Toxicon 182 (2020) 30–33

Contents lists available at ScienceDirect

Toxicon

journal homepage: http://www.elsevier.com/locate/toxicon

# Chemical profile of the parotoid gland secretion of the Amazonian toad (*Rhinella margaritifera*)

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ARTICLE INFO

*Keywords:*
Bufonidae
Bufadienolides
Amazonian anuranfauna

ABSTRACT

The secreted poisonin bufonids (Anura: Bufonidae) include proteins, biogenic amines, toxic bufadienolides and alkaloids. The chemical composition of the methanolic extract of parotoid gland secretions by the Amazonian toad Rhinella margaritifera was evaluated in a UFLC-DAD-micrOTOF system. Of the twenty three compounds found in the methanolic extract, eighteen were identified by the mass/charge ratio as: five arginine diacids, six bufagenins (telocinobufagin, marinobufagin, bufotalin, cinobufotalin, bufalin and cinobufagin), six bufotoxins, and an alkaloid (dehydrobufotenin).

The Bufonidae family, which includes the so-called true toads, is composed of 52 genera with worldwide distribution (AmphibiaWeb, 2020). These animals occur in a wide variety of habitats from deserts to tropical forests.

Bufonids produce very potent toxins in their skin, especially concentrated in the parotoid glands, dorsal structures located in the post-orbital region, and can be fatal to predators when ingested (Clarke, 1997; Toledo et al., 1992). However, several studies have been exploring these compounds in pharmacological models for antitumor and cytotoxic, cardiotonic, antifungal, antimicrobial and antiparasitic activity (Barnhart et al., 2017; Chen et al., 2018; Cunha-Filho et al., 2010; Medeiros et al., 2019; Ferreira et al., 2013; Banfi et al., 2016; He et al., 2019; Kowalski et al., 2018; Meng et al., 2019; Nalbantsoy et al., 2016; Perera-Córdova et al., 2016; Rodríguez et al., 2017; Sousa et al., 2017).

The *Rhinella margaritifera* Laurenti (*R. margaritifera) (*Laurenti, 1768*)* species has wide distribution throughout the Amazon basin, which includes Colombia, Venezuela, Peru, Bolivia, Brazil and the Guyana. It is a terrestrial species with daytime and nighttime activity, which lives in the litterfall of primary forest (Ávila et al., 2010). It is a brown toad (Fig. 1) of medium size (males measure between 40 and 67 mm and females between 46 and 76 mm), with conspicuous parotoid glands and developed cranial ridges (especially in females). The morphology and brown coloration of *R. margaritifera* allows them to mix with the litterfall. Individuals climb to low vegetation up to 1.5 m above the ground at night, where they remain inactive (Moravec et al., 2014). Adults feed on arthropods, especially ants (Lima and Magnusson, 2000).

Parotoid glands from toads can secrete several different compounds, including proteins, biogenic amines, alkaloids and steroids (Clarke, 1997; Daly et al., 2005; Mariano et al., 2018; Rash et al., 2011; Zhao et al., 2006). The components abundance and diversity of each of these compounds class can vary according to the life history of the amphibian in question, gender, and season (Sherman et al., 2009).

Bufadienolides are the major components of bufonid parotoid secretions. These molecules are polyhydroxy-steroids with 24 carbon atoms, characterized by an unsaturated lactone ring and an α-pyrone group linked to C-17. They can be found in free form or in conjunction

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https://doi.org/10.1016/j.toxicon.2020.04.106
Received 7 February 2020; Received in revised form 8 April 2020; Accepted 28 April 2020
Available online 6 May 2020

**Fig. 1.** *R. margaritifera*. Photography by domingos rodrigues.

with sulfates, dicarboxylic esters and amino acids at the C-3 position (Sousa et al., 2017). These possibilities lend this group of compounds to have enormous structural diversity (Gao et al., 2010).

These steroids are potent inhibitors of $Na^+/K^+$-ATPase activity, and if ingested they cause a bitter taste in the mouth, nausea or heart failure. In addition, they repel and may even kill predators. Bufadienolides can also contribute to the toads' immunological defense against pathogens (Barnhart et al., 2017).

Although several trivial nomenclatures for bufadienolides can be found in the literature, we have adopted the nomenclature of the bufagenins and bufotoxins subclass for the purposes of this work. Thus, the smaller hydrolyzed bufadienolide molecules are called bufagenins (free bufadienolides), and the larger bufadienolide molecules which have a side chain of amino acids are called bufotoxins.

About 100 bufadienolides have been reported in the literature, being bufonid poisons (Steyn and Van Heerden, 1998). Although amphibian skin secretions have proven to be a rich source of unique molecules, they remain largely unexplored and represent great potential for developing new molecular models for pharmacological and toxicological evaluations and even for synthesis and medicinal chemistry. Therefore, the objective of this work was to analyze the chemical composition of *R. margaritifera* and to trace the bufagenin and bufotoxin profile by chromatographic and spectrometric analyzes for the first time.

