https://dx.doi.org/10.21577/0103-5053.20210009

J. Braz. Chem. Soc., Vol. 32, No. 5, 1060-1069, 2021

Article

Chemical Constituents and Cytotoxic Activity of *Rhinella jimi* (Anura: Bufonidae)

Evaldo S. Monção Filho,[a] Yara P. F. Pio,[a] Mariana H. Chaves,[a] Paulo M. P. Ferreira,[b] Mariluce G. Fonseca,[c] Cláudia Pessoa,[d] Daisy J. B. Lima,[d] Bruno Q. Araújo[e] and Gerardo M. Vieira Jr. *,[a]

[a]*Laboratório de Produtos Naturais, Departamento de Química, Universidade Federal do Piauí, 64049-550 Teresina-PI, Brazil*

[b]*Laboratório de Cancerologia Experimental, Departamento de Biofisica e Fisiologia, Universidade Federal do Piauí, 64049-550 Teresina-PI, Brazil*

[c]*Curso de Ciências Biológicas, Universidade Federal do Piauí, 64607-670 Picos-PI, Brazil*

[d]*Laboratório de Oncologia Experimental, Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, 60020-181 Fortaleza-CE, Brazil*

[e]*Programa de Pós-Graduação em Química, Universidade Federal do Espírito Santo, 29075-910 Vitória-ES, Brazil*

Rhinella jimi toads (Stevaux, 2002) belong to the Bufonidae family, are endemic in the Brazilian Northeast and are commonly found during rainy periods. In general, amphibians of this family have in their poisons different metabolites that show a diversity of pharmacological activities. The isolation and identification of these compounds are of great importance, and techniques such as high-performance liquid chromatography coupled to mass spectrometry are widely used for the discovery of novel and known compounds in these poisons. For *R. jimi* poison, the ethyl acetate and methanolic extracts were obtained and thirty compounds were identified by combining ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) with direct infusion atmospheric pressure chemical ionization mass spectrometry (DI-APCI-MS/MS) and direct infusion electrospray mass spectrometry (DI-ESI-MS/MS) for each extract, respectively. Marinobufagin (**2**) and marinobufotoxin (**19**) were the majorities of each extract, respectively. In addition, other bufadienolides mainly present in the ethyl acetate extract, such other bufotoxins, alkaloids and arginine diacid derivatives were identified in the methanol extract. In a cytotoxic assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the extracts and compound **2** demonstrated half-maximal inhibitory concentration (IC_{50}) values better than the positive control doxorubicin, evidencing excellent cytotoxic. This is the most complete study of the chemical composition of *R. jimi* toad poison and its respective cytotoxic activity, promoting the enrichment of knowledge about this family and species.

Keywords: bufadienolides, *Rhinella jimi*, DI-APCI-MS/MS, DI-ESI-MS/MS, pharmacological potential

Introduction

Natural products of animals have been studied with very promising results for action against diseases. Some metabolites have been extensively investigated, such as the fixed oils of serpents (*Spilotes pullatus*), chelonians (*Phrynops geoffroanus*) and amphibians from the Leptodactylidae family.[1] Among other toxin-producing animals, some are part of traditional medicine in many countries around the world.[2] Amphibians, especially of the Bufonidae family, present a range of pharmacologically active molecules in their skin and venom, such as steroids, alkaloids, biogenic amines, guanidine derivatives, proteins and peptides, produced as defenses against microorganisms, predators and infections, with the major biological activity attributed to the secondary metabolites.[3] These crude venoms are used in traditional extracts known as "Chan'Su" and are widely used as therapeutic agents in traditional Chinese medicine and against symptoms such as abdominal pain, fevers, high blood pressure,

*e-mail: magela@ufpi.edu.br

inflammation, sinusitis, common cold and heart disease.[2,4,5] Especially for amphibians of this family, the study of their poisons presents a variety of biological activities, such as trypanocidal, leishmanicidal, bactericidal, antifungal,[1,6,7] antiproliferative,[8-10] insecticide,[11] antiviral,[12,13] and cardiotonic properties.[14]

One of the 84 species from the Bufonidae family is *Rhinella jimi*.[15] As with other species of genus *Rhinella*, they have the characteristic glands present in most amphibians of this family, parotid glands, possess nocturnal habits and are distributed in the Northeastern region of Brazil.[1] The main active compounds of these toads are the bufadienolides.[16] These compounds are 24-carbon polyhydroxylated steroids related to cholesterol, characterized by containing an unsaturated lactone ring attached at the C-17 position of the perhydrocyclophenanthrene nucleus.[17] In past years, studies[8,18] have demonstrated cytotoxic activity of bufadienolides.

The isolation, characterization and identification of the chemical constituents in toad venoms are essential for the scientific investigation of pharmacological activities and to direct the therapeutical potentials of the extracts and isolated compounds.[19,20] Liquid chromatography-mass spectrometry (LC-MS) is one of the main instruments for identifying compounds from mixtures and extracts, which facilitates the determination of novel and known compounds in crude extracts, promoting a generic investigation of minor and major compounds in extracts.[21] In light of this, the present work had as an objective to identify compounds from the ethyl acetate and methanolic extracts by combining ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS) and direct insertion mass spectrometry (DI-MS/MS), as well as to evaluate the cytotoxic activity of the extracts and a pure compound from *R. jimi* poison.

Experimental

Reagents

The analytical solvents methanol and ethyl acetate were purchased from Labsynth (Diadema, São Paulo, Brazil). The ultrapure water was prepared by a Milli-Q Water purification Master System MS2000 from Gehaka (São Paulo, Brazil), and the high-performance liquid chromatography (HPLC) grade solvent acetonitrile was purchased from Tedia Company Inc. (Fairfield, Ohio, USA). The extracts were concentrated on Heidolph rotary evaporator (Laborota 4000, Darmstadt, Germany), and the residual water was removed in a Thermo Electric ModulyoD freeze dryer (Milford, Massachusetts, USA).

Gathering of poisons

The poison of twenty specimens of *R. jimi*, SisGen cadaster No. AE58A09 and IBAMA SISBIO 55970-1 (permanent license to collect zoological material), were collected between January and February of 2016 in Teresina (Piauí State, Brazil), semi-arid region in Brazil. Voucher specimen (*R. jimi* CHCJ#0669) was deposited in the Coleção Científica de Herpetologia Jorge Jim, CHCJ, Federal University of Piauí (Picos, Piauí, Brazil). These poisons were secreted from parotid glands of the frogs by manual compression and after this procedure, the animals were returned to nature. The poison collected was placed in desiccators with silica at room temperature, to remove the water, and after dried, crushed using pistil and mortar to obtain the poison powder.

Secretion extract preparation

The poison powder (3.9 g) was extracted with ethyl acetate (3 × 75 mL) for 10 min each in an ultrasound bath at room temperature, to give the ethyl acetate extract (EARJ, 149.7 mg), and then extracted using methanol under the same conditions to obtain the methanolic extract (EMRJ, 334.1 mg). Compound **2** was isolated and identified as marinobufagin as described by Garcia *et al.*[22] and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data is shown in Supplementary Information (SI) section (Figures S1 and S2).

