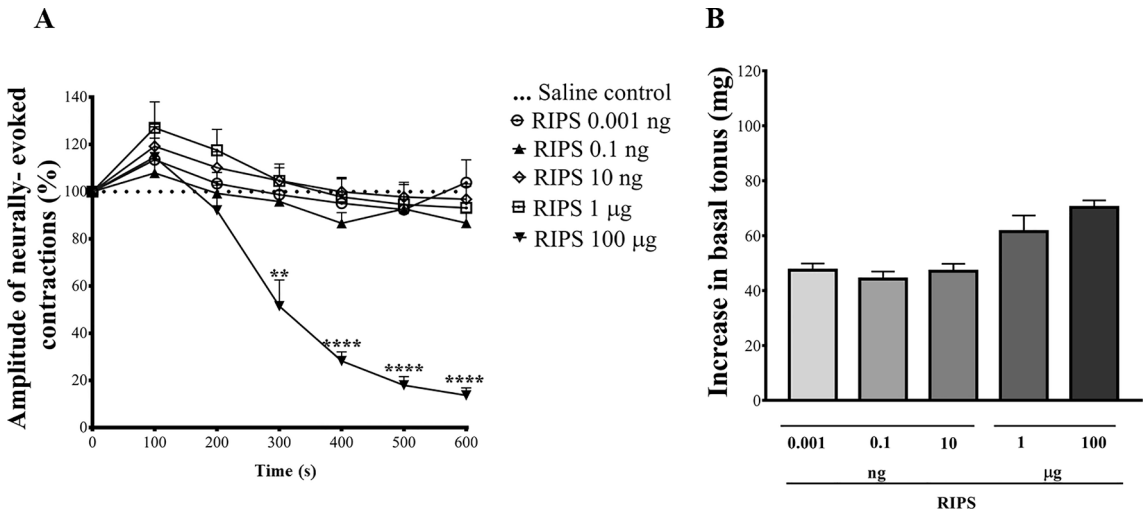

Fig. 5. RIPS-induced changes in the amplitude of neurally-evoked contractions of *L. migratoria* oviduct muscle. (**A**) Changes in contraction amplitude in response to RIPS. The highest dose of RIPS (100 µg) caused a dramatic time-dependent decrease in contractile force. The changes in amplitude were monitored for 10 min after secretion application and expressed as a percentage (%) of control (saline) responses (dotted line). (**B**) Increases in basal tonus after incubation with varying amounts of RIPS for 10 min. The increase in basal tonus was significant ($p < 0.001$) for all quantities of RIPS, with the highest amount causing greater contraction than the three lowest quantities ($p < 0.001$). In all cases, RIPS was applied in a fixed volume of 200 µL. The points in (**A**) and columns in (**B**) represent the mean ± S.E.M. (n = 6). $^{**}p < 0.01$ and $^{****}p < 0.001$ compared to control values (100%) in panel **A**.

