https://dx.doi.org/10.21577/0103-5053.20230052

J. Braz. Chem. Soc., Vol. 34, No. 10, 1398-1409, 2023

Article

Unraveling the Metabolomic Profile and Bioactivities of the Paratoid Gland Secretion from *Rhinella granulosa*

***Elcio Daniel S. Barros,[a,b] Evaldo S. Monção Filho,[b] Mariluce G. Fonseca,[c] Patrícia S. Alves,[d] Antônia Laíres S. Santos,[d] Chistiane M. Feitosa,[d] Dulce Helena S. Silva,[e] Pedro Mikael S. Costa,[f] Cláudia Pessoa,[f] Carmem D. L. Campos,[g] Cristina A. Monteiro,[g] Mariana Helena Chaves*[*,b] *and Gerardo M. Vieira Júnior*[*,b]**

[a]*Departamento de Ensino, Pesquisa e Extensão, Instituto Federal de Educação, Ciência e Tecnologia do Maranhão, 65620-000 Coelho Neto-MA, Brazil*

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

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

[d]*Laboratório de Produtos Naturais e Neuroquímica Experimental, Núcleo de Tecnologia Farmacêutica, Universidade Federal do Piauí, 64049-550 Teresina-PI, Brazil*

[e]*Instituto de Química de Araraquara, Departamento de Química Orgânica, Universidade Estadual Paulista, 14800-900 Araraquara-SP, Brazil*

[f]*Laboratório de Oncologia Experimental, Núcleo de Pesquisa e Desenvolvimento de Medicamentos, Universidade Federal do Ceará, 60430-275 Fortaleza-CE, Brazil*

[g]*Departamento de Biologia, Instituto Federal de Educação, Ciência e Tecnologia do Maranhão, 65030-005 São Luís-MA, Brazil*

Toads of the *Rhinella* genus have a pair of paratoid glands that store biological secretions of high toxicity and varied chemical composition, rich in biologically active compounds. The present work aimed to carry out the investigation of the metabolomic profile and evaluation of the biological potential of the secretion paratoid glands (PGS) from *Rhinella granulosa*. The paratoid secretion was collected in the Piauí state (Brazil), extracted with methanol and the extract was analyzed by ultra-performance liquid chromatography with quadrupole time-of-flight mass spectrometry. Fifty chemical constituents were identified. The extract showed cytotoxicity against tumor cell lines of the central nervous system (half maximal inhibitory concentration (IC_{50}) = 1.9 μg mL^{-1}) and prostate (IC_{50} = 1.6 μg mL^{-1}), unsatisfactory antimicrobial potential (minimal inhibitory concentration (MIC) > 312 μg mL^{-1}) and inhibited the enzyme acetylcholinesterase (IC_{50} = 5.119 mg mL^{-1}). The results presented relevant information about the PGS and contributed to the understanding of the metabolomic and biological potential of *R. granulosa*.

Keywords: *Rhinella granulosa*, toad secretion, UPLC-QToF-MS/MS, fingerprint, biological activities

Introduction

Natural products have been widely used by humanity since ancient times, mainly in the prevention, treatment, and cure of various diseases.[1] The use of animal body parts and products of their metabolism (biological secretions and excrements) for the treatment of diseases, as well as for hunting, defense, and execution of prisoners, was a common practice among peoples of the Ancient Age.[2,3] Brazil has a rich herpetofauna, with a large number of amphibian species.[4] A total of 1026 species of amphibians have already been recorded in the country, of which 988 belong to the order Anura, representing the largest known

*e-mail: mariana@ufpi.edu.br; magela@ufpi.edu.br
Editor handled this article: Paulo Cezar Vieira

anurofauna in the world, distributed in all Brazilian biomes.[5,6]

The Bufonidae family has a cosmopolitan geographic distribution, being present on all continents except Antarctica.[7,8] In Brazil, the Bufonidae family is represented by eight genera, totaling 85 species, with the *Rhinella* genus being the most common.[9] Toads of the *Rhinella* genus have a worldwide distribution, totaling about 200 species, most frequently in humid and tropical regions.[10,11]

Anurans of the Bufonidae family have a pair of paratoid glands, present in the dorsolateral region of the body, which store biological secretions of high toxicity and varied chemical composition, having the function of defense against infections, microorganisms, and predators.[12] A wide variety of compounds such as steroids (bufadienolides and bufotoxins), arginine diacids, alkaloids, peptides and proteins can be found in the paratoid secretions of amphibians.[13] Bufadienolides have proven cardiotonic activity, while peptides and proteins are biomolecules, commonly referred to as toxins, which generally act on the central nervous system.[14] About 2000 peptides have already been identified in amphibian glandular secretions. Among them are angiotensins, neuropeptides, myotropic peptides, antimicrobials and several others with a wide variety of biological activities.[15]

The biological secretions produced through glands found in the skin of anurans are rich in biologically active components and have great biotechnological potential.[16] In ancient civilizations, secretion paratoid glands (PGS) of toads was already used as a diuretic, cardiac stimulant, expectorant, analgesic, and anti-inflammatory.[17,18] *In vitro* studies showed compounds in paratoid secretions of these animals with several biological activities, such as antimalarial, antifungal, antitrypanosomal, antiviral, antileishmanial, antibacterial, insecticidal, anesthetic, and cytotoxic.[4,11,19]

Rhinella granulosa (Figure 1) is a toad found in different ecosystems with a wide distribution in the Brazilian Northeast and some southeastern Brazilian states, occurring mainly in the Caatinga biome, being more easily evidenced in the vicinity of streams, ponds, and water puddles.[20,21] It is a small animal (48 to 53 mm) that has a nocturnal habit, explosive reproduction, and a diet consisting of arthropods, ants, and coleopterans.[22] These toads have a back covered by small irregular granules (wart-like), varied coloration (from burnt yellow to brown), a whitish belly (cream), small dark spots scattered along the body, and inconspicuous paratoid glands, located just behind the eyes.[23]

Considering the significant occurrence of *R. granulosa* in the southern region of the state of Piauí (Northeast Brazil) and the lack of scientific research on the PGS of this species, the present work aimed to carry out the investigation of the metabolomic profile and evaluation of the cytotoxicity, antimicrobial and anticholinesterase activities of the paratoid gland secretion from *R. granulosa.*

Figure 1. Picture of a *Rhinella granulosa.*

Experimental

Obtaining the PGS

The toads of the species *Rhinella granulosa* were identified by biologists of the Federal University of Piauí, Picos campus, under the supervision of herpetologist, Prof Dr Mariluce Gonçalves Fonseca (IBAMA/SISBIO No. 22508-2). Through manual compression of the animal's paratoid glands in their natural habitat, the biological secretion of interest in this study was obtained. PGS was collected from toads distributed in the city of Picos (7°04'48"S, 41º26'10"W), located in the southern region of the Piauí state (Northeast Brazil), during the month of February 2022. After collection, the animals were returned to their natural habitat without injuries and/or bruises.

The collection of PGS from the animals was carried out after authorization from the Ethics Committee on the Use of Animals of the Federal University of Piauí (CEUA/UFPI No. 52107-2), supported by the research registration (SisGen No. AE58A09) and the permanent license for collection of zoological material (IBAMA/SISBIO No. 55970-1).

Preparation of extract from paratoid secretion

During collection, the biological secretion was deposited in disposable plastic bottles and stored in a desiccator with silica for 72 h at room temperature (under vacuum). After this period, the dry PGS was transferred to a glass bottle and placed in a freezer at 4 °C. The extract was prepared by adding 50 mL of methanol (MeOH, Synth, Diadema, Brazil) to 1 g of PGS powder. The mixture was subjected to sonication in an ultrasonic (Ultronic, Indaiatuba, Brazil) bath for 15 min (four times), followed by simple filtration.