UPLC-MS and DI-MS/MS analysis

The samples EARJ and EMRJ were analyzed separately by ultra-performance liquid chromatography coupled to mass spectrometry using atmospheric pressure chemical ionization (APCI) source for EARJ (UPLC-APCI-MS) and electrospray (ESI) source for EMRJ (UPLC-ESI-MS), only to obtain the full extracted ion chromatogram (EIC) with the respective retention time of each compound.

The separation was performed in an UPLC (Shimadzu Corporation, Kyoto, Japan) consisting of an LC-20AT pump, an SPD-M20A UV diode array detector, CTO-20AC column oven. The separation was carried out in a reverse phase C-18 column of Agilent technologies (4.6 × 50 mm, 2.7 μm). The analysis was performed in an exploratory gradient with a mobile phase that consisted of ultrapure water (A) and acetonitrile (B) as follows: 5% B to 100% B from 0.1 to 35 min. The flow rate was 0.5 mL min^{-1}, detection was 296 nm, and the volume injected was 5 μL. For the mass spectrometry parameters (coupled to the UPLC) was used an Amazon X (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer equipped with APCI

and ESI source, for EARJ and EMRJ, respectively. The extract ion chromatograms (EIC) for both extracts was acquired in positive-ion mode.

The source ionization conditions for APCI were a vaporizer temperature of 300 °C, with a drying gas flow of 5.0 L min^{-1}, a capillary voltage (–) 4000 V, a nebulizer at 40 psi and the current corona of 4000 nA, and for ESI source were a drying gas temperature 300 °C and flow of 12.0 L min^{-1}; a capillary voltage (–) 4500 V and a nebulizer at 44 psi. The mass spectrometer was operated with a range of *m/z* 100-1000 for both analyses.

After obtaining their respective extracted ion chromatogram (EIC), the extracts were reanalyzed by direct infusion (DI) using the same mass spectrometer (Amazon X, ion trap, Bruker Daltonics, Bremen, Germany) and the same parameters to obtain the fragmentation of ions previously observed for each extract in the UPLC-MS analysis.

Cytotoxicity activity

Evaluation of cytotoxicity on tumor cells

The cytotoxicity of the EARJ, EMRJ and compound **2** was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[23] Lines were plated in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin at 0.1 × 10^6 cells mL^{-1} for PC3 (prostate), L-929 (murine fibroblast), HEK-293 (human embryonic kidney), SF-295 (glioblastoma), and SNB19 (central nervous system), 0.3 × 10^6 cells mL^{-1} for HL-60 (leukemia), 0.5 × 10^5 cells mL^{-1} for NCI-460 (lung carcinoma), and 0.7 × 10^5 cells mL^{-1} for HCT-116 (colorectal) cells. The plates were incubated in atmosphere with 5% CO_2 at 37 °C for 69 h (Shel Lab CO_2 Incubator, Cornelius, USA). Afterwards, the plates were centrifuged and the supernatant was removed. Then, 150 μL of 10% MTT solution (tetrazolium salt) were added, and the cells were incubated again for additional 3 h. Formazan salt was dissolved in 150 μL of pure dimethyl sulfoxide (DMSO) and absorbance measures at 595 nm were used to determine cell proliferation. Values of half-maximal inhibitory concentration (IC_{50}) and their 95% confidence intervals were obtained by nonlinear regression using the GraphPad program version 6.0.[24]

Results and Discussion

In the chemical composition of the extracts from *R. jimi* poison investigation, maninobufagin (**2**) and marinobufotoxin (**19**) were identified in the EARJ and EMRJ as the major constituents, respectively. Both compounds had been previously identified in *R. marina* and *R. schneideri* species.[25,26] The ion trap MS/MS analysis for both extracts showed the presence of more bufadienolides and bufotoxins, indolic alkaloids and argininyl diacid derivatives. This variety of composition was observed in chromatograms, with different *m/z* and retention times. The structures of the thirty identified compounds (Figure 1) were based on the MS/MS

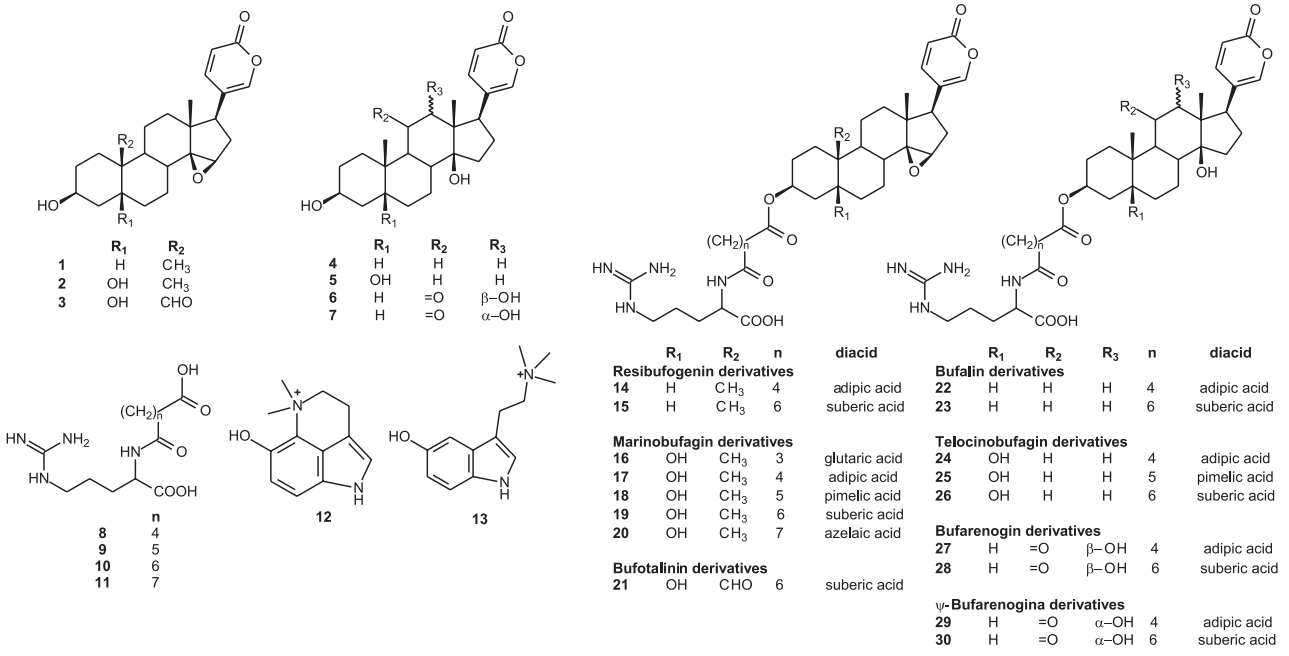

Figure 1. Structure of identified compounds in EARJ and EMRJ from *R. jimi* venom.

fragmentations and its stereochemistry were suggested based on the biosynthesis of the steroids and in the spectroscopic data (NMR) of compounds related in the literature.[5,6,17,25-30]

Identification of chemical constituents

Ethyl acetate extract (EARJ)

By UPLC-DAD, peaks with a wavelength of maximum absorption of 296 nm characteristic of α-pirone ring present in the bufadienolides were detected, the chromatogram is shown in Figure 2. In the literature,[31] the APCI source was demonstrated to perform better than ESI for bufadienolide metabolites. The analysis of EARJ by UPLC-APCI-MS combined with DI-APCI-MS/MS resulted in the identification of seven compounds.

