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INVESTIGATION OF CRUDE METHANOLIC EXTRACT FROM POISON SECRETED BY THE *Rhaebo gutattus* ON STATUS REDOX ANTIOXIDANT IN MICE

Sheila Rodrigues do Nascimento PELISSARI[1], Valéria Dornelles Gindri SINHORIN[1], Domingos de Jesus RODRIGUES[1], Lee Yun SHENG[2], Lindsey CASTOLDI[3], Adilson Paulo SINHORIN[1]

[1] Integrated Chemistry Research Laboratories, Postgraduate Program in Environmental Sciences, Institute of Natural, Human and Social Sciences, Universidade Federal de Mato Grosso (UFMT), Sinop, Mato Grosso, Brazil.
[2] Institute of Natural, Human and Social Sciences, Universidade Federal de Mato Grosso (UFMT), Sinop, Mato Grosso, Brazil.
[3] Institute of Health Sciences, Universidade Federal de Mato Grosso (UFMT), Sinop, Mato Grosso, Brazil.

Corresponding author:
Adilson Paulo Sinhorin
adilson.sinhorin@ufmt.br

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Abstract

The study evaluated the antioxidant properties of a crude methanolic extract (CME) from *Rhaebo guttatus* poison in mice over a period of 7 and 30 days. The mice were divided into groups and treated with different concentrations of the extract (0; 8 µg mL^{-1}; 16 µg m^{L-1} and 32 µg mL-1 or vehicle; 100 µL/animal/day; via gavage). The liver samples were analyzed for status redox parameters as catalase (CAT), glutathione-S-transferase (GST), reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). The results showed that the CME caused changes in the levels of various antioxidants and oxidative stress markers. At 7 days, there was an increase in TBARS levels (8 µg mL^{-1} dose) and GST activity (16 µg mL^{-1} dose), and a reduction in GSH levels (32 µg mL^{-1} dose) compared to the control group. At 30 days, TBARS and GSH levels returned to control values in the same period, but GSH increased (32 µg mL^{-1} dose) compared at 7 days; GST activity remained high after 30 days for 32 µg mL^{-1} dose compared other groups and time of treatment (7 days). Overall, the study suggests that the extract modulates antioxidant properties *per se* that can affect various markers of status redox in the liver of mice, mainly 16 µg mL^{-1} dose demonstrated to act under antioxidant enzymes in different times (7 or 30 days).

Keywords: Antioxidants. Biodiversity. Poison Toad*. Rhaebo guttatus.*

1. Introduction

The Brazilian Amazon is a biodiverse region that contains a vast array of flora and fauna, some of which are unique to the region (Coe et al. 2013). The presence of this biodiversity has attracted the attention of researchers and bioprospectors, as many of the species in this region contain bioactive compounds that have potential use in medicine, cosmetics, and other industries (Harvey et al. 2015; Bolzani 2016; Newman et al. 2020).

Anuran species are a type of amphibian that includes frogs, toads, and tree frogs (Segalla et al. 2016). These animals are known for secreting toxins from their skin as a defense mechanism against predators. However, these toxins also have potential therapeutic applications in medicine, making them a

promising subject of study for researchers in the field of molecular bioprospecting (Uetanabaro et al. 2008; Sciani et al. 2013; Segalla et al. 2016).

Rhaebo guttatus (R. guttatus) is a toad species in the Bufonidae family that inhabits Central and South America (Schneider, 1799; Hinkson and Baecher 2019; Frost 2020). This species is unique compared to other toads as it can voluntarily release its poison through the parotoid glands as a defense mechanism against predators (Jared et al. 2011; Mailho-Fontana et al. 2014). The venom of this toad has been found to contain alkaloids and steroids, with the composition varying depending on the animal's environment and diet (Souza et al. 2020).

There are studies that have demonstrated the biological activity as the effects that extracts from the secretion of the parotoid gland of *R. guttatus* exhibit hemolytic and cytotoxic activity against tumor and non-tumor cells (Ferreira et al. 2013); toxic activity against *Plasmodium falciparum* and phytopathogenic fungi (Banfi et al. 2016; Raasch-Fernandes et al. 2021); mutagenic and antimutagenic (Oliveira et al. 2019); nociception properties (Mailho-Fontana et al. 2014) and immunomodulatory activity (Pelissari et al. 2023).