The MeOH extract of PGS (yield of 55%) was obtained after rotoevaporation (Heidolph, Schwabach, Germany) of the solvent.

UPLC-QToF-MS/MS analysis

Ultra-performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS/MS) analysis was performed on a Waters Acquity UPLC Xevo G2-XS Q-TOF instrument (Waters, Milford, USA) with an electrospray ionization interface (ESI). The chromatographic separation was performed using an Acquity UPLC®HSS T3 column (2.1 × 100 mm, 1.8 µm), with a mobile phase composed of ultrapure H_2O + 0.1% formic acid (Merck, Darmstadt, Germany) (A) and MeCN (Sigma-Aldrich, Saint Louis, USA) + 0.1% formic acid (B). The elution gradient used was: 10 to 100% B in 8 min, maintaining the condition of 100% B for 0.2 min, returning to 10% B at t = 8.20 min, and maintaining this gradient until the time of 10 min. 0.1 µL aliquots of the samples were injected at a flow rate of 0.5 mL min^{-1}, and the samples were solubilized (0.5 mg mL^{1}) in H_2O/MeCN (3:7, v/v).

UPLC-MS data were obtained in positive ion detection mode. The parameters defined for MS were mass range *m/z* between 100-1500 Da, source temperature of 100 °C, capillary voltage of 2500 V, desolvation temperature of 250 °C, 40 V cone voltage, desolvation gas flow of 550 L h^{-1}, cone gas flow of 50 L h^{-1}, centroid mode, and 0.2 s^{-1} scan time. The MS/MS mode analysis was performed based on the application of a collision-induced dissociation energy gradient ranging from 10 to 30 eV.

Cytotoxic assay

The tumor lines used, HCT-116 (human colon), SNB-19 (glioblastoma), and PC-3 (prostate), were provided by the National Cancer Institute (USA), cultivated in RPMI-1640 medium (Thermo Fisher Scientific, Paisley, UK), and the non-tumor lineage L929 (murine fibroblast) was cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Thermo Fisher Scientific, Paisley, UK), supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Paisley, UK) and 1% antibiotics (Penicillin-Streptomycin, Thermo Fisher Scientific, New York, USA), and kept in an oven at 37 ºC and an atmosphere containing 5% CO_2. The sample was diluted in pure sterile dimethyl sulfoxide (DMSO, Tedia, Fairfield, USA), obtaining a stock solution with a concentration of 50 mg mL^{-1}, which was further diluted in working solutions to concentrations between 250 and 1.95 µg mL^{-1}.

The cytotoxicity of the MeOH extract from *R. granulosa* was evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, Saint Louis, USA) colorimetric method, using serial sample dilution.[24] Cells were plated at concentrations of 7.0×10^4, 1.0×10^5, 1.0×10^5, and 7.0×10^4 cells mL^{-1} for the HCT-116, SNB-19, PC-3, and L929 strains, respectively. The plates were incubated with the MeOH extract for 72 h in an oven at 5% CO_2 at 37 ºC. After this period, the plates were centrifuged and the supernatant removed. Then, 100 µL of the MTT solution (tetrazolium salt) was added, and the plates were incubated for 3 h. After incubation, the plates were centrifuged again to remove the MTT solution. The absorbances were measured after dissolving the formazan precipitate with 100 µL of pure DMSO in a plate spectrophotometer (PerkinElmer, Pontyclun, UK) at 595 nm.

The absorbances obtained in the test were used to calculate the concentration capable of inhibiting 50% of cell growth (IC_{50}) of the sample through non-linear regression using the GraphPad Prism program (version 8.0).[25] The MeOH extract from *R. granulosa* was tested in triplicate in three independent experiments. Doxorubicin (Sigma-Aldrich, Saint Louis, USA) was used as a positive control.

Antimicrobial assay

Staphylococcus aureus (ATCC 29213), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 90028), and *Candida krusei* (ATCC 6258) were kindly provided by the Biology Laboratory Microorganism Collection of the Federal Institute of Maranhão, Monte Castelo Campus. Bacteria were cultured on Mueller-Hinton agar (MH, Merck, Darmstadt, Germany) at 37 ºC for 24 h, and the yeasts were cultured onto Sabouraud dextrose agar (SDA, Merck, Darmstadt, Germany) at 37 ºC for 48 h before tests. During the experiments, each culture medium was kept at 4°C.

The minimal inhibitory concentration (MIC) of the MeOH extract from *R. granulosa* was determined using the broth dilution method, as recommended by the Clinical Laboratory Standards Institute (CLSI).[26] For this method, 190 µL *per* well of MH broth or 200 µL *per* well RPMI-1640 (Sigma-Aldrich, Poole, UK) buffered with 0.165 mol L^{-1} morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich, Saint Louis, USA) for bacteria or yeasts, respectively, were added to 96-well microplates.

Before experiments, extract powder dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) was diluted in RPMI-1640 or MH broth medium, depending on whether the tests were against yeast or

bacteria, respectively. Then, an aliquot of the MeOH extract (100 µL *per* well) was added to the first well of 96-well microplates, and serial dilutions were carried out in subsequent wells. Tested concentrations of MeOH extract were 2500-4.88 µg mL^{-1}. Fluconazole (FLZ, Sigma-Aldrich, Poole, UK) and ciprofloxacin (CPR, Sigma-Aldrich, Poole, UK) were used as positive controls.

Following that, 100 µL of RPMI-diluted *Candida* inoculum (1×10^3 colony forming units (CFU) mL^{-1}) or 10 µL of saline-diluted bacteria inoculum (1.5×10^8 CFU mL^{-1}) was added to each well and incubated at 37 ºC for 24 h in RPMI-1640 medium. After the incubation period, the MIC was defined as the lowest concentration that visibly inhibited fungal growth. MIC values were confirmed after adding 10 µL of resazurin 0.03% (Thermo Fisher Scientific, Waltham, USA) to each well and incubating for 4 h in the dark at 37 ºC. RPMI-1640 or MH (100 µL) plus standardized inoculum was used as a negative control. Sterile DMSO (1% in saline) was also used as a negative control. The results were obtained from three independent assays performed in triplicate.

Acetylcholinesterase enzyme inhibiting assay

The inhibition potential of the acetylcholinesterase enzyme (AChE, Sigma-Aldrich, Saint Louis, USA) was determined in 96-well microplates by the method of Ellman, modified by Rhee *et al.*[27] Initially, solutions (50 mmol L^{-1}) of buffer A (tris(hydroxymethyl)aminomethane (Sigma-Aldrich, São Paulo, Brazil) + HCl (Synth, Diadema, Brazil), at pH 8; Tris/HCl; buffer B (Tris/HCl + 0.1% bovine albumin (ACS Scientific, Sumaré, Brazil)); buffer C (Tris/HCl + NaCl (Neon, São Paulo, Brazil) 0.1 mol L^{-1} + 0.02 mol L^{-1} $MgCl_2 \cdot 6H_2O$ (Vetec, Rio de Janeiro, Brazil)); 15 mmol L^{-1} iodide of acetylcholine (ATCI, Merck, Darmstadt, Germany); 3 mmol L^{-1} 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Thermo Fisher Scientific, Waltham, USA) and AchE 0.22 U mL^{-1} (10 units of AchE enzyme diluted in 45.45 mL in buffer B) were prepared.