The extracted ions chromatogram (EIC) in positive mode is shown in Figure 3 and the fragmentation and identification are shown in Table 1. Compounds **1** to **7** were attributed to the bufadienolides class, and according to the fragmentation pattern, they were separated into two groups.

Group I: resibufogenin (**1**), marinobufagin (**2**), and bufotalinin (**3**)

Compound **1** detected at *m/z* 385.2 $[M + H]^+$, showed a base peak at *m/z* 366.2 $[M - H_2O]^+$. The compound presented ions at *m/z* 349.2 $[M + H - 2H_2O]^+$ and at 253.2 $[M + H - 2H_2O - 96]^+$ with good abundance in the MS/MS spectra, joined to peaks at *m/z* 331.2 $[M + H - 3H_2O]^+$, *m/z* 321.1 $[M + H - 2H_2O - CO]^+$ and *m/z* 303.1 $[M + H - 3H_2O - CO]^+$. This compound was attributed as resibufogenin, which had been previously identified in *R. jimi* toads and other species of the *Rhinella* and *Bufo* genera.[1,26,27]

Compound **2** is a mono-hydroxylated derivative of resibufogenin. The compound detected at *m/z* 401.3 $[M+H]^+$, showed an ion at *m/z* 365.2 $[M + H - 2H_2O]^+$ that appears as a base peak, indicating the presence of ions at *m/z* 383.2 $[M + H - H_2O]^+$, *m/z* 347.2 $[M + H - 3H_2O]^+$, *m/z* 329.2 $[M+H-4H_2O]^+$, *m/z* 319.1 $[M+H-3H_2O-CO]^+$, *m/z* 301.2 $[M + H - 4H_2O - CO]^+$ characteristic of successively losses of H_2O and CO and *m/z* 251.1 $[M + H - 3H_2O - C_5H_4O_2]^+$ characteristic of the loss of an α-pirone ring, the main fragmentation route for bufadienolides (Table 1). This compound was identified as marinobufagin (**2**), and its

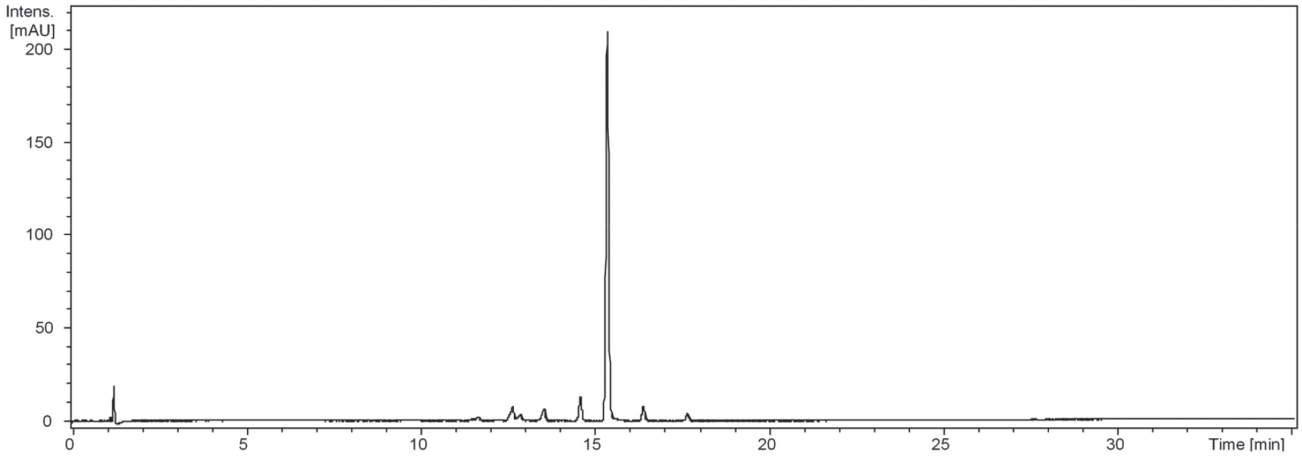

Figure 2. UPLC chromatogram of EARJ in wavelength absorption of 296 nm.

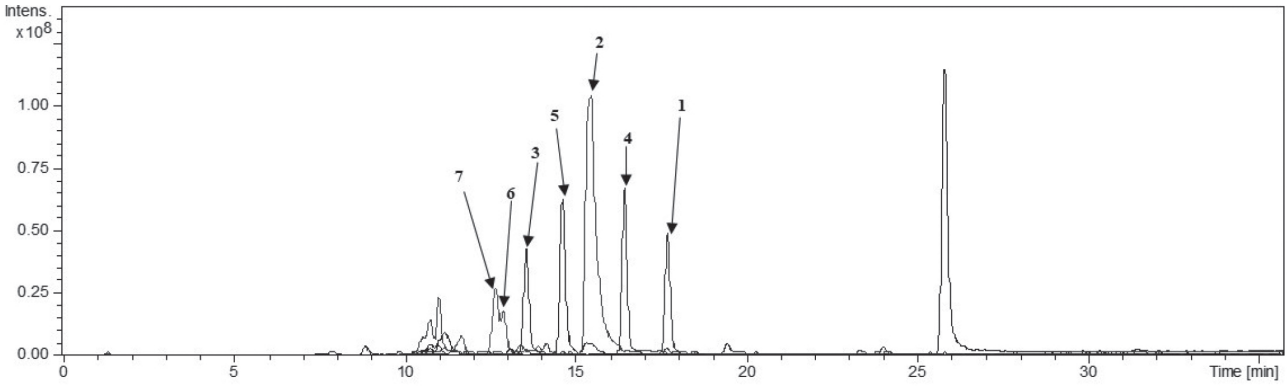

Figure 3. Extracted ion chromatogram (EIC) of identified compounds in EARJ.

Table 1. Identification of the EARJ compounds by UPLC-APCI-MS and DI-APCI-MS/MS in positive ion mode

Retention time / min	Compound	$[M + H]^+$	MS/MS (percentage abundance / %)	Identification
12.7/12.9	**7/6**	417.3	399.2 (100), 381.1 (15), 371.2 (11), 363.2 (21), 353.2 (10), 335.1 (22)	ψ-bufarenogin[27]/ bufarenogin[27]
13.5	**3**	415.3	397.2 (64), 379.1 (63), 369.2 (28), 361.2 (65), 351.2 (100), 333.2 (84), 237.1 (46)	bufotalinin[27]
14.6	**5**	403.3	385.2 (17), 367.2 (45), 349.2 (100), 339.2 (22), 331.2 (15), 321.2 (28), 303.1 (15), 253.1 (18)	telocinobufagin[27]
15.4	**2**	401.3	383.2 (27), 365.2 (100), 347.2 (87), 329.2 (19), 319.1 (35), 301.2 (15), 251.1 (30)	marinobufagin[27]
16.4	**4**	387.3	369.2 (46), 351.2 (100), 333.2 (35), 323.2 (27), 305.2 (35), 255.2 (74)	bufalin[27]
17.7	**1**	385.2	366.2 (100), 349.2 (58), 331.2 (20), 321.1 (20), 303.1 (13), 253.2 (38)	resibufogenin[27]

fragmentation pattern, that followed the same pattern as the other compounds of this group, is shown in Figure 4. The compound identified as bufotalinin (**3**) showed a similar fragmentation pattern to compound **2**, with the difference of a formyl group at position C-19 of the steroidal nucleus. Both compounds have been identified in other species of the genus *Rhinella* and *Bufo*.[26,27]

Group II: bufalin (**4**), telocinobufagin (**5**), bufarenogin (**6**) and ψ-bufarenogin (**7**)

The compounds **4**, **5**, **6** and **7** are different from the compounds of group I due to the absence of an epoxy group and the additional presence of a hydroxyl and/or carbonyl, when their structures are compared to group I.