The *R. margaritifera* specimen was captured in January 2018 in the municipality of Cotriguaçu (9°49′20.00″S 58°17′16.00″ W) in Mato Grosso state, Brazil, identified by the team of biologists from the Federal University of Mato Grosso, under the coordination of Prof. Dr. Domingos de Jesus Rodrigues (IBAMA, SISBIO: 30034-1). The poison was collected by manually compressing the animal's parotoid glands and then stored in glass flasks containing silica gel. The toad was returned to nature after collecting the poison. The access to Brazilian Genetic Heritage was registered with SisGen, in compliance with the provisions of Law No. 13,123/2015 and its regulations, under No. ACC9622. The dried poison was crushed with a mortar and pestle and extracted in an ultrasonic bath with 1:10 methanol for 60 min (Kerkhoff et al., 2016). The obtained methanolic extract was dried by rotary evaporation at reduced pressure of 600 mmHg at 40 °C and finished in a high vacuum pump. The obtained extract was subsequently stored at 4 °C. For chromatographic analysis, 1 mg of methanolic extract was solubilized in 1 mL of HPLC grade methanol and filtered through a filter membrane with porosity of 0.22 μm.

The analyzes were performed in a Liquid Chromatography System with Detection by Ultraviolet Spectroscopy and Mass Spectrometry (UFLC-DAD-micrOTOF) and the method was adapted from the one developed by Schmeda-Hirschmann et al. (2016) and had the following conditions: Injection Volume: 1 μL; Chromatographic column: Kromasil 100-5-C18 column, 250 × 4.6 mm, 5 μm; Pre-Column: Kromasil Guard Column 100-5-C18, 4.6 × 10 mm, 5 μm; Chromatographic Conditions: Mobile Phase (Solvent A: 0.5% Formic Acid Solution; Solvent B: Acetonitrile 0.5% formic acid); Elution Mode: Gradient (following the following schedule: 0–45 min with 8–64% Solvent B); Flow: 1.0 mL/min; Column Temperature: 40 °C.

Chromatographic analysis of the methanolic extract obtained from the *R. margaritifera* parotoid secretion detected 23 compounds (Fig. 2), in which 18 of these were identified by the mass/charge ratio (Table 1). Compounds **1**-**9** are smaller, polar molecules which correspond to the amino acid arginine and arginine diacids, in addition to an alkaloid, dehydrobufotenin. The compounds 10–23 are those derived from cardiotonic steroids, of bufagenin and bufotoxin types, with less polarity.

Compound **1** was identified as the amino acid Arginine, with the molecular formula of $C_6H_{14}N_4O_2$ and m/z 175.1187 (Shek et al., 2006). Compounds **2**, **3**, **5** and **6** have been identified as arginine diacids. The compounds belong to a homologous series which varies in the size of the diacid carbon chain, between 6 and 9, and have been designated as adipoyl-, pimeloyl-, suberoyl- and azelayl-argininyl. This identification proposal is in accordance with the results of the study by Schmeda-Hirschmann (2014).

Compound **4** was identified as the Dehydrobufotenin alkaloid ($C_{12}H_{14}N_2O$; m/z 203.1169), a tricyclic metabolite of serotonin and has been identified in several species of the Bufonidae family (Schmeda-Hirschmann et al., 2017).

The bufagenins identified in this work correspond to the compounds **10**, **15**, **16**, **18, 20** and **23,** and are described as follows: compound **10** was identified as Telocinobufagin ($C_{24}H_{34}O_5$; m/z 403.2449); compound **15** corresponds to Marinobufagin ($C_{24}H_{32}O_5$; m/z 401.2319); compound **16** was identified as Bufotalin ($C_{26}H_{36}O_6$; m/z 445.2578); compound **18** was identified as Cinobufotalin ($C_{26}H_{34}O_7$; m/z 459.2381); compound **20** was assimilated to Bufalin ($C_{24}H_{34}O_4$; 387.2535); and compound **23** was identified as Cinobufagin ($C_{26}H_{34}O_6$; m/z 443.2419) (He et al., 2019). All ions and fragments are consistent with those found in the work of Zhang et al. (2016), who performed chromatographic analyzes of 64 bufadienolides found in bufonids. These molecules have already been described in several studies which investigated the chemical composition of bufonid poisons, such as *Rhinella marina*, *Rhinella schneideri*, *Rhinella ornata*, *Rhinella arenarum*, *Bufo gargarizans*, and *Peltophryne fustiger* (Chen et al., 2018; Cunha-Filho et al., 2010; Perera-Córdova et al., 2016; Petroselli et al., 2018; Schmeda-Hirschmann et al., 2017, 2016).