The quantitative test for AchE enzyme inhibition was performed, in triplicate, by dissolving 10 mg of MeOH extract in buffer A and 10% ethanol (Synth, Diadema, Brazil) (stock solution). From this solution, working solutions were prepared (dilution in buffer A) in concentrations of 10, 5, 2.5, 1.25, and 0.625 mg mL^{-1}. 25 µL of MeOH extract solution, 50 µL of buffer B, and 25 µL of AchE 0.22 U mL^{-1} were added to the microplate wells. The blank was prepared with 25 µL of buffer A with 10% ethanol in 50 µL of buffer B with 25 µL of AchE 0.22 U mL^{-1}. The microplate with the extract solutions was kept in an oven for 15 min at 37 °C. Subsequently, 125 µL of 3 mmol L^{-1} DTNB and 25 µL of 15 mmol L^{-1} ATCI were added to each investigated extract solution, and the plate was measured at λ = 405 nm, at 0 and 5 min, using a microplate reader model Polaris (Celer, Belo Horizonte, Brazil). Rivastigmine (Exelon, Basel, Switzerland) was used as the positive control.

Results and Discussion

Identification of chemical constituents of PGS

The investigation of the chemical profile of the methanolic extract of PGS from *Rhinella granulosa*, collected in the city of Picos (Caatinga biome) in the southern region of the state of Piauí (Brazil), allowed the identification of 50 constituents (Figure 2), distributed in six classes of compounds: one amino acid, one carboxylic acid, three indole alkaloids, five arginine derivatives, 16 bufadienolides, and 24 bufotoxins. The compounds were identified by comparison with data reported in the literature, considering the relative error, the elution order of the chromatographic column (retention time), and the main fragment ions of the protonated molecules (Table 1). The compounds evidenced in the matrix studied (Figure 3) are reported in glandular secretions of other anuran species of the genera *Bufo*, *Rhinella* and *Rhaebo*.[28-30] However, this is the first time that the amino acid arginine (**1**) and martinelic carboxylic acid (**50**) are identified in the PGS of toads of the genus *Rhinella*, especially from the South American continent.

Compound **1** was identified as the amino acid L-arginine, being reported for the first time in the secretion of the paratoid glands of toads of the *Rhinella* genus, Cao *et al.*[28] identified this amino acid in Chansu, a commercial product from the glandular secretions of *Bufo gargarizans* Cantor,

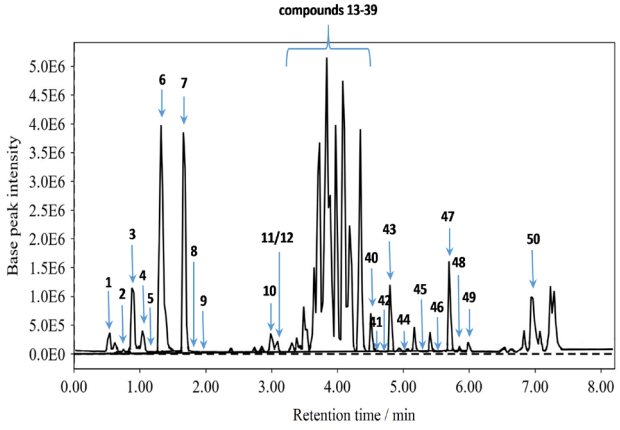
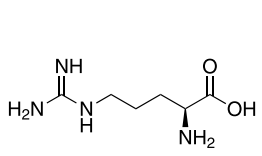
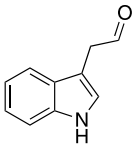


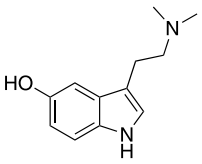
Figure 2. Total ion chromatogram of methanolic extract of the paratoid secretion from *R. granulosa.*



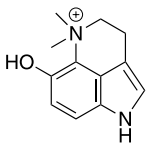
1



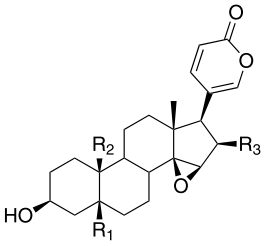
3



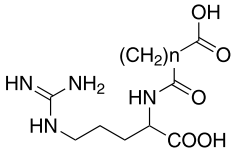
5



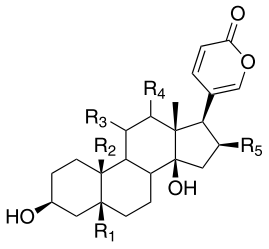
6



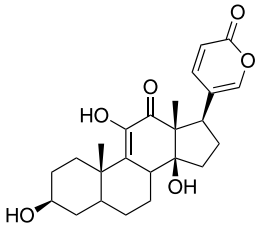
compound	R_1	R_2	R_3
10	H	CH_3	OAc
35	H	CH_2OH	H
36	OH	CHO	H
43	OH	CH_3	H
47	H	CHO	H
48	H	CH_3	H



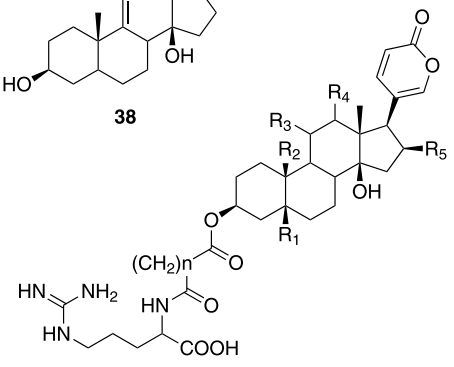
compound	n
2	4
4	5
7	6
8	7
9	8



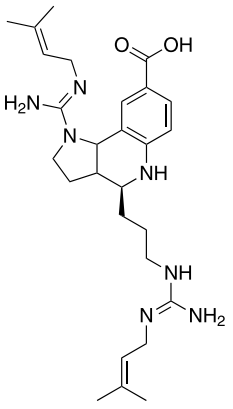
compound	R_1	R_2	R_3	R_4	R_5
15	H	CH_3	=O	β-OH	H
16	H	CH_3	=O	α-OH	H
23	H	CH_3	α-OH	=O	H
29	OH	CHO	H	H	H
33	H	CH_3	α-OH	H	H
40	H	CH_3	H	H	OH
42	OH	CH_3	H	H	H
46	H	CH_3	H	H	H



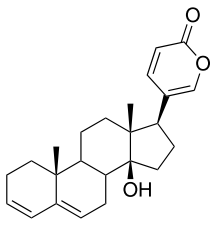
38



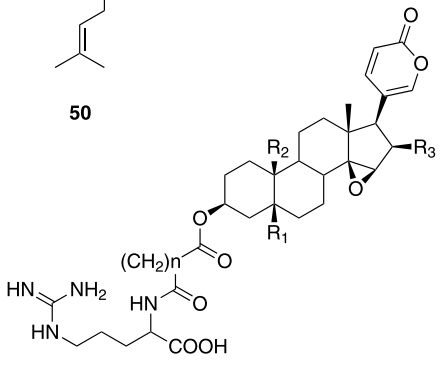
compound	R_1	R_2	R_3	R_4	R_5	n
11	H	CH_3	=O	β-OH	H	4
12	H	CH_3	=O	α-OH	H	4
13	H	CH_3	=O	β-OH	H	5
14	H	CH_3	=O	α-OH	H	5
17	H	CH_3	α-OH	=O	H	4
19	OH	CHO	H	H	OH	6
20	OH	CHO	H	H	H	4
21	H	CH_3	α-OH	=O	H	5
22	H	CH_3	α-OH	=O	H	6
24	OH	CH_2OH	H	H	H	6
26	OH	CHO	H	H	H	5
27	H	CH_3	α-OH	H	H	5
28	OH	CHO	H	H	H	6
30	H	CH_3	α-OH	H	H	6
32	OH	CH_3	H	H	H	5
37	OH	CH_3	H	H	H	6
44	H	CH_3	H	H	H	6



