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Comparative study of the chemical profile of the parotoid gland secretions from Rhaebo guttatus from different regions of the Brazilian Amazon

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ABSTRACT

Amphibian cutaneous secretion has great potential for bioprospection and is a great tool in the development of bioproducts. Thus, the objective of the present work was to evaluate the comparative study of the chemical profile parotoid gland secretions from Rhaebo guttatus collected in two distinct regions of the Brazilian Amazon. For this, the chemical composition of six methanolic extracts of this species were analyzed by Liquid Chromatography in UV and MS Detection Ultra-Chromatography Systems (UFLC-DAD-micrOTOF). All obtained chromatograms presented two distinct regions; one referring to the more hydrophilic molecules (alkaloids), while the other refers to the more hydrophobic compounds (steroids). The steroid region resembles all samples, regardless of where they were collected. In the alkaloid region, there was a standardized variation for the samples from the southern Brazilian Amazon, but the same was not true for the samples collected in the Amazon-Cerrado transition region. Thus, the data suggest that the environment and diet of R. guttatus may be important in alkaloid production, but do not influence steroid content. These results add new information about the poison of the toad R. guttatus and raises new questions to be further investigated, thus contributing to the knowledge of the anuran fauna of the Brazilian Amazon.

1. Introduction

Brazil is home to the largest biodiversity of amphibians on the planet with great emphasis on the Brazilian Amazon (Noronha et al., 2015; Hoogmoed, 2016; Prudente, 2016; Segalla et al., 2016; AmphibiaWeb, 2019). The cutaneous secretion of these animals has great potential for bioprospection and has therefore attracted the interest of many researchers (Ferreira et al., 2013; Schmeda-Hirschmann et al., 2017; Li et al., 2015; Oliveira et al., 2019).

Anuran skin secretion is a complex mixture of substances produced and stored in the glands of the whole skin or in glandular accumulations such as parotoids. These substances are used mainly against attacks by predators and pathogenic microorganisms, ensuring amphibians' surviving in different habitats (Daly et al., 2004; Jared et al., 2009; Pinto et al., 2009; Mailho-Fontana et al., 2018). Within most bufonids this complex mixture of compounds was developed through evolutionary pressures, depending on predators and/or microorganisms. However, in some species (Dendrobatidae, Mantellidae, Myobatrachidae, Bufonidae and Eleutherodactylidae) they are acquired from their diet through the sequester of arthropod precursors substances which they feed on. In

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these cases the environment in which the anuran is inserted directly influences the skin glands chemical composition (Saporito et al., 2012; Savitzky et al., 2012).

The main classes of compounds found in amphibian skin secretion with pharmacological actions are amines, alkaloids, peptides, proteins and steroids (Cunha-Filho et al., 2010; Schmeda-Hirschmann et al., 2017). These classes are associated with several biological effects such as antiviral (Vigerelli et al., 2014), antiparasitic (Tempone et al., 2008), cytotoxic, antimutagenic (Oliveira et al., 2019), and antitumor (Sciani et al., 2013b), as well as neurotoxic, cardiotoxic, hemotoxic, myotoxic and immunomodulatory actions (Anjolete et al., 2015), and strong antiproliferative effect (Schmeda-Hirschmann et al., 2014).

In Latin America, common toads are divided into two genera, *Rhinela* and *Rhaebo* (Frost et al., 2006) belonging to the Bufonidae family, which is composed of 54 genera and 621 species (Frost, 2019). Alkaloids, steroids and proteins are the main classes present in *Rhinela* and *Rhaebo* poison (Sciani et al., 2013a). Alkaloids and steroids are classified as toxic agents which contribute to the chemical defense of the anuran against predators (Daly et al., 2004). Such substances act on the cardiovascular system by increasing blood pressure and/or increasing the heart contraction force. On the other hand, proteins basically act as homeostatic agents (Daly et al., 2005). Although species belonging to these two genera are related, they may present significant differences depending on the habitat in which they live (Jared et al., 2011). *Rhaebo guttatus* (Schneider, 1799), is an example of a naturally distributed anuran in the Amazon basin (Lötters et al., 2000). Only individuals of this species are able to release secretion from their parotoid glands voluntarily as a defense strategy (Jared et al., 2011; Mailho-Fontana et al., 2014). Some studies on *Rhaebo guttatus* (*R. guttatus*) have already been performed (Jared et al., 2011; Ferreira et al., 2013; Sciani et al., 2013a; Mailho--Fontana et al., 2014; Kerkhoff et al., 2016; Oliveira et al., 2019), but this species remains poorly known.