Compound **4** showed ions at *m/z* 369.2 $[M + H - H_2O]^+$, *m/z* 351.2 $[M + H - 2H_2O]^+$, *m/z* 333.2 $[M + H - 3H_2O]^+$, *m/z* 323.2 $[M + H - 2H_2O - CO]^+$, *m/z* 305.2 $[M + H - 3H_2O - CO]^+$ and *m/z* 255.2 $[M + H - 2H_2O - C_5H_4O_2]^+$. These fragments are characteristic of the successive loss of $-H_2O$ and $-CO$ joined to a complete loss of the α-pirone group, which is the typical fragmentation of bufadienolides.[27] The compound was identified as bufalin (**4**), and the fragmentation data observed (as the same for compounds **5**, **6** and **7**) follows the same as showed by Ye and Guo.[27] The compounds **4**, **5**, **6** and **7** have been identified in other species of genus *Rhinella* and *Bufo*.[26,27]

Methanolic extract (EMRJ)

In the analysis of the methanolic extract by UPLC-ESI-MS, a large variety of ions with different *m/z* were observed, and combined with DI-ESI-MS/MS were identified 28 compounds. Some of these compounds were observed in the EARJ. In this case, bufadienolides were observed in both extracts, but the methanolic extract showed other classes joined to these bufadienolides. These results demonstrated that ethyl acetate had a better performance to extract bufadienolides and corroborates with the extraction proposed in the literature.[31]

Besides the bufadienolides, bufotoxins, indole alkaloids and argininyl diacid derivatives were also

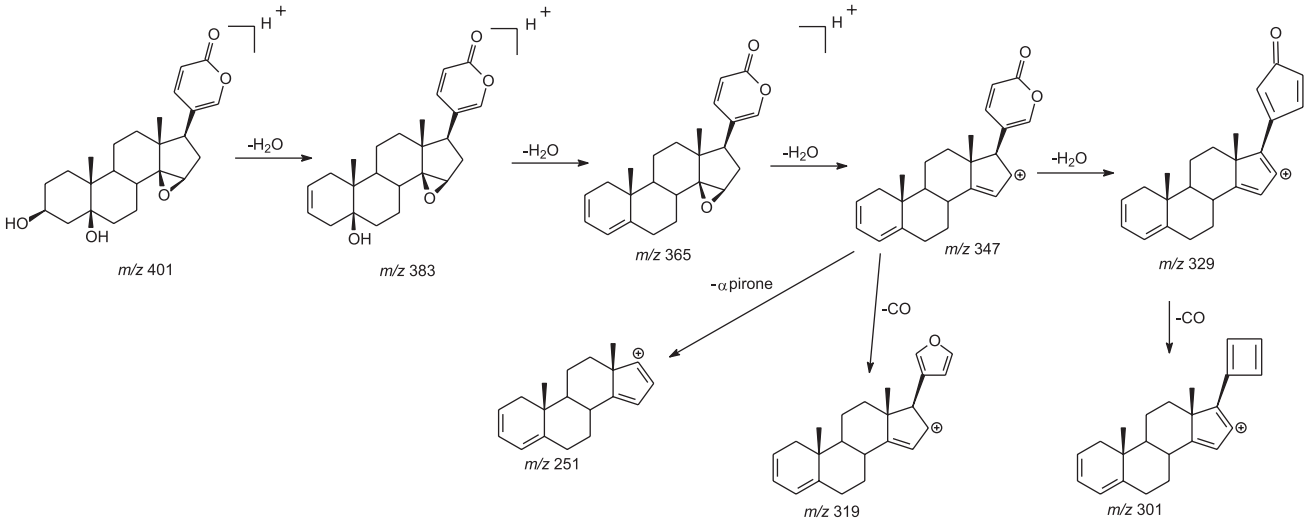

Figure 4. Proposed fragmentation pattern mechanism of the compound **2** (group I) (adapted from reference 26).

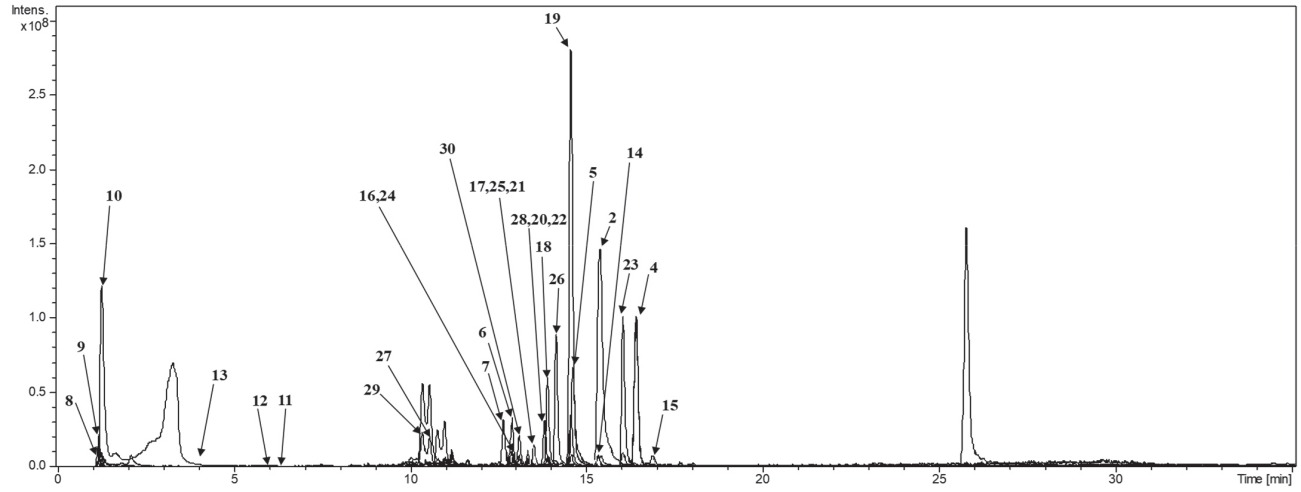

Figure 5. Extracted ion chromatogram (EIC) of identified compounds in EMRJ.

identified. According to the class and fragmentations, the compounds were divided into groups of compounds that showed a similar fragmentation pattern. The extracted ion chromatogram (EIC) in positive mode for all substances is showed in Figure 5, differentiating the respective retention time designed for each compound.