50



49



compound	R_1	R_2	R_3	n
18	OH	CHO	OH	6
25	OH	CHO	H	5
31	OH	CHO	H	6
34	OH	CH_3	H	5
39	OH	CH_3	H	6
41	H	CH_3	H	4
45	H	CH_3	H	6

Figure 3. Chemical constituents identified in the methanolic extract of PGS from *R. granulosa.*

Table 1. Identification of compounds of the MeOH extract of paratoid secretion from *R. granulosa* by UPLC-QToF-MS/MS fragmentation pattern in positive ion mode

Compound	t_R / min	$[M + H]^+$ measured	$[M + H]^+$ theorical	Error / ppm	MS/MS fragments	Molecular formula	Identification
1	0.54	175.1196	175.1190	3.43	158.9619, 141.9586, 128.9511, 116.0704	$C_6H_{15}N_4O_2$	arginine[28]
2	0.75	303.1664	303.1663	0.33	175.1186, 158.9617, 141.9585, 128.9509	$C_{12}H_{22}N_4O_5$	adipoyl arginine[29,31]
3	0,88	160.0763	160.0757	3.75	141.9586, 132.0811, 117.0574, 115.0541	$C_{10}H_9NO$	indole-3-acetaldehyde[32,33]
4	1.04	317.1828	317.1820	2.52	300.1547, 236.1122, 158.9619, 141.9585	$C_{13}H_{24}N_4O_5$	pimeloyl arginine[29,31]
5	1.06	205.1340	205.1335	2.44	160.0762, 141.9585, 128.9511, 115.0544	$C_{12}H_{16}N_2O$	bufotenin[31,34]
6	1.32	203.1183	203.1179	1.97	188.0947, 173.0712, 155.0593, 146.0600	$C_{12}H_{14}N_2O$	dehydrobufotenine[31,34]
7	1.66	331.1975	331.1976	0.30	314.1722, 250.1528, 158.9613, 141.9589	$C_{14}H_{26}N_4O_5$	suberoyl arginine[34,35]
8	1.81	345.2137	345.2133	1.16	264.1477, 236.1130, 158.9611, 141.9594	$C_{15}H_{28}N_4O_5$	azelayl arginine[29,36]
9	1.92	359.2294	359.2289	1.39	331.1974, 303.8829, 158.9616, 141.9591	$C_{16}H_{30}N_4O_5$	sebarcyl arginine[28,29]
10	3.09	443.2426	443.2428	0.45	415.2170, 379.1856, 361.1796, 343.8801	$C_{26}H_{35}O_6$	cinobufagin[28,29]
11 **12**	3.25	701.3763	701.3756	0.99	399.3544, 381.7643, 363.8644, 303.8815	$C_{36}H_{52}N_4O_{10}$	3-(*N*-adipoyl argininyl) bufarenogin[37,38] 3-(*N*-adipoyl argininyl) Ψ-bufarenogin[37,38]
13 **14**	3.30	715.3911	715.3913	0.28	697.3804, 679.3756, 399.2114, 317.1352	$C_{37}H_{54}N_4O_{10}$	3-(*N*-pimeloyl argininyl) bufarenogin[37,38] 3-(*N*-pimeloyl argininyl) Ψ-bufarenogin[37,38]
15 **16**	3.44	417.2281	417.2277	0.95	399.2153, 381.2068, 363.1902, 335.1985	$C_{24}H_{32}O_6$	bufarenogin[29,31] Ψ-bufarenogin[29,31]
17	3.46	701.3760	701.3756	0.57	683.3641, 399.2112, 371.0919, 303.8858	$C_{36}H_{52}N_4O_{10}$	3-(*N*-adipoyl argininyl) arenobufagin[28,29]
18	3.49	743.3870	743.3862	1.08	715.3915, 687.3599, 331.1930, 158.9615	$C_{38}H_{55}N_4O_{11}$	3-(*N*-suberoyl-argininyl) hydroxybufotalinin[29]
19	3.59	745.4013	745.4018	0.67	717.4047, 699.3597, 331.1956, 158.9617	$C_{37}H_{54}N_4O_{11}$	3-(*N*-suberoyl-argininyl) hydroxyhellebrigenin[28,29]
20	3.61	701.3782	701.3756	3,70	683.3600, 399.2170, 353.2113, 303.8827	$C_{36}H_{52}N_4O_{10}$	3-(*N*-adipoyl argininyl) hellebrigenin[28,29]
21	3.62	715.3912	715.3913	0.14	697.3796, 399.2170, 353.2113, 317.1860	$C_{37}H_{54}N_4O_{10}$	3-(*N*-pimeloyl argininyl) arenobufagin[28,29]
22	3.64	729.4069	729.4069	0.00	711.3950, 701.3782, 683.3600, 331.1974	$C_{38}H_{56}N_4O_{10}$	3-(*N*-suberoyl-argininyl) arenobufagin[28,29]
23	3.66	417.2274	417.2277	0.72	399.2170, 381.2043, 363.1939, 345.1824	$C_{24}H_{32}O_6$	arenobufagin[28,29]
24	3.73	731.4221	731.4226	0.68	713.4095, 695.3983, 383.2206, 331.1961	$C_{38}H_{58}N_4O_{10}$	3-(*N*-suberoyl-argininyl) hellebrigenol[28,31]
25	3.78	713.3758	713.3756	0.28	695.3670, 667.3652, 317.1356, 158.9617	$C_{37}H_{52}N_4O_{10}$	3-(*N*-pimeloyl argininyl) bufotalinin[29]
26	3.86	715.3936	715.3913	3.21	697.3795, 399.2171, 381.2044, 317.1851	$C_{37}H_{54}N_4O_{10}$	3-(*N*-pimeloyl argininyl) hellebrigenin[28,29]
27	3.87	701.4128	701.4120	1.14	683.4017, 665.3861, 317.1851, 158.9616	$C_{37}H_{56}N_4O_9$	3-(*N*-pimeloyl argininyl) gamabufotalin[28,29]
28	3.88	729.4080	729.4069	1.50	701.4128, 693.3851, 399.2171, 331.1968	$C_{38}H_{56}N_4O_{10}$	3-(*N*-suberoyl-argininyl) hellebrigenin[28,29]
29	3.89	417.2283	417.2277	1.44	399.2169, 381.2065, 363.1957, 335.2004	$C_{24}H_{32}O_6$	hellebrigenin[28,29]

Table 1. Identification of compounds of the MeOH extract of paratoid secretion from *R. granulosa* by UPLC-QToF-MS/MS fragmentation pattern in positive ion mode (cont.)