Oxidative stress is a physiological condition that occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of the body to neutralize them through antioxidants. This imbalance can lead to damage to lipids, proteins, and DNA, causing various diseases such as liver disease, cancer, and neurodegenerative disorders. Therefore, the search for natural antioxidants, such as those found in *R. guttatus* poison, is of great interest to the scientific community (Leonarduzzi et al. 2010; Stauffer et al. 2012; Carocho and Ferreira 2013). Studies realized by our group investigated the potential antioxidant effects of *R. marina* poison and understand how it may modulate oxidative stress in animals (Pelissari et al. 2021). In line with this view, we decided to study the possible antioxidant effects *per se* caused by treating animals orally with the crude methanolic extract for both 7 and 30 days. This study may also help determine the long-term effects of the extract and provide insight into any potential therapeutic applications.

2. Material and Methods

Poison collection and extract preparation

The adult animals (males and females) were captured and identified by D. J. Rodrigues (IBAMA, SISBIO: 30034-1). Registration number in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) A313DC9. The collection took place in the municipality of Cotriguaçu/MT (9°49'26.00"S 58°15'26.00" W) in November and December 2016. The toad poison was obtained through manual compression of the parotoid glands. The poison was dried, crushed and extracted by maceration with methanol 99% using an ultrasonic bath (Unique, Indaiatuba, São Paulo, Brazil) for two hours in order to obtain the crude methanolic extract (CME). Afterwards, it was filtered through filter paper (Unifil), and the poison was macerated twice more under the same conditions. Finally, the extracts were grouped and the solvent was rotary evaporated (IKA, Staufen, Germany) at 40 °C and kept under vacuum in a desiccator at room temperature for 48 h. The obtained CME was stored at 4 °C. The experimental conditions are in accordance with the work done by the research group in (Kerkhoff et al. 2016).

Animals and experimental design

This study was registered and approved by the UFMT Ethics Committee on the Use of Animals (CEUA) under No. 23108.918243/2017-50. Male Swiss mice with an average weight of 35 g (70 days old) obtained from the Central Biotério of the Federal University of Mato Grosso, Campus of Cuiabá, were used in the experiment. The animals were maintained in polyethylene boxes with a stainless steel grid during the acclimatization (14 days) and experimental period. They were divided into 5 groups of 6 animals each, with a 12-hour light/dark cycle in a temperature-controlled room (24 ± 1 °C and relative humidity of 55 ± 2%), and provided with food and filtered water ad libitum. The animals were treated with water (control),

0.5% Tween 20 solution (vehicle), or different doses of the CME (8 µg mL^{-1}, 16 µg m^{L-1}, and 32 µg mL^{-1}; 100 µL/animal/day; via gavage) for 7 or 30 days. The doses were defined according to the study by Oliveira et al. (2019).

The aliquots were prepared in microtubes, diluted in 0.5% Tween 20 solution, and stored at -4 °C. At the end of the treatment (24 hours later), the animals were euthanized by cervical dislocation, and their livers were removed and kept frozen in an ultra-freezer (-80 °C) for the analysis of oxidative stress parameters.

Oxidative stress parameters

Catalase (CAT) activity was assayed according to Nelson and Kiesow's method, which involves the spectrophotometric measurement of the change in H_2O_2 absorbance over 60 seconds at 240 nm. Liver tissues were homogenized in 20 mM potassium phosphate buffer (PPB), pH 7.5 and centrifuged at 10,000 g for 20 min at 4 °C. The assay mixture consisted of 1.0 mL potassium phosphate buffer (50 mM, pH 7.0), 0.025 mL H_2O_2 (0.3 M), and 0.025 mL homogenate. CAT activity was calculated using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} and expressed in µmol min^{-1} mg $protein^{-1}$ (Nelson and Kiesow 1972).

Glutathione-S-transferase (GST) activity was determined according to Habig et al.'s method, which involves the spectrophotometric measurement of the increase in absorbance at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Liver samples were homogenized in 20 mM potassium phosphate buffer (PPB), pH 7.5 and centrifuged at 10,000 g for 15 min at 4 °C. The assay mixture consisted of 1.250 mL PPB (50 mM, pH 7.0), 0.150 mL GSH (10 mM), 0.075 mL CDNB (20 mM) and 0.025 mL homogenate. GST activity was expressed in µmol GS-DNB min^{-1} mg $protein^{-1}$. The molar extinction coefficient used for CDNB was 9.6 mM cm^{-1} (Habig et al. 1974).