Given the above and the lack of studies on *R. guttatus*, more research is necessary to contribute with the scientific knowledge on the species. Thus, the objective of the present work was to evaluate the chemical profile of the methanolic extract obtained from *R. guttatus* parotoid secretion collected in two distinct regions of the Brazilian Amazon aiming to contribute to the knowledge of the local anurofauna.

2. Material and methods

2.1. Animal capture and poison collection

Adult animals (male and female) were captured and identified by a team of biologists from the Federal University of Mato Grosso – campus of Sinop, under the coordination of Professor Doctor Domingos de Jesus

Fig. 1. Municipalities of *R. guttatus* origin captured in the Brazilian Amazon.

Rodrigues (IBAMA, SISBIO: Number 30034–1) in three municipalities: RG1 (n = 2) and RG2 (n = 2) samples were obtained from anurans collected in Colniza/Mato Grosso/Brazil (9°13′46.71″S 60°17′41.75″W) located in the south of the Brazilian Amazon; RG3 (n = 3) and RG4 (n = 3) samples were obtained from anurans collected in Nova Ubiratã/Mato Grosso/Brazil (13°6′16.20″S 54°25′51.01″W), and RG5 (n = 2) and RG6 (n = 2) samples were obtained from anurans collected in Sinop/Mato grosso/Brazil (11°31′30.80″S 55°36′6.05″W), both located in the Amazon-Cerrado transition region (Fig. 1). The collections were performed between October and December 2016.

The captured toads were sanitized with running water and their poisons were collected by manual compression of the animals' parotoid glands. The cutaneous secretions were subsequently dried for two days using a desiccator at room temperature and in the absence of light. The procedure for collecting the parotoid secretion of the animals did not cause death or damage to any of them, which enabled returning all toads to their natural habitat.

2.2. Preparation of methanolic extracts

The dried poison from each sample (RG1 to RG6) was ground with the aid of mortar and pistil and submerged in analytical grade methanol. Then, each sample was submitted to an ultrasonic bath extraction process (Unique) for 2 h, filtered on filter paper (Unifil) and recoiled (twice) under the same conditions. The extracting solvent was removed in a vacuum pump coupled to a rotary evaporator (Diagtech) (Prismatec) at a constant temperature of 40 °C. The extract was kept in the desiccator for 2 day at room temperature and protected from light for total methanol removal. After total drying the sample was then stored at 4 °C. The procedure above, performed for each sample, resulted in six extracts. The experimental conditions were chosen according to the work previously done by our group (Kerkhoff et al., 2016).

2.3. QTOF-analysis

Liquid Chromatography analyzes were performed on UV and MS Detected Ultra-Chromatography Systems (UFLC-DAD-micrOTOF) under the following conditions: Injection volume: 1.00 μL; Chromatographic Column: (Brand: Kromasil 100-5-C18) with dimension: 250 × 4.6 mm; Particle Size: 5 μm; Stationary Phase: C18 Reverse Phase; Separation Mode: Mobile Phase (Solvent A: 0.5% aqueous Formic Acid Solution; Solvent B: Acetonitrile Acidified with 0.5% Formic Acid); Elution Mode: Gradient (0–45 min with 8–64% Solvent B); Flow: 1.0 mL/min with 50% flow divider at the column outlet, i.e. 0.5 mL/min reached the mass detector, while the remainder was discarded; Column temperature: 40 °C; Ion Polarity: Positive; Scan Mode: MS; Mass Range: 50 to 1000 mz; Rolling Average: 2 x; Spectra Rate: 2.00 Hz; Capillary Voltage: 4500 V; Nebulizer Gas: 4.0 Bar; Dry Gas: 9.0 L/min; Dry Temperature: 200 °C.

Samples were prepared with 1 mg dry methanolic extract and 1 mL HPLC grade methanol. They were subsequently filtered through a 0.45 μm pore PTFE syringe filter. The experimental conditions were developed according to Schmeda-Hirschmann et al. (2014). The equipment used belongs to the department of chemistry of the Federal University of Mato Grosso, campus of Cuiabá, Brazil.