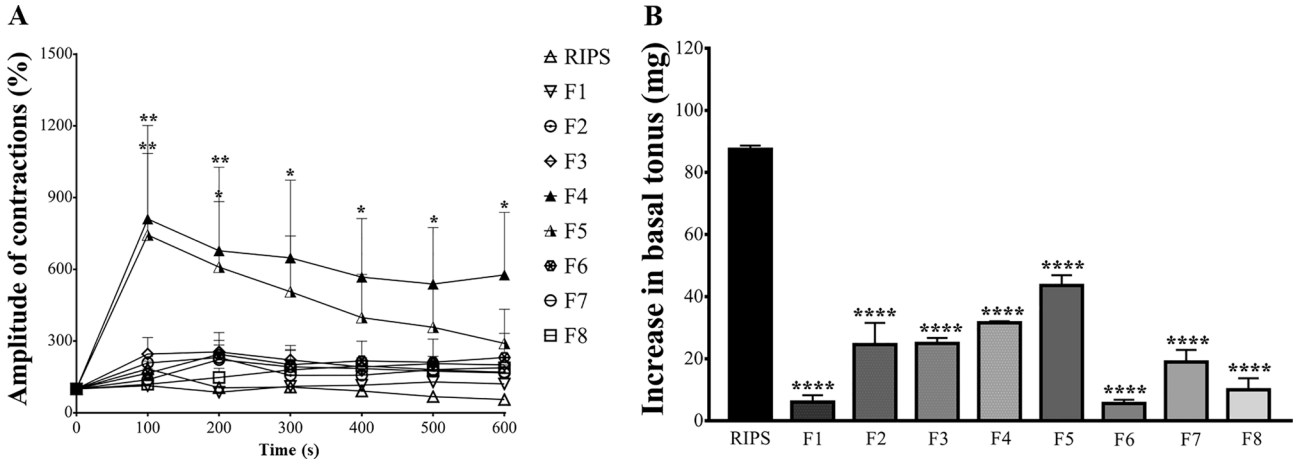

Fig. 6. Screening of RP-HPLC fractions of RIPS for activity on *L. migratoria* oviduct contraction. (**A**) Changes in amplitude of spontaneous contractions after application of RIPS. Note that only fractions F4 and F5 enhanced the contractile activity above that of RIPS. The changes in amplitude were expressed as a percentage (%) of the amplitude of spontaneous contractions in saline (considered 100%). (**B**) Increase in basal tonus after a 10 min incubation with RIPS and the RP-HPLC fractions. Note that all fractions caused a smaller increase in tonus than RIPS. The increase in tonus was expressed relative to the basal tonus before sample addition. In all assays, a fixed amount of RIPS and fraction was used (100 µg in 200 µL). The points in (**A**) and the columns in (**B**) represent the mean ± S.E.M. (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.001$ compared to RIPS.

system for examining the role of calcium in invertebrate visceral muscle contractions. In the locust oviduct, extracellular calcium is necessary for muscle contraction and the presence of inositol-3-phosphate (IP_3)-sensitive calcium stores has been suggested (Lange et al., 1987). As shown here, in calcium free-high magnesium saline, RIPS still caused an increase in basal tonus, a finding suggestive of the release of intracellular calcium, a phenomenon characteristic of striated muscle in low calcium solution (Irwin and Hein, 1963).

The possible mechanisms involved in the action of RIPS in the two preparations studied here were investigated primarily in oviduct muscle. The stimulatory effect on oviduct muscle was not mediated by PLA_2 since the secretion was devoid of this activity. Phospholipases A_2, some of which are neurotoxic, are widely distributed in animal venoms (Dal Belo et al., 2005; Tonello and Rigoni, 2017; Schütter et al., 2019). However, toad parotoid secretions generally do not possess PLA_2 neurotoxins, although some PLA_2 have been identified in the skin secretions of *Rhinella* spp. (Arantes et al., 2018). The absence of PLA_2 activity indicated that the neurotoxicity of RIPS in insect preparations must be attributable to other compounds in the secretion. In agreement with this conclusion, mass spectrometric analysis of fractions F4 and F5 demonstrated the presence of a variety of bufadienolides that could possibly be involved in the effects of RIPS.

Several classes of compounds have been identified in the parotoid or skin gland secretions of toads, including peptides, steroids, indole alkaloids, bufogargarizanines and organic acids (Garg et al., 2008; Gao et al., 2010; Anjolette et al., 2015; Zulfiker et al., 2016; Arantes et al., 2018). Bioactive compounds from anuran secretions exhibit a number of pharmacological activities (Das et al., 2001; Lewis and Garcia, 2003; Siano et al., 2014; Hong Ling et al., 2016), including antiproliferative (Cunha-Filho et al., 2010; de Oliveira et al., 2019), antiparasitic (Tempone et al., 2008), antiviral (Vigerelli et al., 2014; da Mata et al., 2017), antimicrobial (Toledo and Jared, 1995; Pinto et al., 2009; Siano et al.,

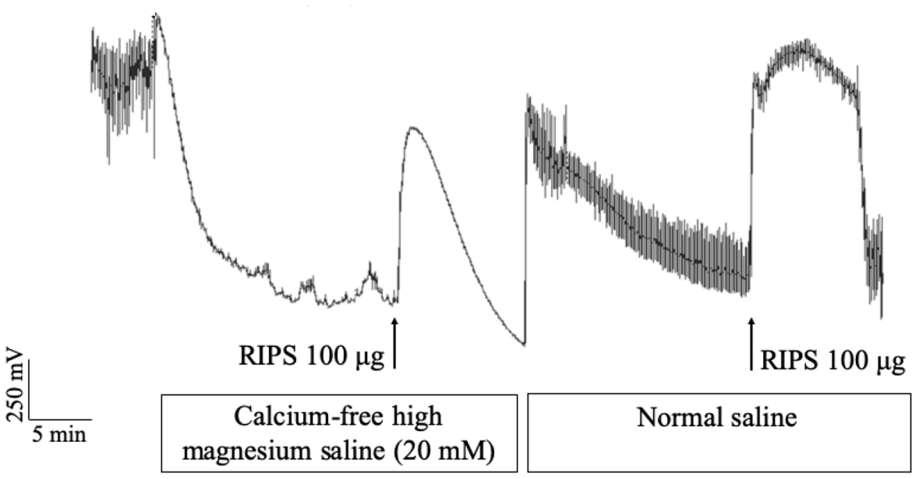

Fig. 7. Activity of RIPS on neurally-evoked contractions of *L. migratoria* oviduct in saline with a modified ionic composition. The addition of RIPS to calcium-free saline containing a high concentration of magnesium (20 mM) lead to a slight initial increase in basal tension followed by marked tissue relaxation. Note that neurally-evoked contractions were abolished by the absence of extracellular calcium. The addition of RIPS (100 µg) after maximal relaxation resulted in a strong muscle contraction. Neurally-evoked contractions returned when the preparation was placed in normal saline (right half of the recording) and RIPS produced a larger and more sustained contraction. These traces are representative of six experiments for each condition.