Group III: alkaloids

Two alkaloids were identified in the EMRJ. This class of secondary metabolites, in general, is found in plants, but it can be encountered in amphibians, including toads.[32] Their structures are of the indole alkylamine type and are characterized by the presence of the indole ring, typically bounded with alkyl or alkylamine groups in the third carbon of the five ring members and a hydroxyl group in five-carbons of the aromatic ring.[19] The fragments can be generated through the loss of a side chain, which can have different *m/z*. Compound **13** presented a partial fragmentation of the side chain observing an ion at *m/z* 160.0. Compound **12** is an ammonium quaternary cyclic compound in salt form, and it was observed with a loss of 15 Da, characteristic of a methyl group loss, generating an ion at *m/z* 188.1. The respective fragmentation patterns are shown in Figure 6. Both compounds have already been reported in species of the genus *Rhinella*.[26,33]

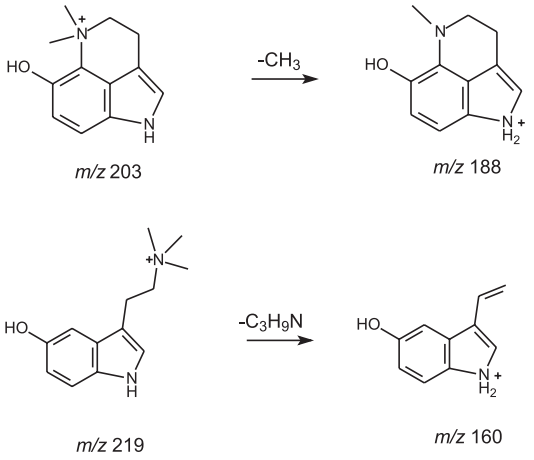

Figure 6. Proposed fragmentation pattern mechanism of compounds **12** and **13** (group III) (adapted from reference 19).

Group IV: diacids argininyl derivatives

Compound **8** presented ions at *m/z* 285.2 $[M + H - H_2O]^+$, *m/z* 268.2 $[M + H - H_2O - NH_3]^+$ and *m/z* 250.1 $[M + H - 2H_2O - NH_3]^+$. For both cyclization reactions at *m/z* 285 and 250, the carbonyl groups can act as internal nucleophiles, following the Baldwin rules in the formation of five ring members being more favorable.[34] It has showed ions at *m/z* 175.1 $[M + H - C_8H_{13}O_3]^+$ and 159.1 $[M + H - C_8H_{17}O_3N]^+$, characteristic of the arginine portion. Its fragmentation is shown in Figure 7. Compounds **9**, **10** and **11** showed similar ions, but differentiated from compound **8** by + 14 Da, + 28 Da and + 42 Da, respectively, characteristic of the presence of five, six or seven CH_2 in the diacid chain, presenting the same ions at *m/z* 175 and 158 of the arginine portion. All of these compounds have already been reported in other species of the genus *Rhinella*.[26,30,35]

Group V: bufotoxins

Most of the metabolites identified in the methanolic extract were bufotoxins. Their structures are proposed while taking into account the different diacid argininyl derivatives identified and that several bufadienolides have the same molecular mass. The assignment for each different bufadienolide was made for those identified in EARJ, which gives, in some cases, different retention times and the same *m/z*. The general scheme of the fragmentation pattern is shown in Figure 8.

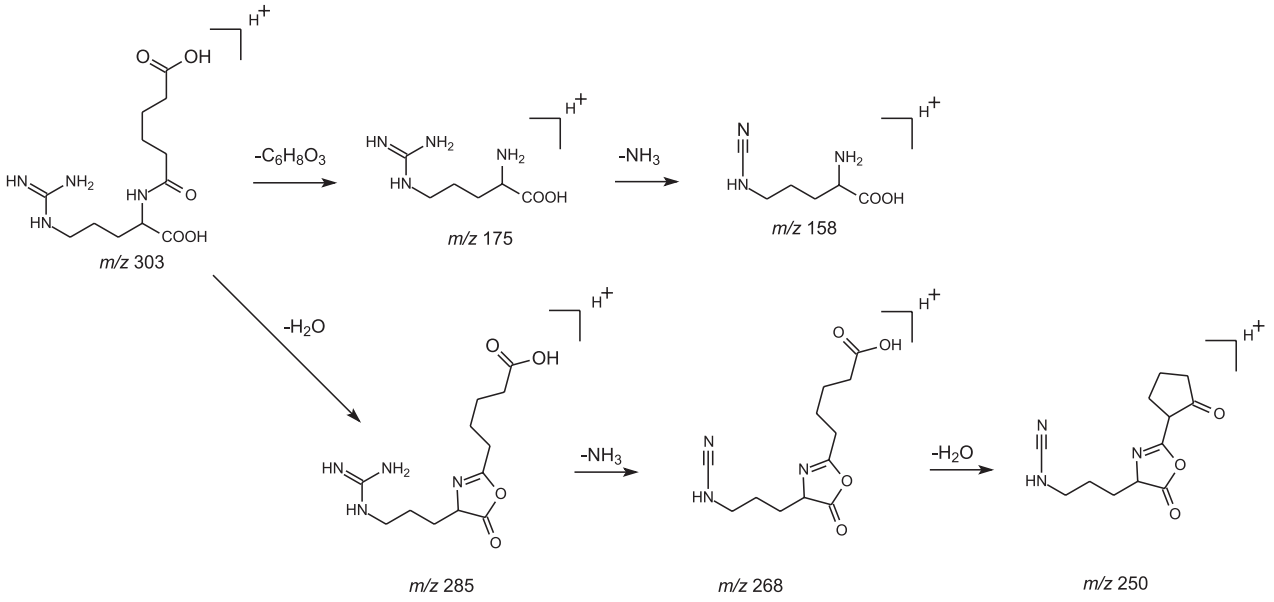

Figure 7. Proposed fragmentation pattern mechanism of the compound **8** (group IV).

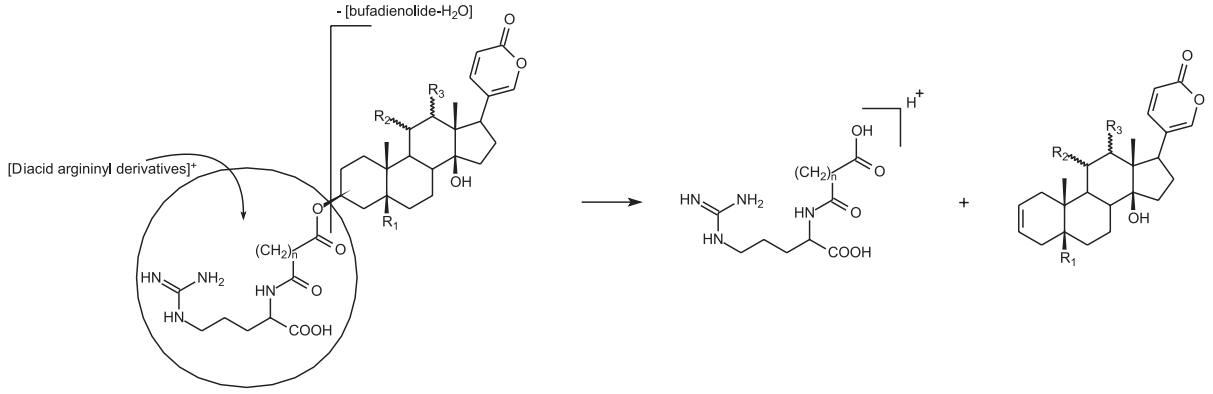

Figure 8. Proposed fragmentation pattern mechanism of general bufotoxins (group V) (adapted from reference 36).