3. Results and discussion

The parotoid secretions of fourteen *R. guttatus* specimens distributed in two distinct regions of the Brazilian Amazon were analyzed to carry out a comparative study of methanolic extract composition. The peaks of the chromatograms obtained by LC-MS were selected with a relative percentage equal to or greater than 1.0% according to Fig. 2, showing the presence of 9 compounds, of which 6 were identified. It is noteworthy that the signals produced by the UV/Vis detector in this analysis were not extracted/acquired because the information obtained via Mass Spectrometry is more reliable. The experimental physical data obtained with this technique and the identifications/attempts to identify the compounds present in the extracts are summarized in Table 1. The structures of the identified compounds are shown in Fig. 3.

It was possible to identify the six compounds present in *R. guttatus* samples through the analysis of the experimentally obtained physical data (Table 1) together with the literature information, namely: compound **1** ($C_9H_{11}NO_2$) identified as ethenzamide according to the study by Zulfiker et al. (2016), compound **2** as N′-methyl-5-hydroxytryptamine ($C_{11}H_{14}N_2O$) (Rodríguez et al., 2017), compound **4** identified as bufotenine ($C_{12}H_{16}N_2O$) (Rodríguez et al., 2017), compound **5** described as dehydrobufotenine ($C_{12}H_{15}N_2O$) (Schmeda-Hirschmann et al., 2016), compound **6** identified as 3β, 16β-dihydroxybufa-8(14),20,22-trienolide ($C_{24}O_4H_{32}$) according to the study by Meng et al. (2016), and finally compound **7** as a bufatrienolide ($C_{24}H_{30}O_5$). All the identifications were confirmed by the fragmentation mechanism, meaning the experimental values of m/z of the fragments were confirmed through a theoretical analysis about the possible bond breaks in the structures. The other compounds were not identified due to a lack of data in the literature. As *R. guttatus* in an endemic species from the Amazon basin, which is a region still poorly studied mainly regarding the bioprospection of biomolecules, information about the chemical composition of the cutaneous secretion of this species is still very poor (Ferreira et al., 2013; Sciani et al., 2013a; Mailho-Fontana et al., 2014; Kerkhoff et al., 2016).

In general, it was observed that the chromatograms showed two distinct regions, with the first being composed of peaks referring to the most hydrophilic molecules, while peaks in the second region referring to the most hydrophobic compounds were noted (Fig. 2). These data are in agreement with the physicochemical characteristics of the analysis and also with the work of Sciani et al. (2013a). It was also noted that there are compounds belonging to the alkaloid class among the most hydrophilic molecules, while steroids are among the most hydrophobic compounds (Sciani et al., 2013a).

While RG1, RG2 e RG6 extracts presented one identified alkaloid (compound **5**), in RG3 and RG5 presence of alkaloid was not observed. Finally, the RG4 sample presented the largest number of compounds of this class. Regarding steroids, all samples presented the compounds identified in this class as shown in Fig. 2.

Compound **9** was the secondary metabolite with the highest relative proportion, presenting percentages equal to 73.5%, 67.9%, 67.7%, 56.7%, 78.0% and 74.2% for RG1, RG2, RG3, RG4, RG5 and RG6, respectively (Table 1). In the work of Sciani et al. (2013a) it was observed that the biggest peaks in the chromatograms corresponded to the alkaloids.

The data here presented showed that in relation to the steroids the samples are similar regardless of the collection site, but the same did not occur in the region of the alkaloids for most of the samples (RG3, RG4, RG5 and RG6) (Fig. 2). In other words, the data from a comparative analysis suggest that individuals collected in southern Brazilian Amazonia presented standardized variation in the chemical composition of the *R. guttatus* poison. Meanwhile, for specimens collected in the transition region of vegetation (Amazon-Cerrado), a standardized variation was observed only for steroids, but the same was not observed for the production of alkaloids. It is important to point out that both regions have naturally distinct ecological contexts: while the collection site in the south of the Brazilian Amazon is an area of fully recovered vegetation and with very little human intervention, the collection site of the Amazon-Cerrado region has suffered several anthropic actions.

The results of the present study suggest that the environment in which *R. guttatus* inhabits and its diet may be important for alkaloid production, but do not influence the steroid content of the parotoid gland. Studies from other authors have shown that the diet can influence the composition of amphibian poison, which include some species of the families Dendrobatidae, Bufonidae, Mantellidae, Myobatrachidae and Eleutherodactylidae (Saporito et al., 2012). However, it is known that skin poisons for the vast majority of amphibians are the result of