Compound	t_R / min	$[M + H]^+$ measured	$[M + H]^+$ theorical	Error / ppm	MS/MS fragments	Molecular formula	Identification
30	3.95	715.4268	715.4277	1.25	697.4122, 331.1970, 158.9615, 141.9592	$C_{38}H_{58}N_4O_9$	3-(*N*-suberoyl argininyl) gamabufotalin[28,29]
31	3.97	727.3929	727.3913	2.20	699.3959, 681.3848, 331.1976, 158.9614	$C_{38}H_{54}N_4O_{10}$	3-(*N*-suberoyl argininyl) bufotalinin[28,29]
32	3.99	701.4106	701.4120	1.99	683.3997, 665.3888, 317.1852, 158.9614	$C_{37}H_{56}N_4O_9$	3-(*N*-pimeloyl argininyl) telocinobufagin[29,31]
33	4.00	403.2485	403.2479	1.48	385.2386, 367.2290, 349.2179, 331.1966	$C_{24}H_{34}O_5$	gamabufotalin[28,29]
34	4.13	699.3953	699.3964	1.57	681.3846, 663.3691, 331.2026, 158.9616	$C_{37}H_{54}N_4O_9$	3-(*N*-pimeloyl argininyl) marinobufagin[31,34]
35	4.15	401.2320	401.2326	1.49	383.2213, 365.2110, 347.1998, 329.2545	$C_{24}H_{32}O_5$	resibufaginol[28,29]
36	4.17	415.2106	415.2115	2.16	397.1990, 379.1890, 361.1779, 343.1675	$C_{24}H_{30}O_6$	bufotalinin[31,34]
37	4.19	715.4265	715.4277	1.68	697.4149, 679.4058, 331.1949, 158.9615	$C_{38}H_{58}N_4O_9$	3-(*N*-suberoyl-argininyl) telocinobufagin[29,31]
38	4.32	415.2109	415.2115	1.45	397.1995, 379.1903, 361.1811, 343.1740	$C_{24}H_{30}O_6$	argentinogenin[28]
39	4.35	713.4135	713.4120	2.10	695.4001, 677.3895, 365.2106, 331.1959	$C_{38}H_{56}N_4O_9$	3-(*N*-suberoyl argininyl) marinobufagin[29,31]
40	4.48	403.2487	403.2479	1.98	385.2374, 367.2277, 349.2156, 331.2044	$C_{24}H_{34}O_5$	desacetylbufotalin[28]
41	4.59	669.3854	669.3858	0.60	367.2312, 349.2105, 303.8810, 158.9622	$C_{36}H_{52}N_4O_8$	3-(*N*-adipoyl argininyl) resibufogenin[29,37]
42	4.74	403.2495	403.2479	3.96	385.2342, 367.2282, 349.2163, 331.1655	$C_{24}H_{34}O_5$	telocinobufagin[29,31]
43	4.79	401.2346	401.2326	4.98	383.2236, 365.2130, 347.2020, 329.1909	$C_{24}H_{32}O_5$	marinobufagin[28,31]
44	4.82	699.4350	699.4327	3.29	681.4194, 351.2087, 331.8886, 158.9623	$C_{38}H_{58}N_4O_8$	3-(*N*-suberoyl-argininyl) bufalin[28,31]
45	5.06	697.4155	697.4171	2.29	679.4009, 331.1885, 250.1769, 158.9614	$C_{38}H_{56}N_4O_8$	3-(*N*-suberoyl argininyl) resibufogenin[29,37]
46	5.17	387.2547	387.2532	3.87	369.2437, 351.2334, 333.1860, 255.0787	$C_{24}H_{34}O_4$	bufalin[28,31]
47	5.62	399.2177	399.2166	2.75	381.2067, 363.1961, 345.1828, 335.2007	$C_{24}H_{30}O_5$	resibufagin[28,37]
48	5.69	385.2378	385.2373	1.30	367.2284, 349.2144, 329.1716, 321.2029	$C_{24}H_{32}O_4$	resibufogenin[29,37]
49	5.75	367.2267	367.2268	0.27	349.2156, 331.2092, 321.2212, 241.0657	$C_{24}H_{30}O_3$	scillaridin A[28]
50	6.94	496.3394	496.3395	0.20	480.3081, 478.3285, 454.2924, 426.3570	$C_{27}H_{41}N_7O_2$	martinelic acid[34]

t_R: retention time; $[M + H]^+$: protonated molecule; ppm: parts *per* million; Ψ: Psi.

used in traditional Chinese medicine for the treatment of various diseases, including cancer.

Compounds **3**, **5**, and **6** belong to the class of alkaloids. Although they occur mostly in plants, they can be found in animals, especially amphibians, which use these substances for their defense and protection against pathogens and predators.[39,40] Studies[41,42] have shown that the concentration of alkaloids in anurans can vary depending on the species, geographic location, diet, and other associated complex characteristics.

Compound **3** was identified as indole-3-acetaldehyde and was evidenced in the chemical composition of

Bufonis venenum, a product of the glandular secretions of *Bufo gargarizans* Cantor, widely used in traditional Chinese medicine.[32,33] Compound **5** was identified as bufotenin and has already been identified in methanolic extracts from toads of different species distributed on the oceanic continent.[31,34] Compound **6** was identified as dehydrobufotenine and has already been described in the PGS of *Rhinella schneideri* from the Brazilian Pantanal, *Rhinella marina* from Iquitos and from the Amazon region of Brazil, and *Rhinella jimi* from the state of Piauí, Northeast region of Brazil.[37,40]

Compounds **2**, **4**, **7**, **8**, and **9** belong to the class of arginine-derived diacids. As a homologous series, these compounds have a similar fragmentation pattern, characterized by the loss of H_2O, NH_3, and CO, being peculiar in the MS/MS mass spectra of these compounds the presence of fragment ions *m/z* 175 $[C_6H_{14}N_4O_2]^+$, characteristic of the arginine portion and *m/z* 158 $[C_6H_{14}N_4O_2\text{-}NH_3]^+$, referring the loss of the NH_3 group of this amino acid.[37,39] Also, the existence of fragment ions diverging by 14 Da was observed, referring to the CH_2 units of the carbonic portion of the diacid bound to arginine.[37,38] The arginine diacids identified in the PGS of *R. granulosa* were adipoyl arginine (**2**), pimeloyl arginine (**4**), suberoyl arginine (**7**), azelayl arginine (**8**), and sebarcyl arginine (**9**). These compounds are widely described in the glandular secretions of toads of the genera *Bufo*, *Rhinella*, and *Rhaebo*.[43]

Compounds **10**, **15**, **16**, **23**, **29**, **33**, **35**, **36**, **38**, **40**, **42**, **43**, **46**, **47**, **48**, and **49** belong to the class of bufadienolides, which are the major chemical constituents found in the PGS of anurans of the Bufonidae family.[44] These steroids are primarily responsible for the biological activities identified in amphibian glandular secretions.[45] More than one hundred bufadienolides identified in biological secretions of toads are described in the literature.[31] The bufadienolides identified in this work showed a similar fragmentation pattern, evidenced by the successive losses of water molecules $[M + H - H_2O]^+$, the α-pyrone group $[M + H - C_5H_4O_2]^+$, and the carbon monoxide group $[M + H - CO]^+$.