There were 11 different peaks at *m/z* between 669.4 and 729.4 Da, which were attributed to the bufotoxins class by UPLC-ESI-MS and DI-ESI-MS/MS, and their respective retention time and fragmentations are shown in Figure 5 and Table 2, respectively. The detected ions at *m/z* 669.4, 685.5 and 687.5, presented the same characteristic fragment, due to the loss of the diacid argininyl derivative with the peak at *m/z* 303.2 assigned to the species $[M + H]^+$ of adipoyl arginine and for each ion was attributed to a different bufadienolide. In this way, the identifications of compounds were 3-(*N*-adipoyl-argininyl)-resibufogenin (**14**), 3-(*N*-adipoyl-argininyl)-marinobufagin (**17**) and 3-(*N*-adipoyl-argininyl)-telocinobufagin (**24**), respectively. Similarly, the ions detected at *m/z* 696.5, 713.5, 714.5 and 729.5 presented a fragment at *m/z* 331.3, attributed to the species $[M + H]^+$ of suberoyl arginine. The identifications of compounds for each *m/z* were 3-(*N*-suberoyl-argininyl)-resibufogenin (**15**), 3-(*N*-suberoyl-argininyl)-marinobufagin (**19**), 3-(*N*-suberoyl-argininyl)-telocinobufagin (**26**) and an isomer mixture of 3-(*N*-suberoyl-argininyl)-ψ-bufarenogin (**30**) and 3-(*N*-suberoyl-argininyl)-bufarenogin (**28**), respectively (Figure 1).

For each fragmented ion, the base peak appeared at *m/z* of its respective diacid argininyl derivative $[M + H - \text{bufadienolide} + H_2O]^+$. Furthermore, the characteristic fragment from argininyl diacids at *m/z* 250.1 of adipoyl arginine and 278.2 of suberoyl arginine, thus a common fragment at *m/z* $[M + H - H_2O]^+$, generated mainly from hydroxyl groups, was presented in the bufadienolide portion of bufotoxins.

The ion detected at *m/z* 671.4 presented simultaneous fragments correspondent for two diacid argininyl derivatives, at *m/z* 289.2 and 303.2, attributed to the species $[M + H]^+$ of glutaroyl arginine and adipoyl arginine, respectively. By the extracted ion chromatogram (EIC) of this ion, two peaks with different retention times indicate the existence of isomers containing the presence of different bufadienolides assigned as marinobufagin and bufalin. These compounds were identified as 3-(*N*-glutaroyl-

Table 2. Identification of the EMRJ compounds by UPLC-ESI-MS and DI-ESI-MS/MS in positive ion mode

Retention time / min	Compound	$[M + H]^+$	MS/MS (percentage abundance / %)	Identification
1.2	**8**	303.2	285.2 (23), 268.2 (28), 250.1 (100), 175.1 (31), 159.1 (21)	adipoyl arginine[26]
1.3	**9**	317.3	299.2 (18), 282.2 (17), 264.2 (100), 175.1 (19), 158.1 (21)	pimeloyl arginine[26]
1.4	**10**	331.3	313.2 (16), 296.2 (8), 278.2 (100), 175.2 (16), 158.1 (23)	suberoyl arginine[26]
4.1	**13**	219.1	160.0 (100)	bufotenidin[26]
5.9	**12**	203.1	188.1 (100)	dehydrobufotenine[26]
6.2	**11**	345.3	323.2 (46), 308.2 (29), 273.1 (100), 175.1 (19), 158.1 (47)	azelayl arginine[26]
10.3/10.6/13.5	**29/27/25**	701.5	682.5 (100), 317.3 (51), 303.3 (8)	3-(*N*-adipoyl-argininyl)-ψ-bufarenogin[36]/3-(*N*-adipoyl-argininyl)-bufarenogin[36]/3-(*N*-pimeloyl-argininyl)-telocinobufagin[26]
12.6/12.9	**7/6**	417.3	399.3 (100), 381 (11), 372.3 (30), 363.3 (27), 351.3 (15), 335.3 (25)	ψ-bufarenogin[23]/bufarenogin[27]
12.9/ 14.6	**16/22**	671.5	653.5 (86), 303.2 (35), 289.2 (100)	3-(*N*- glutaroyl-argininyl)-marinobufagin[26]/3-(*N*-adipoyl-argininyl)-bufalin[26]
12.9	**24**	687.5	668.5 (73), 303.2 (100),	3-(*N*-adipoyl-argininyl)-telocinobufagin[26]
13.1/ 13.8	**30/28**	729.5	711.5 (41), 331.3 (100), 278.2 (43)	3-(*N*-suberoyl-argininyl)-ψ-bufarenogin[36]/3-(*N*-suberoyl-argininyl)-bufarenogin[36]
13.3	**17**	685.5	667.5 (52), 303.2 (100)	3-(*N*-adipoyl-argininyl)-marinobufagin[26]
13.5/ 15.3	**21/20**	727.5	709.5 (22), 658.3 (100), 345.3 (16), 331.2 (51), 278.1 (11)	3-(*N*-suberoyl-argininyl)-bufotalinin[30]/3-(*N*-azelayl-argininyl)-marinobufagin[26]
13.9/16.0	**18/23**	699.5	681.5 (100), 331.3 (34), 317.3 (50), 278.2 (17)	3-(*N*-pimeloyl-argininyl)-marinobufagin[26]/3-(*N*-suberoyl-argininyl)-bufalin[26]
14.1	**26**	714.5	696.5 (48), 331.3 (100), 278.2 (18)	3-(*N*-suberoyl-argininyl)-telocinobufagin[26]
14.5	**19**	713.5	695.5 (47), 331.2 (100), 278.2 (21)	3-(*N*-suberoyl-argininyl)-marinobufagin[26]
14.6	**5**	403.3	385.3 (18), 349.3 (100), 303.3 (23)	telocinobufagin[27]
15.3	**14**	669.5	651.4 (38), 303.2 (100)	3-(*N*-adipoyl-argininyl)-resibufogenin[36]
15.4	**2**	401.3	383.3 (33), 365.3 (100), 347.3 (91), 329 (18), 319.3 (34), 251.2 (35)	marinobufagin[27]
16.4	**4**	387.3	369.2 (51), 351.3 (100), 333.3 (43), 323 (30), 305.3 (46), 255.2 (84)	bufalin[27]
16.9	**15**	696.5	672.0 (68), 331.3 (100)	3-(*N*-suberoyl-argininyl)-resibufogenin[36]

argininyl)-marinobufagin (**16**) and 3-(*N*-adipoyl-argininyl)-bufalin (**22**). Both compounds presented the same *m/z*, but each diacid argininyl derivatives are bounded to a different bufadienolides, which generated isomeric compounds.[36]