2014; Hong Ling et al., 2016) and insecticidal (Leal et al., 2018) effects that may be related to the presence of bufadienolides (Cunha Filho et al., 2005; Cunha-Filho et al., 2010; Qi et al., 2018).

Bufadienolides are a class of C-24 steroids consisting of a characteristic α-pyrone ring at position C-17 (Cunha-Filho et al., 2010; Kolodziejczyk-Czepas and Stochmal, 2017). These compounds are widely distributed among bufonid toads and act primarily by inhibiting the Na^+/K^+-ATPase pump (Cunha-Filho et al., 2010; Kolodziejczyk-Czepas and Stochmal, 2017). Thus, the presence of bufadienolides such as bufalitoxin in RIPS is indicative of potential cardiotoxicity (Kolodziejczyk-Czepas and Stochmal, 2017) and neurotoxicity (Botha, 2016). Indeed, we have previously demonstrated that RIPS is cardiotoxic and neurotoxic in vertebrates (Oliveira et al., 2018, 2020) and invertebrates (Leal et al., 2018), potentially through its ability to inhibit the Na^+/K^+-ATPase pump. As shown here, mass spectrometric analysis of fractions F4 and F5 of RIPS identified a variety of bufadienolides, including bufalitoxin, hellebrigenin isomer, hellebrigenin 3-suberoyl arginine ester isomer, marinobufagin 3-pimeloyl arginine ester, marinobufagin 3-suberoyl arginine ester, suberoyl arginine, and telocinobufagin 3-suberoyl arginine ester. One or more of these compounds could contribute to the effects of RIPS in the bioassays used in this study.

Proctolin is the main neuropeptide involved in modulating the contractility of oviduct muscle (Cook and Holman, 1985; Lange et al., 1987; Lange, 2009) and the stimulatory effect of RIPS on locust oviduct muscle contraction could possibly be mediated by interaction of the bufadienolides in fractions F4 and F5 with proctolin receptors (Lange et al., 1987). The activation of proctolinergic pathways by RIPS may occur in parallel with the known ability of this secretion to modulate octopaminergic neurotransmission (Leal et al., 2018). Octopamine inhibits contraction of the locust oviduct muscle (Lange and Orchard, 1986). However, confirmation of this would require the use of specific antagonists of these receptors to assess whether receptor blockade would attenuate the activity of RIPS. The purification and identification of the compounds in fractions F4 and F5 would also help to clarify the relationship between molecules capable of activating proctolin signaling pathways and the presence of bufadienolides.

5. Conclusions

The results of this study show that RIPS has stimulatory and inhibitory effects on *L. migratoria* muscle preparations in vitro and modulates locust visceral striated muscle contraction by a mechanism dependent on intracellular calcium but probably independent of PLA_2 activity. The activity profile of RIPS in the *L. migratoria* preparations resembles that induced by bufadienolides. Further chromatographic and chemical analyses of fractions F4 and F5 should lead to identification of the components responsible for this activity in *L. migratoria*.

CRediT authorship contribution statement

Raquel Soares Oliveira: Investigation, Formal analysis, Validation, Writing - original draft. **Bruna Trindade Borges:** . **Allan P. Leal:** Investigation, Formal analysis. **Patrícia de Brum Vieira:** Methodology, Resources, Investigation, Formal analysis, Validation. **Denise Brentan Silva:** Methodology, Resources, Investigation, Formal analysis, Validation. **Stephen Hyslop:** Validation. **Lúcia Vinadé:** Methodology, Supervision, Validation, Funding acquisition. **Tiago Gomes dos Santos:** Resources. **Celia R. Carlini:** Funding acquisition. **Ian Orchard:** Conceptualization, Resources, Supervision. **Angela B. Lange:** . **Cháristo A. Dal Belo:** Conceptualization, Resources, Funding acquisition, Supervision, Project administration.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Authors' contributions

CADB, RSO, AL and IO conceived the investigation, RSO, BTB, APL, DBS and PBV did the experimental work, RSO, DBS, PBV, BTB and LV analyzed and validated the data and prepared the results for publication, CADB, AL, IO, DBS, PBV and TGS provided resources, CADB, CRC and LV provided funding, RSO and CADB wrote the original draft, with subsequent reviewing, editing and validation by SH, IO and AL. CADB, IO, AL and LV provided supervision and CADB was responsible for general administration of the project. All authors read and approved the final version of the manuscript submitted for publication.

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