Reduced glutathione (GSH) levels were determined according to Sedlack and Lindsay's method using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB). Liver samples were homogenized in 20 mM EDTA. A 2.0 mL aliquot of supernatant was mixed with 4.0 mL of Tris–HCl (0.4 M and pH 8.9) and was added to 0.01 M DTNB in 0.05 M phosphate buffer, pH 8.0. The formation of the thiolate anion was determined by spectrophotometric measurement at 412 nm against a GSH standard curve. The results were expressed in µmol of GSH mg $protein^{-1}$ (Sedlack and Lindsay 1968).

Lipid damage was estimated by spectrophotometric determination of the levels of thiobarbituric acid reactive substances (TBARS), a method described by Buege and Aust (1978). Liver samples were homogenized in 20 mM PPB, pH 7.5, centrifuged at 1000 g at 4 °C and the supernatant was removed after centrifugation for examination. The readings were performed at 535 nm, and the values for TBARS concentration were expressed in nmol malondialdehyde (MDA) mg $protein^{-1}$, following the calibration curve for MDA.

Protein content of samples was estimated by spectrophotometry according to Bradford's method using bovine serum albumin as a standard, and the absorbance of the samples was measured at 595 nm (Bradford 1976).

Statistical Analysis

The data for the redox status parameters were subjected to normality and homogeneity of variances tests. For data that showed normal distribution or homogeneous variances, a one-way analysis of variance (ANOVA) was performed, followed by *post hoc* Tukey test (for CAT and GST). On the other hand, for non-parametric data, Kruskal-Wallis analysis was performed, followed by *post hoc* Dunn's test to assess differences between experimental groups (for GSH and TBARS). The results were presented as mean ± standard deviation (SD) or median and total amplitude, and the level of significance for the study was set at 95% ($P<0.05$).

3. Results

In this study, CAT activity did not change among groups after 7 days of treatment, but it was observed that this parameter increased only at the 16 µg mL^{-1} concentration when compared to the 8 µg mL^{-1} concentration for the 30-day treatment. In GST activity, the 16 µg mL^{-1} concentration was different from the control group and the 32 µg mL^{-1} concentration after 7 days of treatment. However, after 30 days of treatment, the 8 µg mL^{-1} concentration elevated this activity when compared to other groups in the same period and also presented an increase when compared to 8 µg mL^{-1} (7 days). For GSH levels, the 32 µg mL^{-1} concentration was reduced when compared to the control group (7 days) and this dose increased GSH levels after 30 days of treatment. Still, in the 30-day treatment, this parameter increased in this same group in relation to the 16 µg mL^{-1} concentration. For TBARS levels, a lipid peroxidation biomarker, the 8 µg mL^{-1} dose (7 days) presented a significant increase when compared to most treatments (except for the 32 µg mL^{-1} dose), but after 30 days, none of the treatments showed significant changes, Figure 1.