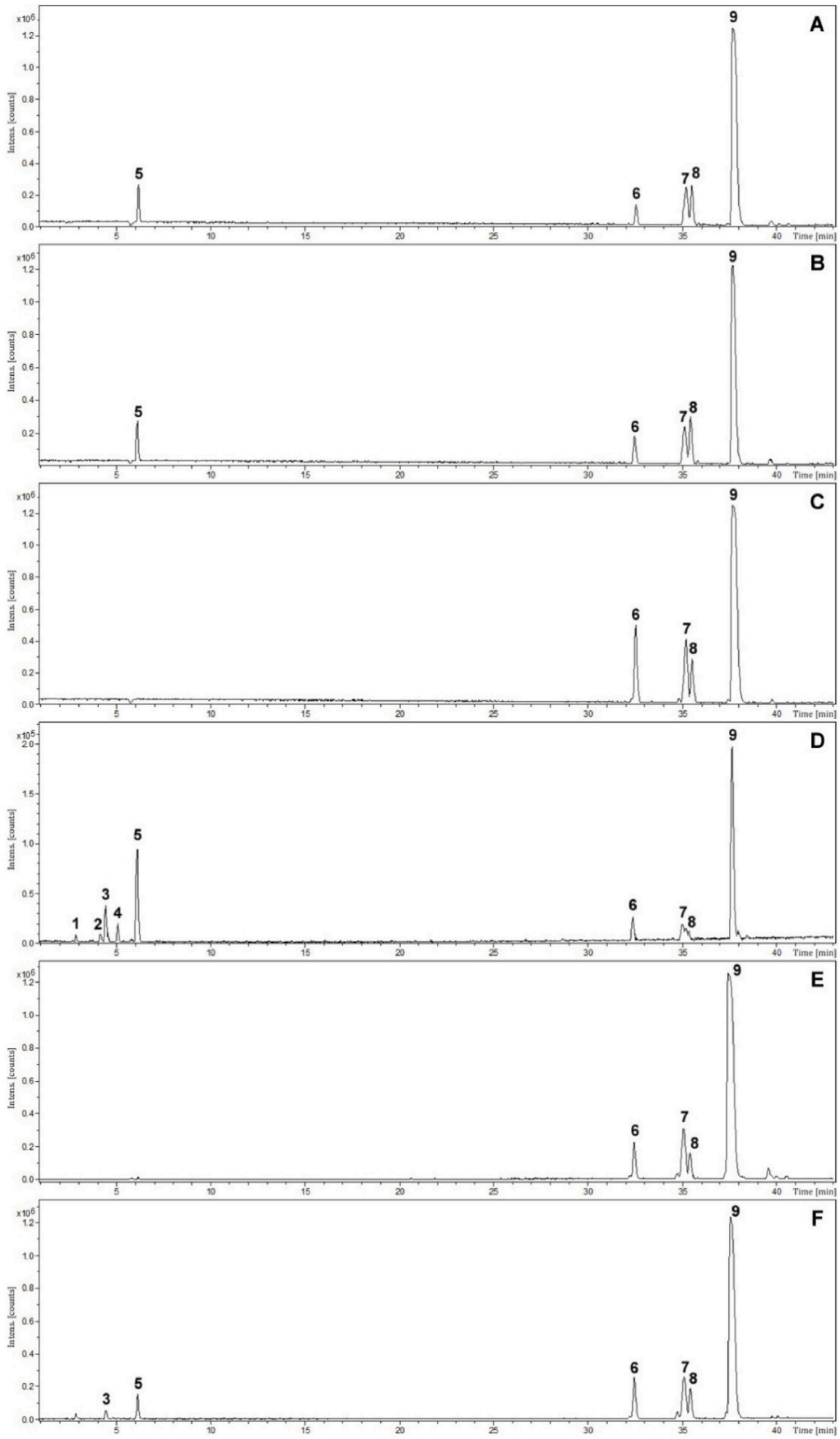

Fig. 2. Chromatograms of the methanolic extracts of the *R. guttatus* poison, namely: RG1 (A), RG2 (B), RG3 (C), RG4 (D), RG5 (E) and RG6 (F).

Table 1
Physical data of *R. guttatus* methanolic extracts by HPLC-MS-MS and identification/attempted identification of compounds.