Bufotoxins are bufadienolides with an amino acid side chain. Thus, there are numerous possible combinations of bufadienolides, diacids and amino acids. A fragmentation pattern was found for the bufotoxins, with the most intense fragment being arginine diacid, followed by the loss of one amine group and two $H_2O$ groups of that fraction, generating the fragments which correspond to [M + H–$NH_2$], [M + H–$NH_2$–$H_2O$] and [M + H–$NH_2$–2$H_2O$], respectively. Thus, compound **11** was identified as 3-(*N*-adipoyl argininyl) bufotalin ($C_{38}H_{56}N_4O_{10}$); compound **12** was also identified as a derivative of bufotalin, 3-(*N*-pimeloyl argininyl) bufotalin ($C_{39}H_{58}N_4O_{10}$; m/z 743.4168); compound **13** was identified as 3-(*N*-suberoyl argininyl) telocinobufagin ($C_{38}H_{60}N_4O_{10}$; m/z 715.4246); **17** was assimilated to 3-(*N*-suberoyl argininyl) bufotalin ($C_{40}H_{60}N_4O_{10}$; m/z 757.4392); compound **19** was identified as 3-(*N*-pimeloyl argininyl) cinobufagin ($C_{39}H_{56}N_4O_{10}$; m/z 741.4064); and compound **21** corresponds to as 3-(*N*- suberoyl argininyl) cinobufagin ($C_{40}H_{58}N_4O_{10}$; m/z 755.4231) (Schmeda-Hirschmann et al., 2017).

The major compounds were the bufadienolides Bufotalin (Compound **16**, 33%), Cinobufagin (Compound **23**, 16%) and Marinobufagin (Compound **15**, 13%), which correspond to more than 60% of the extract. These findings corroborate the high toxicity found in bufonid poisons (Tóth et al., 2019). Altogether, 80% of the constituents of *R. margaritifera* extract were identified. It was not possible to find data in

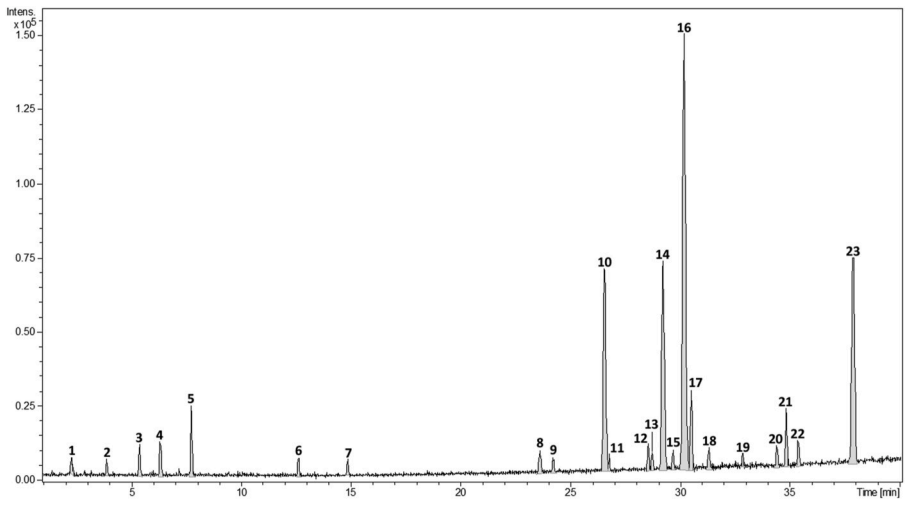


**Fig. 2.** UFLC-DAD-micrOTOF chromatogram of *R. margaritifera* methanolic extract. Chromatographic Conditions: Mobile Phase (Solvent A: 0.5% Formic Acid Solution; Solvent B: Acetonitrile 0.5% formic acid); Elution Mode: Gradient (following the schedule: 0–45 min with 8–64% Solvent B; Flow: 1.0 mL/min; Column Temperature: 40 °C.