The bufadienolides identified in the MeOH extract of *R. granulosa* can be divided into two groups. The compounds cinobufagin (**10**), resibufaginol (**35**), bufotalinin (**36**), marinobufagin (**43**), resibufagin (**47**), and resibufogenin (**48**) have an epoxy group at the C-14 and C-15 carbons, while the compounds bufarenogin (**15**), Ψ-bufarenogin (**16**), arenobufagin (**23**), hellebrigenin (**29**), gamabufotalin (**33**), argentinogenin (**38**), desacetylbufotalin (**40**), telocinobufagin (**42**), bufalin (**46**), and scillaridin A (**49**) have a hydroxyl group at the C-14 carbon. The presence of cyclic ether in the compounds alters the fragmentation pattern, generating distinct fragment ions between the two groups of bufadienolides.[37]

Bufotoxins constitute the largest number of compounds identified in this study, totaling 24 metabolites. The number of bufotoxins identified in the glandular secretions of anurans is increasing, since there are numerous possibilities of combination between diacids, bufadienolides, and amino acids.[46] Bufotoxins are widely described in glandular secretions of toads of the *Bufo*, *Rhinella*, and *Rhaebo* genera distributed around the world.[36] A general fragmentation pattern for bufotoxins is the loss of the arginine-derived diacid $[M + H - \text{arginine diacid}]^+$, followed by the loss of an amine group $[M + H - NH_3]^+$ and one or two water molecules by the generated diacid.[43] A reduction of 28 Da in the mass of the compound was also observed, referring to the loss of the carbonyl group $[M + H - CO]^+$ and the loss of one or more water molecules by the bufadienolide portion.[37,38]

Similarly to the bufadienolides reported in this study, bufotoxins can also be organized into two distinct groups, characterized by the presence or absence of the epoxy group in the steroidal ring of the bufadienolide portion of the bufotoxin. The compounds 3-(*N*-suberoyl argininyl) hydroxybufotalinin (**18**), 3-(*N*-pimeloyl argininyl) bufotalinin (**25**), 3-(*N*-suberoyl argininyl) bufotalinin (**31**), 3-(*N*-pimeloyl argininyl) marinobufagin (**34**), 3-(*N*-suberoyl argininyl) marinobufagin (**39**), 3-(*N*-adipoyl argininyl) resibufogenin (**41**), and 3-(*N*-suberoyl argininyl) resibufogenin (**45**) have an epoxy group in their molecular structure, whereas in bufotoxins 3-(*N*-adipoyl argininyl) bufarenogin (**11**), 3-(*N*-adipoyl argininyl) Ψ-bufarenogin (**12**), 3-(*N*-pimeloyl argininyl) bufarenogin (**13**), 3-(*N*-pimeloyl argininyl) Ψ-bufarenogin (**14**), 3-(*N*-adipoyl argininyl) arenobufagin (**17**), 3-(*N*-suberoyl argininyl) hydroxyhellebrigenin (**19**), 3-(*N*-adipoyl argininyl) hellebrigenin (**20**), 3-(*N*-pimeloyl argininyl) arenobufagin (**21**), 3-(*N*-suberoyl argininyl) arenobufagin (**22**), 3-(*N*-suberoyl argininyl) hellebrigenol (**24**), 3-(*N*-pimeloyl argininyl) hellebrigenin (**26**), 3-(*N*-pimeloyl argininyl) gamabufotalin (**27**), 3-(*N*-suberoyl argininyl) hellebrigenin (**28**), 3-(*N*-suberoyl argininyl) gamabufotalin (**30**), 3-(*N*-pimeloyl argininyl) telocinobufagin (**32**), 3-(*N*-suberoyl argininyl) telocinobufagin (**37**), and 3-(*N*-suberoyl argininyl) bufalin (**44**), this structure is not observed. However, the observed structural difference does not change the fragmentation pattern of this class of compounds.[37,38]

Compound **50** was identified as martinelic acid, being evidenced for the first time in acetic extract from the skin of the Australian cane toad (*Bufo marinus*).[34] There are no reports in the literature of the identification of this carboxylic acid in glandular secretions of toads

in the *Rhinella* genus, distributed in the South American continent. It is the first time that martinelic acid has been detected in PGS from toads occurring in Brazil.

Cytotoxicity

The investigation of the cytotoxic potential of the biological secretions from anurans, as well as isolated metabolites of these fluids, has shown significant growth in recent years, especially the secretions from the paratoid glands of toads from the *Bufo*, *Rhinella*, and *Rhaebo* genera.[28-30] This animal matrix has attracted the attention of the scientific community, mainly due to the richness of compounds and the amount of biologically active metabolites, making it a promising alternative in the development of new therapeutic resources for the treatment of the most varied types of cancer.[47]

The use of the MTT dye reduction method showed that the MeOH extract from the secretion of the paratoid glands of *R. granulosa* produced a considerable cytotoxic effect in central nervous system (SNB-19) and prostate (PC-3) tumor cells, with no similar behavior being observed in the colorectal tumor cell line (HCT-116). Against SNB-19 tumor cells (glioblastoma), the MeOH extract showed similar cytotoxicity (IC_{50} = 1.9 μg mL^{-1}) to the positive control used (doxorubicin, IC_{50} = 2.0 μg mL^{-1}).

The cytotoxicity of the *R. granulosa* MeOH extract was also evaluated in the non-tumor cell line L929 (murine fibroblast) and did not present effect on growth inhibition, even when tested at the highest extract concentration, being more selective for tumoral cells. The IC_{50} value was above 125 μg mL^{-1}. Due to the low cytotoxicity of the extract on the L929 cell line, at the tested concentrations (1-125 μg mL^{-1}), it was not possible to obtain a dose-response curve to determine the exact IC_{50} value. The IC_{50} values, with a confidence interval of 95%, of the methanolic extract and doxorubicin against the tumor and non-tumor cell lines tested in this assay are presented in Table 2.

Antimicrobial activity

The glandular secretions from anurans are complex mixtures of bioactive compounds; however, the biological activities of many of these biomolecules are still unknown, especially regarding the antimicrobial activities of the compounds present in the secretions of the paratoid glands of toads.[48]

Table 2 presents the values of the MIC detected for the MeOH extract from the secretion of the paratoid glands of *R. granulosa* against the standard strains of *S. aureus* (ATCC 29213), *E. coli* (ATCC 25922), *C. albicans* (ATCC 90028), and *C. krusei* (ATCC 6258).

The MeOH extract of *R. granulosa* showed low antibacterial potential against strains of *S. aureus* (MIC = 312 μg mL^{-1}) and *E. coli* (MIC = 625 μg mL^{-1}), not showing relevance for practical uses (MIC of interest < 1.0 μg mL^{-1}). Similarly, the investigated extract did not show a satisfactory antifungal effect (MIC = 1250 μg mL^{-1}) against yeasts of the genus *Candida* (MIC of interest < 8 μg mL^{-1}). There are reports in the scientific literature[48] on the antimicrobial activity of PGS from other species of the genus *Rhinella*; however, this work is pioneer in showing the antibacterial potential of *R. granulosa.*

Potential to inhibit the enzyme acetylcholinesterase

The *in vitro* study carried out with the MeOH extract of PGS from *R. granulosa* at concentrations of 0.625, 1.25,

Table 2. Bioactivities of the MeOH extract of paratoid gland secretion from *Rhinella granulosa*

Sample/positive control	Antimicrobial activity (MIC) / (μg mL^{-1})				Cytotoxicity (IC_{50}) / (μg mL^{-1})				Anticholinesterase activity (IC_{50}) / (mg mL^{-1})
	S. aureus ATCC 29213	*E. coli* ATCC 25922	*C. albicans* ATCC 90028	*C. krusei* ATCC 6258	PC-3	SNB-19	HCT-116	L929	AChE
MeOH extract	312	625	1250	1250	1.6 (0.6-3.7)[a]	1.9 (0.9-3.8)	117.2 (80.0-202.5)	> 125	5.119 (4.411-6.059)[a]
Ciprofloxaxin	0.5	0.0625							
Fluconazole			8	16					
Doxorubicin					0.7 (0.5-0.9)	2.0 (1.7-2.4)	0.2 (0.1-0.3)	0.3 (0.29-0.34)	
Rivastigmine									0.059 (0.052-0.065)

[a]Confidence interval of 95%; MeOH extract: methanolic extract of the paratoid gland secretion from *Rhinella granulosa*; MIC: minimum inhibitory concentration; ATCC: American Type Culture Collection; *S. aureus*: *Staphylococcus aureus*; *E. coli*: *Escherichia coli*; *C. albicans*: *Candida albicans*; *C. krusei*: *Candida krusei*. Tumor lines: PC-3 (prostate), SNB-19 (glioblastoma), HCT-116 (colon), non-tumor lineage: L929 (murine fibroblast); AChE: acetylcholinesterase enzyme; IC_{50}: half maximal inhibitory concentration.