Compound	Rt (min)	$[M+H]^+$ Experiment (m/z)	MS/MS Experiment (m/z)	Sample (Relative area/%)	Identification or Identification attempt
1	2.9	166.0873	137.0599 (100); 121.0653 (54); 103.0556 (80)	RG4 (1.1)	Ethenzamide[a]
2	4.2	189.1023	160.0762 (100); 117.0589 (99)	RG4 (1.5)	N′-methyl-5-hydroxytryptamine[b]
3	4.5	160.0757	117.0582 (66); 115.0548 (100)	RG4 (6.9); RG6 (1.2)	–
4	5.1	203,1180	203,1180 (100)	RG4 (3,0)	Bufotenine[b]
5	6.2	203.1180	188.0949 (100); 173.0713 (81)	RG1 (5.7); RG2 (6.4); RG4 (17.1); RG6 (2.9)	Dehydrobufotenin[c]
6	32.5	384.3581	170.1538 (100); 156.1405 (100)	RG1 (3.3); RG2 (5.6); RG3 (12.8); RG4 (5.5); RG5 (6.3); RG6 (7.8)	3β, 16 β-dihydroxybufa-8 (14),20,22-trienolide[d]
7	35.1	398.3742	240.2335(100); 184.1711(100); 170.1542 (100)	RG1 (9.7); RG2 (9.9); RG3 (12.7); RG4 (2.7); RG5 (11.4); RG6 (9.4)	Bufatrienolides[b]
8	35.4	410.3744	182.1550 (100); 133.0081 (97)	RG1 (7.8); RG2 (10.2); RG3 (6.9); RG4 (1.4); RG5 (4.3); RG6 (5.4)	–
9	37.7	412.3938	227.2000 (62); 184.1714 (100) 147.0152 (57); 88.0787 (50)	RG1 (73.5); RG2 (67.9); RG3 (67.7); RG4 (51.0); RG5 (78.0); RG6 (73.3)	–

[a] Zulfiker et al.(2016).
[b] Rodríguez et al.(2017).
[c] Schmeda-Hirschmann et al.(2016).
[d] Meng et al. (2016).

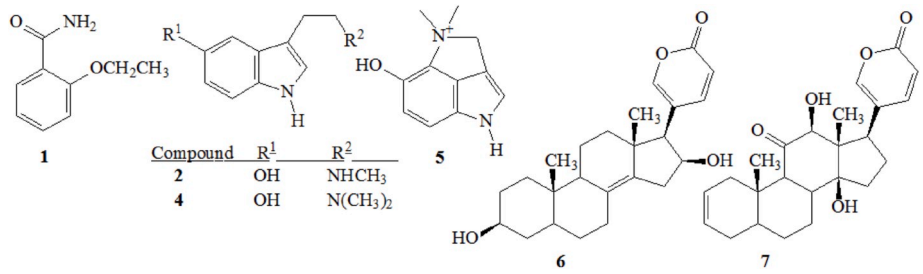

Fig. 3. Chemical structures of compounds identified in methanolic extracts of *R. guttatus*.

endogenous production. In addition, the age and size of the toads can also influence the poison composition in qualitative and quantitative terms (Üveges et al., 2017). However, variation in poison content is expected between different populations, as composition seems not only to depend on the individual's stage of development, but also on the ecological context, as animals are exposed to different predators and/or pathogenic microorganisms (Sciani et al., 2013a; Üveges et al., 2017; Bókony et al., 2019), which seems to be the case of *R. guttatus* for alkaloid production. In addition, studies have shown that the chemical arsenal used in *R. guttatus*' defensive mechanism may differ from other toads (which have a passive defense mechanism) due to its peculiar mode of defense (Jared et al., 2011; Mailho-Fontana et al., 2014).

4. Conclusion

The present work showed that the chromatograms of the six methanolic extracts presented two distinct regions: one referring to the more hydrophilic molecules (alkaloids), and another referring to the more hydrophobic compounds (steroids). Regarding the steroids the results were similar for all samples, regardless of where they were collected. In relation to alkaloids, a standardized variation was observed for samples from southern Brazilian Amazonia, but not for samples collected in the Amazon-Cerrado transition region. The data here presented suggest that the environment as well as the diet of *R. guttatus* may be important for alkaloid production, but do not influence the steroid content of the parotoid secretion. These results add new information about the poison of the toad *R. guttatus* and raises new questions to be further investigated, thus contributing to the knowledge of the anuran fauna of the Brazilian Amazon.

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

Eloana Benassi Ribeiro de Souza: Methodology. **Paulo Teixeira de Sousa Júnior:** Supervision. **Leonardo Gomes de Vasconcelos:** Data curation. **Domingos de Jesus Rodrigues:** Funding acquisition. **Valéria Dornelles Gindri Sinhorin:** Writing - review & editing. **Jacqueline Kerkhoff:** Validation. **Sheila Rodrigues do Nascimento Pelissari:** Methodology. **Adilson Paulo Sinhorin:** Project administration, Writing - review & editing.

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