**Table 1**
Identification of the methanolic extract constituents of *R. margaritifera* poison by UFLC-DAD-micrOTOF.

| Compound | Rt (min) | Relative % | $[M+H]^+$ | Fragmentation | Identification |
|---|---|---|---|---|---|
| **1** | 2.4 | 0.8 | 175.1187 | 130.1568; 116.0473; 112.0278; 105.0385 | Arginine[g] |
| **2** | 4.0 | 0.6 | 303.1652 | 226.0988; 175.1182; 158.0920; 116.0720 | Adipyl arginine[d,f] |
| **3** | 5.4 | 1.3 | 317.1817 | 175.1173; 158.0920; 116.0728; 112.0728 | Pimeloyl arginine[d,f] |
| **4** | 6.4 | 1.7 | 203.1169 | 188.0945; 173.0705; 160.0986; 145.0753 | Dehydrobufotenin[a,b,d,f] |
| **5** | 7.8 | 2.9 | 331.1972 | 175.1148; 158.0926; 130.0970; 116.0707 | Suberoyl arginine[b,d,f] |
| **6** | 12.7 | 0.8 | 345.2134 | 175.1201; 158.0924; 116.0718; 112.0884 | Azelayl arginine[d,f, e] |
| **7** | 14.9 | 0.5 | 197.0778 | 175.0965; 162.0548; 157.0860; 112.0299 | Not assigned |
| **8** | 23.6 | 1.3 | 211.0916 | 189.1108; 173.0792; 171.0973; 169.0601 | Not assigned |
| **9** | 24.2 | 0.8 | 467.2059 | 369.2434; 351.2286; 255.2089; 147.1110 | Not assigned |
| **10** | 26.6 | 13.1 | 403.2449 | 367.2253; 215.1759; 161.1296; 151.0378 | **Telocinobufagin**[a,b,d,f] |
| **11** | 26.8 | 0.5 | 729.4028 | 303.1642; 286.1379; 268.1243; 250.1150 | 3-(*N*-adipoyl argininyl) bufotalin[g] |
| **12** | 28.6 | 1.0 | 743.4168 | 317.1792; 300.1537; 282.1406; 264.1311 | 3-(*N*-pimeloyl argininyl) bufotalin[g] |
| **13** | 28.7 | 0.6 | 715.4246 | 331.1967; 314.1674; 278.1532; 175.1183 | 3-(*N*-suberoyl argininyl) telocinobufagin[b,d] |
| **14** | 29.2 | 15.0 | 431.2413 | 349.2149; 187.0742; 175.1470; 161.1305 | Not assigned |
| **15** | 29.7 | 0.8 | 401.2319 | 365.2129; 215.1770; 157.0955; 149.1283 | **Marinobufagin**[a,b,d,f] |
| **16** | 30.2 | **32.8** | 445.2578 | 367.2244; 349.2155; 241.1212; 161.1309 | **Bufotalin**[g, e] |
| **17** | 30.5 | 3.6 | 757.4392 | 331.1967; 296.1623; 278.1494; 175.1199 | 3-(*N*-suberoyl argininyl) bufotalin[g] |
| **18** | 31.3 | 1.1 | 459.2381 | 381.2106; 363.1969; 255.1000; 201.1627 | **Cinobufotalin**[c] |
| **19** | 32.8 | 0.4 | 741.4064 | 317.1827; 282.1473; 264.1333; 175.1188 | 3-(*N*-pimeloyl argininyl) cinobufagin[f] |
| **20** | 34.4 | 1.0 | 387.2535 | 369.2392; 351.2325; 255.2078; 147.1169 | **Bufalin**[a,b,d,f] |
| **21** | 34.8 | 2.4 | 755.4231 | 331.1986; 314.1724; 278.1512; 175.1198 | 3-(*N*-suberoyl argininyl) cinobufagina[f] |
| **22** | 35.4 | 1.2 | 274.2734 | 274.2702; 256.2652; 106.0889; 102.0928 | Not assigned |
| **23** | 37.9 | 15.8 | 443.2419 | 365.2082; 215.1784; 187.1476; 151.0390 | **Cinobufagin**[c,h] |

[a] Ferreira et al., (2013).
[b] Schmeda-Hirschmann et al., (2014).
[c] Zhang et al., (2016).
[d] Schmeda-Hirschmann et al., (2016).
[f] Schmeda-Hirschmann et al., (2017).
[g] Petroselli et al., (2018).
[h] He et al., (2019).
[e] Perera-Córdova et al., (2016).

the literature for identifying the compounds **7**, **8**, **9**, **14** and **22**, and their fragments.

The chemical analysis of the methanolic extract of the secretions of the parotid glands of *R. margaritifera* resulted in the profile of bufagenins and bufotoxins that composes this poison, with Bufotalin as the major compound. The results point to the relevance of new investigations in toads of the *Rhinella* genus and to establish a reference for other poisons of Amazonian bufonids.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Adilson Paulo Sinhorin:** Project administration, Writing - review & editing. **Jacqueline Kerkhoff:** Methodology, Validation. **Evadro Luiz Dall'Oglio:** Supervision. **Domingos de Jesus Rodrigues:** Funding

acquisition. **Leonardo Gomes de Vasconcelos:** Data curation. **Valéria Dornelles Gindri Sinhorin:** Writing - review & editing.

## Acknowledgements

This study was partly financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (*CAPES*) - Finance Code 001.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2020.04.106.

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