2.50, 5.00, and 10.00 mg mL^{-1} produced inhibitions of 17.45, 18.78, 27.83, 55.35, and 63.69% in the activity of the enzyme AChE, respectively. From this data set, the IC_{50} of the MeOH extract was calculated, which corresponded to 5.119 mg mL^{-1}, ranging from 4.411 to 6.059 mg mL^{-1}, with a confidence interval of 95%. These data are reported in Table 2.

Although this scientific investigation represents a preliminary study of bioprospecting of the paratoid secretion of *R. granulosa* from Northeast Brazil, future research may lead to the isolation of identified metabolites and investigation of the biological potential of these substances, like the studies developed by Tempone *et al*.,[19] Machado *et al*.[11] and Monção Filho *et al*.[37,40]

Conclusions

The investigation of the chemical profile of the methanolic extract of PGS from *Rhinella granulosa* allowed the identification of 50 constituents, distributed in six classes of compounds: one amino acid, one carboxylic acid, three indole alkaloids, five arginine-derived diacids, 16 bufadienolides, and 24 bufotoxins. The compounds evidenced in the matrix studied are reported in glandular secretions of other anuran species of the genera *Bufo*, *Rhinella* and *Rhaebo*. However, this is the first time that the compounds arginine (**1**) and martinelic acid (**50**) are identified in the PGS of toads of the genus *Rhinella*, mainly from the South American continent. The extract from the secretion of the paratoid glands of *R. granulosa* showed promising cytotoxicity, mainly against tumor cell lines of the central nervous system (SNB-19) and prostate (PC-3), unsatisfactory antimicrobial potential and considerable potential to inhibit the enzyme acetylcholinesterase. This study presents relevant information about the PGS analyzed and contributes to the understanding of the chemical and biological potential of *R. granulosa*, expanding the knowledge about the anurofauna of the state of Piauí (Brazil).

Supplementary Information

Supplementary information (Figures S1-S47) is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The authors are grateful to Prof Dr Everton Leandro de França Ferreira (Federal University of Vale do São Francisco-UNIVASF) and doctoral student Leonardo Santos Alexandre (USP-São Carlos), for obtaining the MS/MS spectra; to master student Felipe Pereira da Silva Santos (Federal University of Piauí-UFPI), for support in the AChE enzyme inhibition assay, to graphic designer Elton Maykow Sousa Barros (SEDUC-PI), for the elaboration of the graphical abstract and to the development agencies INCTBioNat (465637/2014-0), CAPES (1776790/2017-0), CNPq (302197/2017-6; 402302/2021-4) and FAPEMA (004348/2021) for financial support and scholarships.

Author Contributions

Elcio Daniel S. Barros was responsible for investigation, methodology, writing original draft, visualization and writing-review and editing; Evaldo S. Monção Filho for investigation, validation and writing original draft; Mariana Helena Chaves for resources and supervision; Mariluce G. Fonseca for investigation, resources and supervision; Patrícia S. Alves for formal analysis and data curation; Antônia Laíres S. Santos for formal analysis, validation and writing original draft; Chistiane M. Feitosa for supervision; Dulce Helena S. Silva for supervision; Pedro Mikael S. Costa for data curation, validation and writing original draft; Cláudia Pessoa for supervision; Carmem D. L. Campos for formal analysis and data curation; Cristina A. Monteiro for formal analysis, validation and writing original draft; Gerardo M. Vieira Júnior for resources and supervision; conceptualization, project administration and writing review and editing.

References

1. Costa Neto, E. M.; *Ciênc. Saúde Coletiva* **2011**, *16*, 1639. [Crossref]
2. Kerkhoff, J.; Noronha, J. C.; Bonfilio, R.; Sinhorin, A. P.; Rodrigues, D. J.; Chaves, M. H.; Vieira Jr., G. M.; *Toxicon* **2016**, *119*, 311. [Crossref]
3. Viegas Jr., C.; Bolzani, V. S.; Barreiro, E. J.; *Quim. Nova* **2006**, *29*, 326. [Crossref]
4. Ferreira, P. M. P.; Lima, D. J. B.; Debiasi, B. W.; Soares, B. M.; Machado, K. C.; Noronha, J. C.; Rodrigues, D. J.; Sinhorin, A. P.; Pessoa, C.; Vieira Jr., G. M.; *Toxicon* **2013**, *72*, 43. [Crossref]
5. Saporito, R. A.; Donnelly, M. A.; Spande, T. F.; Garraffo, H. M.; *Chemoecology* **2012**, *22*, 159. [Crossref]
6. Coutinho, L. M.; *Acta Bot. Bras.* **2006**, *20*, 13. [Crossref]
7. Pramuk, J. B.; *Zool. J. Linn. Soc.* **2006**, *146*, 407. [Crossref]
8. de Sousa, L. Q.; Machado, K. C.; Oliveira, S. F. C.; Araújo, L. S.; Monção-Filho, E. S.; Melo-Cavalcante, A. A. C. M.; Vieira Jr., G. M.; Ferreira, P. M. P.; *Toxicon* **2017**, *127*, 63. [Crossref]
9. Pyron, R. A.; Wiens, J. J.; *Mol. Phylogenet. Evol.* **2011**, *61*, 543. [Crossref]
10. Gadelha, I. C. N.; Melo, M. M.; Soto-Blanco, B.; *Rev. Bras. Hig. Sanid. Anim.* **2015**, *9*, 195. [Crossref]