Similarly, the detected ion at *m/z* 699.5 showed peaks at *m/z* 317.3 and 331.3, correspondent to the species $[M + H]^+$ of pimeloyl and suberoyl arginine, and another ion at *m/z* 701.4 exhibited peaks at *m/z* 303.2 and 317.3, attributed to the species $[M + H]^+$ of adipoyl and pimeloyl arginine and the ion at *m/z* 727.5 presented peaks at *m/z* 331.3 and 345.3, attributed to the species $[M + H]^+$ of suberoyl and azelayl arginine, respectively. By their respective EICs, two or more peaks with different retention times could be observed, indicating the presence of isomers. In this way, for the ions at *m/z* 699.5 were identified as the bufotoxins 3-(*N*-pimeloyl-argininyl)-marinobufagin (**18**) and 3-(*N*-suberoyl-argininyl)-bufalin (**23**), at *m/z* 701.5 the compounds 3-(*N*-adipoyl-argininyl)-ψ-bufarenogin (**29**), 3-(*N*-adipoyl-argininyl)-bufarenogin (**27**) and 3-(*N*-pimeloyl-argininyl)-telocinobufagin (**25**) and at *m/z* 727.5 the compounds 3-(*N*-suberoyl-argininyl)-bufotalinin (**21**) and 3-(*N*-azelayl-argininyl)-marinobufagin (**20**).

All of these ions indicated the existence of isomeric compounds and showed a base peak of $[M + H - H_2O]^+$, a generic fragment observed in all bufotoxins identified. This MS information demonstrates that due to the presence of two or more compounds at the same *m/z*, this fragment has a relatively intense growth when compared with ions that

Table 3. Cytotoxic activity (IC_{50}) against tumor cell lines of compound **2**, ethyl acetate (EARJ) and methanolic extract (EMRJ)

Lineage	IC_{50} / (μg mL^{-1})			
	Compound **2**	EARJ	EMRJ	Doxorubicin
PC3	–	0.09 ± 0.02	0.90 ± 0.09	0.76 ± 0.12
HCT-116	0.07 ± 0.02	0.22 ± 0.06	1.78 ± 0.53	0.21 ± 0.03
SF-295	0.18 ± 0.02	–	> 5	0.41 ± 0.07
NCI-460	–	–	0.09 ± 0.02	0.15 ± 0.03
L-929	–	–	> 5	1.72 ± 0.9
HL-60	0.07 ± 0.01	0.02 ± 0.01	0.07 ± 0.02	0.02 ± 0.01
HEK-293	–	0.03 ± 0.04	0.02 ± 0.02	0.77 ± 0.12
SNB19	–	0.41 ± 0.19	0.35 ± 0.15	2.07 ± 0.29

PC3: prostate; HCT-116: colorectal; SF-295: glioblastoma; NCI-460: lung carcinoma; L-929: murine fibroblast; HL-60: leukemia; HEK-293: human embryonic kidney; SNB19: central nervous system.

presented only one compound, in which the base peak was their respective diacid argininyl derivatives. The retention times and the respective identification of compounds in methanolic extract is shown in Table 2. Mass spectra for all identified compounds of both extracts are shown in Figures S3 to S29.

Cytotoxicity activity

The cytotoxic activity of compound **2**, EARJ and EMRJ were determined by MTT assay and was evaluated against eight different tumor cell lines: PC3 (prostate), HCT-116 (colorectal), SF-295 (glioblastoma), NCI-460 (lung carcinoma), L-929 (murine fibroblast), HL-60 (leukemia), HEK-293 (human embryonic kidney) and SNB19 (central nervous system) as shown in Table 3. Compound **2** and EARJ, when compared with doxorubicin, presented similar or better IC_{50} against all tumor cell lines tested, demonstrating cytotoxic action equal to or greater than this positive control. The EMRJ showed lower IC_{50} values in only three lineages (NCI-460, HEK-293 and SNB19), indicating a potent cytotoxic activity when compared to doxorubicin.

The observed values demonstrate that compound **2** presented potent action against tumor cell lines tested. The ethyl acetate extract presented higher cytotoxicity than the methanolic extract for most tested lineages. This activity may be associated with the large amount of bufadienolides present in this extract, which can be demonstrated with the excellent activity of compound **2** that belongs to this class, while the methanolic extract had bufadienolides joined to a variety of different structures in its composition, together with other compounds.

Conclusions

The study of the two complementary extracts of *R. jimi* poison has led to the identification of 30 known compounds: 7 bufadienolides, 2 alkaloids, 5 diacids arginynil derivatives and 16 bufotoxins. The bufadienolides and bufotoxins were the main components of EARJ and EMRJ, respectively. All samples (EARJ, EMRJ and compound **2**) presented excellent cytotoxicity activity, with the EARJ being the most effective sample, furthermore, studies are needed to address the cytotoxic potential of the poison. The UPLC-MS combined with DI-MS/MS techniques performed well in identifying the composition of this amphibian poison. This work presents a quite extensive and detailed study about the chemical composition of the species and contributes to better comprehension of biochemical and pharmacological molecular knowledge of this native amphibian from Northeast of Brazil.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The authors are thankful for financial support to CNPq and CNPq/INCTBioNat (465637/2014-0) and CAPES (1776790/2017-0).

Author Contributions

Mariluce G. Fonseca contributed in the collect of the venom and identification of the species. Evaldo S. Monção Filho, Yara P. F. Pio, Gerardo M. Vieira Jr., Mariana H. Chaves and Bruno Q. Araújo contributed in the procedures of the extract's preparation and identification of the compounds. Paulo M. P. Ferreira, Cláudia Pessoa and Daisy J. B. Lima contributed with cytotoxic activity of the extracts and compound **2** by MTT assay. Evaldo S. Monção Filho also contributed with the draft of the article. Gerardo M. Vieira Jr. contributed with revision and critical reading of the

manuscript. Gerardo M. Vieira Jr. and Mariana H. Chaves has coordinated and supervised the laboratory work. All the authors have read the final manuscript and approved the submission.