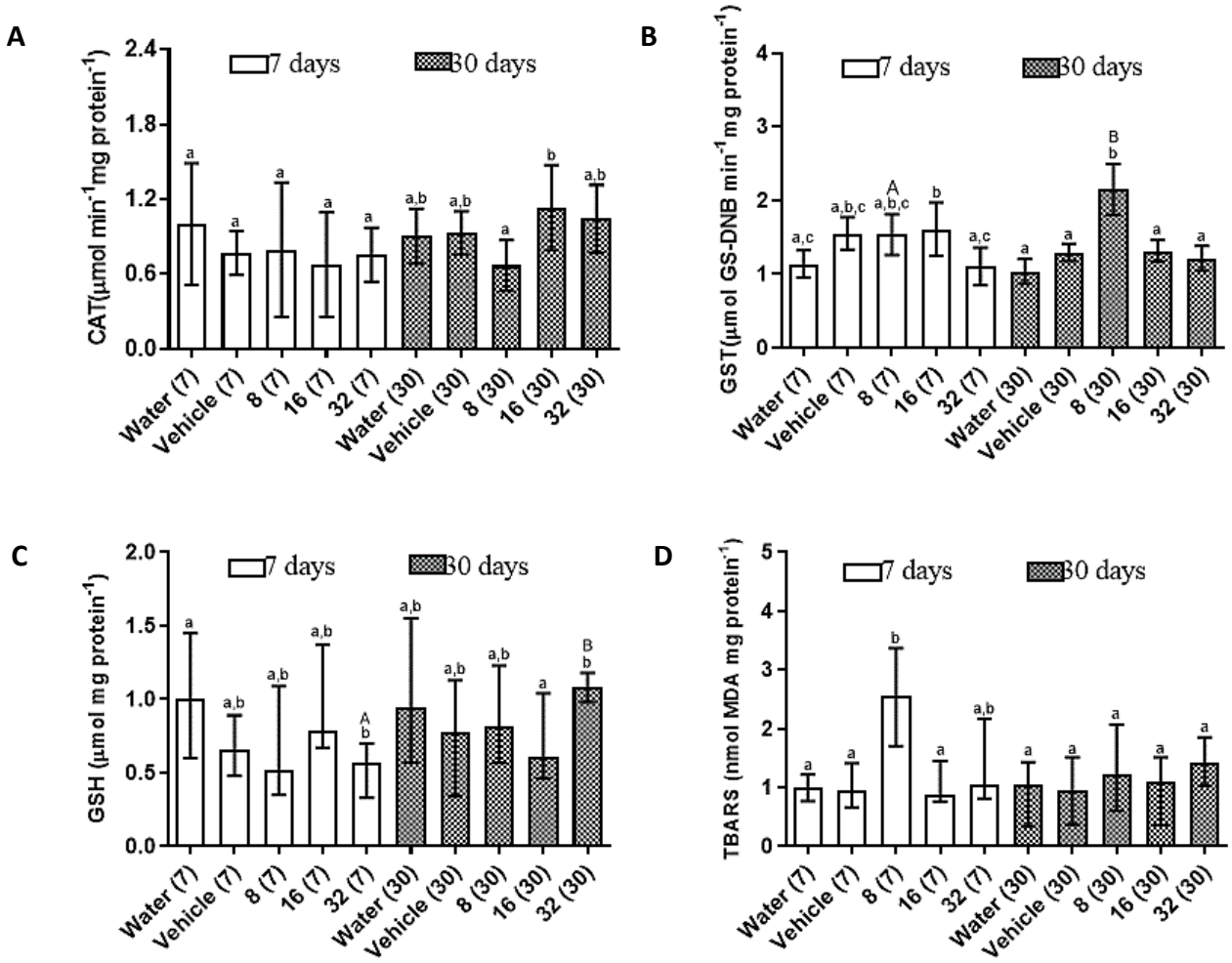

Figure 1. Effect of methanolic extract of *R. guttatus* poison on status redox parameters in mice liver during 7 or 30 days of treatment. Catalase (CAT, graph A), Glutathione-S-transferase (GST, graph B), Reduced glutathione (GSH, graph C) and Thiobarbituric acid reactive substances (TBARS, graph D). Different lowercase letters indicate significant differences between treatments in the same period. Different uppercase letters indicate significant differences between different times. For CAT and GST, a parametric analysis (ANOVA), followed by *Tukey*'s test. For GSH and TBARS, a non-parametric one-way analysis (Kruskal-Wallis), followed by Dunn's test; $P<0.05$; (n = 6).

4. Discussion

This is the first study to investigate the effects of the CME from *R. guttatus* poison on antioxidant or pro-oxidant activity. We considered the doses used by Oliveira et al. in their study on antimutagenic and cytotoxic activity (Oliveira et al. 2019). Our results showed that the 8 µg mL^{-1} dose increased TBARS levels

after 7 days, while the 32 µg mL^{-1} dose reduced GSH levels, an important non-enzymatic antioxidant, during the same period, but returned to control levels after 30 days. Notably, there were significant alterations observed in GST activity with an increase at different doses and exposure times. In contrast, the CAT activity did not change after 7 days, but there was an increase in animals treated with the 16 µg mL^{-1} concentration compared to those treated with the 8 µg mL^{-1} dose, although there was no significant difference compared to the control group. Overall, our findings suggest that the CME from *R. guttatus* poison induces changes in the status of redox parameters.

In this study, we chose to evaluate the hepatic tissue because of its importance as a vital organ in the human body. The liver plays essential roles in metabolism, immune response, and detoxification (Aizarani et al. 2019). The liver is highly susceptible to oxidative stress, and inflammation is a key component of several liver diseases (Schemitt et al. 2019; Zakaria et al. 2021; Ijaz et al. 2022). Metabolites of hepatotoxic drugs can bind to the toll-like receptor complex, which activates nuclear factor kappa B (NFκB), triggering the production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, leading to liver injury (Schemitt et al. 2019; Ijaz et al. 2022). Under stress conditions, the nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the redox balance, promoting the expression of antioxidant enzymes such as GST and CAT (Schemitt et al. 2019).