11. Machado, K. C.; de Sousa, L. Q.; Lima, D. J. B.; Soares, B. M.; Cavalcanti, B. C.; Maranhão, S. S. A.; Noronha, J. C.; Rodrigues, D. J.; Militão, G. C. G.; Chaves, M. H.; Vieira Jr., G. M.; Pessoa, C.; de Moraes, M. O.; e Sousa, J. M. C.; Cavalcante, A. A. C. M.; Ferreira, P. M. P.; *Toxicol. Lett.* **2018**, *285*, 121. [Crossref]
12. Alexandre, L. S.; Braga, F. M. S.; de Oliveira, P. K.; Coelho, T. L. S.; Fonseca, M. G.; de Sousa, R. W. R.; Dittz, D.; Sousa, J. M. C.; Ferreira, P. M. P.; Dantas, C.; Barbosa, H. S.; Chaves, M. H.; Lopes Jr., C. A.; Vieira Jr., G. M.; *Toxicon* **2021**, *192*, 32. [Crossref]
13. Clarke, B. T.; *Biol. Rev. Cambridge Philos. Soc.* **1997**, *72*, 365. [Crossref]
14. Utkin, Y. N.; *World J. Biol. Chem*. **2015**, *6*, 28. [Crossref]
15. Xu, X.; Lai, R.; *Chem. Rev.* **2015**, *115*, 1760. [Crossref]
16. Oliveira, A. F.; Castoldi, L.; Vieira Jr., G. M.; Monção Filho, E. S.; Chaves, M. H.; Rodrigues, D. J.; Sugui, M. M.; *Acta Amaz.* **2019**, *49*, 145. [Crossref]
17. Chen, K. K.; Kovariková, A.; *J. Pharm. Sci.* **1967**, *56*, 1535. [Crossref]
18. Chen, Y.-L.; Bian, X.-L.; Guo, F.-J.; Wu, Y.-C.; Li, Y.-M.; *Fitoterapia* **2018**, *131*, 215. [Crossref]
19. Tempone, A. G.; Pimenta, D. C.; Lebrun, I.; Sartorelli, P.; Taniwaki, N. N.; Andrade-Júnior, H. F.; Antoniazzi, M. M.; Jared, C.; *Toxicon* **2008**, *52*, 13. [Crossref]
20. Narvaes, P.; Rodrigues, M. T.; *Arq. Zool.* **2009**, *40*, 1. [Crossref]
21. Santana, A. S.; Juncá, F. A.; *Braz. J. Biol.* **2007**, *67*, 125. [Crossref]
22. Pereyra, M. O.; Baldo, D.; Blotto, B. L.; Iglesias, P. P.; Thomé, M. T.; Haddad, C. F. B.; Barrio-Amorós, C.; Ibáñez, R.; Faivovich, J.; *Cladistics* **2016**, *32*, 36. [Crossref]
23. Sabagh, L. T.; Carvalho-e-Silva, A. M. P. T.; *Rev. Bras. Zool.* **2008**, *25*, 247. [Crossref]
24. Mossman, T.; *J. Immunol. Methods* **1983**, *65*, 55. [Crossref]
25. Motulsky, H.; *Software Prism*, version 8.0; GraphPad Software, USA, 1989.
26. Clinical and Laboratory Standards Institute (CLSI); *CLSI Guide M100: Performance Standards for Antimicrobial Susceptibility Testing*; Clinical and Laboratory Standards Institute: USA, 2019.
27. Rhee, I. K.; Van de Meent, M.; Ingkaninan, K.; Verpoorte, R.; *J. Chromatogr.* **2001**, *915*, 217. [Crossref]
28. Cao, Y.; Wu, J.; Pan, H.; Wang, L.; *Molecules* **2019**, *24*, 3595. [Crossref]
29. Schmeda-Hirschmann, G.; de Andrade, J. P.; Soto-Vasquez, M. R.; Alvarado-García, P. A. A.; Palominos, C.; Fuentes-Retamal, S.; Mellado, M.; Correa, P.; Urra, F. A.; *Toxins* **2020**, *12*, 608. [Crossref]
30. de Souza, E. B. R.; Sousa Jr., P. T.; Vasconcelos, L. G.; Rodrigues, D. J.; Sinhorin, V. D. G.; Kerkhoff, J.; Pelissari, S. R. N.; Sinhorin, A. P.; *Toxicon* **2020**, *179*, 101. [Crossref]
31. Barros, E. D. S.; Monção Filho, E. S.; Pio, Y. P. F.; de Amorim, M. R.; Berlinck, R. G. S.; Moura, R. C.; Fonseca, M. G.; Dantas, C.; Coelho, R. C.; Silva, G. R.; Chaves, M. H.; Vieira Jr., G. M.; *Toxicon* **2022**, *214*, 37. [Crossref]
32. He, R.; Ma, H.; Zhou, J.; Zhu, Z.; Li, X.; Li, Q.; Wang, H.; Yan, Y.; Luo, N.; Di, L.; Wu, Q.; Duan, J.; *Molecules* **2019**, *24*, 1943. [Crossref]
33. Wei, W.-L.; Hou, J.-J.; Wang, X.; Yu, Y.; Li, H. J.; Li, Z. W.; Feng, Z. J.; Qu, H.; Wu, W.-Y.; Guo, D. A.; *J. Ethnopharmacol.* **2019**, *237*, 215. [Crossref]
34. Zulfiker, A. H. M.; Sohrabi, M.; Qi, J.; Matthews, B.; Wei, M. Q.; Grice, I. D.; *J. Pharm. Biomed. Anal.* **2016**, *129*, 260. [Crossref]
35. Sinhorin, A. P.; Kerkhoff, J.; Dall'Oglio, E. L.; Rodrigues, D. J.; Vasconcelos, L. G.; Sinhorin, V. D. G.; *Toxicon* **2020**, *182*, 30. [Crossref]
36. Schmeda-Hirschmann, G.; Gomez, C. V.; Arias, A. R.; Burgos-Edwards, A.; Alfonso, J.; Rolon, M.; Brusquetti, F.; Netto, F.; Urra, F. A.; Cárdenas, C.; *J. Ethnopharmacol.* **2017**, *199*, 106. [Crossref]
37. Monção Filho, E. S.; Pio, Y. P. F.; Chaves, M. H.; Ferreira, P. M. P.; Fonseca, M. G.; Pessoa, C.; Lima, D. J. B.; Araújo, B. Q.; Vieira Jr., G. M.; *J. Braz. Chem. Soc.* **2021**, *32*, 1060. [Crossref]
38. Petroselli, G.; Raices, M.; Jungblut, L. D.; Pozzi, A. G.; Erra-Balsells, R.; *J. Mass Spectrom.* **2018**, *53*, 465. [Crossref]
39. 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. [Crossref]
40. Monção Filho, E. S.; Chaves, M. H.; Ferreira, P. M. P.; Pessoa, C.; Lima, D. J. B.; Maranhão, S. S. A.; Rodrigues, D. J.; Vieira Jr., G. M.; *Toxicon* **2021**, *194*, 37. [Crossref]
41. Sciani, J. M.; Angeli, C. B.; Antoniazzi, M. M.; Jared, C.; Pimenta, D. C.; *Sci. World. J.* **2013**, *2013*, ID 937407. [Crossref]
42. 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. [Crossref]
43. Pelissari, S. R. N.; Sinhorin, V. D. G.; Castoldi, L.; de Vasconcelos, L. G.; Rodrigues, D. J.; Ribeiro, E. B. S.; Kerkhoff, J.; Sinhorin, A. P.; *J. Braz. Chem. Soc.* **2021**, *32*, 1584. [Crossref]
44. Schmeda-Hirschmann, G.; Quispe, C.; Theoduloz, C.; Sousa Jr., P. T.; Parizotto, C.; *J. Ethnopharmacol*. **2014**, *155*, 1076. [Crossref]
45. Shimada, K.; Ishii, N.; Nambara, T.; *Chem. Pharm. Bull.* **1986**, *34*, 3453. [Crossref]
46. Lebedev, D. S.; Ivanov, I. A.; Kryukova E. V.; Starkov, V. G.; *Dokl. Biochem. Biophys.* **2017**, *474*, 178. [Crossref]

47. Oliveira, L. P.; Pinheiro, R. C.; Vieira, M. S.; Paula, J. R.; Bara, M. T. F.; Valadares, M. C.; *Rev. Bras. Farmacogn.* **2010**, *20*, 201. [Crossref]
48. Sales, D. L.; Morais-Braga, M. F. B.; dos Santos, A. T. L.; Machado, A. J. T.; Araújo Filho, J. A.; Dias, D. Q.; da Cunha, F. A. B.; Saraiva, R. A.; de Menezes, I. R. A.; Coutinho, H. D. M.; Costa, J. G. M.; Ferreira, F. S.; Alves, R. R. N.; Almeida, W. O.; *Biomed. Pharmacother.* **2017**, *92*, 554. [Crossref]

Submitted: November 9, 2022
Published online: April 13, 2023