References

1. Sales, D. L.; Morais-Braga, M. F. B.; dos Santos, A. T. L.; Machado, A. J. T.; de Araújo-Filho, J. A.; Dias, D. Q.; da Cunha, F. A. B. D.; Saraiva, R. A.; de Menezes, I. R. A.; Coutinho, H. D. M.; Costa, J. G. M.; Ferreira, F. S.; Alves, R. R. D. N.; Almeida, W. O.; *Biomed. Pharmacother.* **2017**, *92*, 554.
2. Córdova, W. H. P.; Leitão, S. Z.; Cunha-Filho, G.; Bosch, R. A.; Alonso, I. P.; Pereda-Miranda, R.; Gervou, R.; Touza, N. A.; Quintas, L. E. M.; Noel, F.; *Toxicon* **2016**, *110*, 27.
3. Mariano, D. O. C.; Prezotto-Neto, J. P.; Spencer, P. J.; Sciani, J. M.; Pimenta, D. C.; *J. Proteomics* **2019**, *209*, 103525.
4. Toledo, R. C.; Jared, C.; *Comp. Biochem. Physiol.* **1995**, *111*, 1.
5. Sousa, L. Q.; Machado, K. D.; Oliveira, S. F.; Araújo, L. D.; Monção-Filho, E. S.; Melo-Cavalcante, A. A.; Vieira-Júnior, G. M.; Ferreira, P. M. P.; *Toxicon* **2017**, *172*, 63.
6. Cunha-Filho, G. A.; Schwartz, C. A.; Resck, I. S.; Murta, M. M.; Lemos, S. S.; Castro, M. S.; Kyaw, C.; Pires-Júnior, O. R.; Leite, J. R. S.; Bloch-Júnior, C.; Schwartz, E. F.; *Toxicon* **2005**, *45*, 777.
7. Tempone, A. G.; Pimenta, D. C.; Lebrun, I.; Sartorelli, P.; Taniwaki, N. N.; de Andrade-Júnior, H. F.; Antoniazzi, M. M.; Jared, C.; *Toxicon* **2008**, *52*, 13.
8. Ferreira, P. M. P.; Lima, D. J. B.; Debiasi, B. W.; Soares, B. M.; Machado, K. C.; Noronha, J. C.; Rodrigues, D. J.; Shinhorin, A. P.; Pessoa, C.; Vieira-Junior, G. M.; *Toxicon* **2013**, *72*, 43.
9. Sciani, J. M.; De-Sá-Júnior, P.; Ferreira, A. K.; Pereira, A.; Antoniazzi, M. M.; Jared, C.; Pimenta, D. C.; *Biomed. Prev. Nutr.* **2013**, *1*, 10.
10. Oliveira, A. F.; Castoldi, L.; Vieira-Júnior, G. M.; Monção-Filho, E. S.; Chaves, M. H.; Rodrigues, D. J.; Sugui, M. M.; *Acta Amazonica* **2019**, *49*, 149.
11. Supratman, U.; Fugita, T.; Akiyama, K.; Hayashi, H.; *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1310.
12. Wang, D. L.; Qi, F. H.; Tang, W.; Wang, F. S.; *Chem. Biodiversity* **2011**, *8*, 559.
13. Vigerelli, H.; Sciani, J. M.; Jared, C.; Antoniazzi, M. M.; Caporale, G. M. M.; Silva, A. C. R.; Pimenta, D. C.; *J. Venomous Anim. Toxins* **2014**, *20*, 45.
14. Mijatovic, T.; Dufrasne, F.; Kiss, R.; *Pharm. Pat. Anal.* **2012**, *1*, 91.
15. Stevaux, M. N.; *Rev. Bras. Zool.* **2002**, *19*, 235.
16. Daly, J. W.; Noimai, N.; Kongkathip, B.; Kongkathip, N.; Wilham, J. M.; Garraffo, H. M.; Kaneko, T.; Spande, T. F.; Nimit, Y.; Nabhitabhata, J.; Chan-Ard, T.; *Toxicon* **2004**, *44*, 805.
17. Krenn, L.; Kopp, B.; *Phytochemistry* **1998**, *48*, 1.
18. Machado, K. C.; Sousa, L. Q.; Lima, D. J. B. L.; Soares, B. M.; Cavalcanti, B. C.; Maranhão, S. S.; Noronha, J. C.; Rodrigues, D. J.; Militão, G. C. G.; Chaves, M. H.; Vieira-Júnior, G. M.; Pessoa, C.; Moraes, M. O.; Sousa, J. M. C.; Cavalcante, A. A. C. M.; Ferreira, P. M. P.; *Toxicol. Lett.* **2018**, *285*, 121.
19. Zulfiker, A. H. M.; Sohrabi, M.; Qi, J.; Matthews, B.; Wei, M. Q.; Grice, I. D.; *J. Pharm. Biomed. Anal.* **2016**, *129*, 260.
20. Vigerelli, H.; Sciani, J. M.; Pereira, P. M. C.; Lavezo, A. A.; Silva, A. C. R.; Collaço, R. C. O.; Rocha, T.; Bueno, T. C.; Pimenta, D. C.; *J. Venomous Anim. Toxins* **2020**, *26*, 20190050.
21. Liu, Y.; Xiao, Y.; Xue, X.; Zhang, X.; Liang, X.; *Rapid Commun. Mass Spectrom.* **2010**, *24*, 667.
22. Garcia, I. J. P.; Oliveira, G. C.; Valadares, J. M. M.; Banfi, F. F.; Andrade, S. N.; Freitas, T. R.; Monção-Filho, E. S.; Santos, H. L.; Vieira-Júnior, G. M.; Chaves, M. H.; Rodrigues, D. J.; Sanchez, B. A. M.; Varotti, F. P.; Barbosa, L. A.; *Steroids* **2019**, *152*, 108490.
23. Mossman, T.; *J. Immunol. Methods* **1983**, *65*, 55.
24. *GraphPad*, version 6.0; Intuitive Software for Science, San Diego, CA, USA, 2013.
25. Zhao, H.; Wu, X.; Wang, H.; Gao, B.; Yang, J.; Si, N.; Bian, B.; *J. Liq. Chromatogr. Relat. Technol.* **2014**, *37*, 1163.
26. Schmeda-Hirschmann, G.; Gomez, C. V.; de Arias, A. R.; Burgos-Edwards, A.; Alfonso, J.; Rolon, M.; Brusquetti, F.; Netto, F.; Urra, F. A.; Cárdenas, C.; *J. Ethnopharmacol.* **2017**, *199*, 106.
27. Ye, M.; Guo, D.; *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1881.
28. Dewick, P. M.; *Medicinal Natural Products*, 3rd ed.; John Wiley & Sons Ltd: Chichester, UK, 2009.
29. Kasai, H.; Tsubuki, M.; Shimada, K.; Nambara, T.; Honda, T.; *Chem. Pharm. Bull.* **2009**, *57*, 948.
30. Schmeda-Hirschmann, G.; Quispe, C.; Arana, G. B.; Theoduloz, C.; Urra, F. A.; Cárdenas, C.; *Toxicon* **2016**, *121*, 119.
31. Kerkhoff, J.; Noronha, J. C.; Bonfilio, R.; Sinhorin, A. P.; Rodrigues, D. J.; Chaves, M. H.; Vieira-Júnior, G. M.; *Toxicon* **2016**, *119*, 311.
32. Daly, J. W.; Myers, C. W.; Whittaker, N.; *Toxicon* **1987**, *25*, 1023.
33. Maciel, N. M.; Schwartz, C. A.; Pires-Júnior, O. R.; Sebben, A.; Castro, M. S.; Sousa, M. V.; Fontes, W.; Schwartz, E. N. F.; *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **2003**, *134*, 641.
34. Carey, F. A.; Sundberg, R. J.; *Advanced Organic Chemistry*, Part B, 4th ed.; Plenum Press: New York, USA, 2007.
35. Schemeda-Hirschmann, G.; Quispe, C.; Theoduloz, C.; Sousa-Júnior, P. T.; Parizotto, C.; *J. Ethnopharmacol.* **2014**, *155*, 1076.
36. Petroselli, G.; Raices, M.; Jungblut, L. D.; Pozzi, A. G.; Erra-Balsells, R.; *J. Mass Spectrom.* **2018**, *53*, 465.

Submitted: September 18, 2020
Published online: January 18, 2021