Based on the susceptibility of the liver to oxidative stress, we hypothesized that the observed increase in TBARS levels is likely due to the production of free radicals mediated by substances present in the poison. These substances may stimulate the lipid peroxidation process and promote an imbalance in the protective antioxidant action of GSH and GST. In line with this view, a recent study conducted by Pelissari et al. (2023) showed crude methanolic extract from the parotoid gland secretion of *Rhaebo guttatus* stimulates the production of reactive species and pro-inflammatory cytokines by peritoneal macrophages, corroborating with our data.

Among the various compounds found in *R. guttatus* there are alkaloids (N-methyl-5-hidroxytryptamine, bufotenine e dehydrobufotenin) and steroids (3β,16β-dihydroxybufa-8(14),20,22-trienolided and bufatrienolides) (Souza et al. 2020). In the literature, bufotenine is described for its anti-inflammatory and analgesic effect, being able to inhibit the production of TNF- α and IL-6, through the inhibition of NFκB activation (Zulfiker et al. 2016; Wang et al. 2021). Dehydrobufotenin presents antimalarial activity (Banfi et al. 2021) and bufatrienolides have strong antiproliferative activity against tumor cells (Dai et al. 2013). Furthermore, these three compounds may not have cytotoxic action against normal cells (Dai et al. 2013; Zulfiker et al. 2016; Wang et al. 2021; Banfi et al. 2021).

It is indeed important to consider that isolated compounds may have a different response profile compared to that observed in the crude extract (Oliveira et al. 2019). Therefore, despite the anti-inflammatory profile of the isolated compounds, the combined action of the compounds present in the crude extract or their metabolites may have stimulated a hepatic inflammatory response, resulting in tissue damage (indicated by TBARS levels). Additionally, the inflammatory mediators produced may have inhibited the activation of the antioxidant defenses of the liver. During inflammation, the overproduction of reactive oxygen species (ROS) can decrease the activity of antioxidant enzymes due to deactivation or reduction in enzymatic synthesis (Zakaria et al. 2021). This could explain the lack of CAT activity and the reduction of GSH, in this case, due to the intense consumption in response to the overproduction of ROS. Thus, the increase in GST alone would not have been sufficient to maintain the redox balance.

In a study conducted by our research group using a different species, the CME from *R. marina* poison, we observed similar results for CAT but different responses for the antioxidants GST and GSH, as well as for TBARS (Pelissari et al. 2021). In the study by Pelissari et al. (2021), the levels of hepatic GSH and TBARS of the treated animals showed no significant difference compared to the control groups. However, an increase in the GSH levels was observed at a 32 µg mL^{-1} dose on the 7th day. The difference in the results obtained may reflect the combination of bioactive compounds present in the extracts, as marinobufagin, telecinobufagin, and bufalin derivatives were found in the CME from *R. marina*, in addition to dehydrobufotenine and arginine diacids (Pelissari et al. 2021).

5. Conclusions

Our results demonstrate that the CME from *R. guttatus* poison has an effect on the redox balance, as evidenced by changes in the lipid damage marker (TBARS) and in the antioxidants (CAT, GST and GSH) in different doses and times of treatment. This is the first study to investigate the oxidant profile of the CME from *R. guttatus* poison, and the findings support the biotechnological potential of the active compounds found in the toad's venom. However, further research is necessary to identify and isolate the compounds present in the crude extract and to investigate the mechanisms of action underlying the observed effects.

Authors' Contributions: PELISSARI, S.R.N.: conception and design, acquisition of data, analysis and interpretation of data, and drafting the article; CASTOLDI, L.: drafting the article and critical review of important intellectual content; SINHORIN, A.P.: analysis and interpretation of data, drafting the article, and critical review of important intellectual content; SINHORIN, V.D.G.: analysis and interpretation of data, drafting the article, and critical review of important intellectual content; RODRIGUES, D.J. Acquisition of data, Poison collection and animal identification; SHENG, L.Y.: analysis and interpretation of data. All authors have read and approved the final version of the manuscript.

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Ethics Approval: It is important to note that all animal experiments detailed in this research adhered to the ethical guidelines set forth by the UFMT Ethics Committee on the Use of Animals (CEUA). The experimentation was carried out under protocol No. 23108.918243/2017-50.

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