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Evaluation of the biological activity of venom extracts from the toads *Rhinella marina* and *Rhaebo guttatus* on peripheral blood monocytes from healthy human subjects

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Abstract. Skin secretions from toads of the Bufonidae family contain immunomodulatory and antioxidant substances and have pharmaceutical potential for treating cancer, degenerative diseases, and cardiovascular conditions. Previous studies have demonstrated that the methanolic extracts of *Rhinella marina* and *Rhaebo guttatus* have an immunomodulatory effect on murine macrophages, stimulating the production of pro-inflammatory cytokines and hydrogen peroxide. However, little is known about its effect on human monocytes. Therefore, the aim of this study was to evaluate the *in vitro* effect of these extracts on the function of human peripheral blood monocytes by assessing cytotoxicity and the production of nitric oxide and hydrogen peroxide. The amphibians were collected in Mato Grosso, Brazil. In the laboratory, their paratoid glands were compressed to extract the venom. Voucher specimens were deposited in the Biological Collection of the Southern Amazon (Sinop/MT). To obtain monocytes, peripheral blood samples were collected from 16 healthy individuals (aged 30–45 years) who had not used any class of medication for the previous 15 days. The monocytes were cultured (5% CO_2 at 37°C) with different extract concentrations for 18 h (for cytotoxicity assessment) or 24 h (for activity assessment). For *R. marina* venom, the most toxic concentration was 1000 µL mL^{-1}, whereas non-toxic concentrations were 62.5 µL mL^{-1}, 15.63 µL mL^{-1}, and 7.8 µL mL^{-1}. For *R. guttatus*, concentrations ranging from 1000 µL mL^{-1} to 31.25 µL mL^{-1} were the most toxic, and the non-toxic concentrations were 15.63 µL mL^{-1} and 7.8 µL mL^{-1}. The cells did not produce nitric oxide or hydrogen peroxide in response to stimulation with the extracts. We conclude that extracts from the skin secretions of *R. marina* and *R. guttatus* exhibit a concentration-dependent cytotoxic effect on human monocytes but do not modulate the cells' *in vitro* ability to produce nitric oxide and hydrogen peroxide.
Keywords: *Rhinella marina*, *Rhaebo guttatus*, human monocytes, cytotoxicity, immunomodulation.

Introduction

Natural products and their derivatives exhibit valuable characteristics for the pharmaceutical industry, including structural diversity, biochemical specificity, structural flexibility, a wide range of biological activities, low toxicity, minimal side effects, and abundant sources (Deng et al., 2020; Leal et al., 2020; Sousa et al., 2017). These advantages explain why approximately 60% of new chemical compounds are directly or indirectly derived from natural products sourced from animals, plants, fungi, bacteria, or marine algae (Ferreira et al., 2013; Leal et al., 2020; Sousa et al., 2017; Sousa et al., 2023). Notable examples include morphine isolated from *Papaver somniferum*, bradykinin from the venom of *Bothrops jararaca*, and azidothymidine from marine algae nucleosides, as well as a wide range of antibiotics such as penicillin obtained from bacteria and fungi, and antiparasitic drugs such as artemisinin (*Artemisia annua*)(Banfi et al., 2016;Rodriguez et al., 2024; Sousa et al., 2023).

Brazil is home to the world's greatest biodiversity and largest equatorial and tropical rainforest, particularly the Brazilian Amazon (Pelissari et al., 2021; Raasch-Fernandes et al., 2021). Amphibians, such as toads and frogs, exemplify this vast potential source of chemical compounds with biological and industrial potential. The skin of these animals contains parotoid glands, which store and secrete venom comprising a variety of biomolecules, including peptides, alkaloids,

proteins, biogenic amines, and bufadienolides(Ferreira et al., 2013;Filho et al., 2021; Ibarra-Vega et al., 2023; Kerkhoff et al., 2016; Medeiros et al., 2019; Souza et al., 2020). Amphibians use these secretions as a defense mechanism against predators, parasites, and microorganisms (Ferreira et al., 2013; Pelissari et al., 2021). However, studies have shown that extracts from these animal venoms exhibit a wide range of biological activities, such as trypanocidal, leishmanicidal, antibacterial, antifungal, insecticidal, antitumor, antiproliferative, antiviral, cardiotonic, immunomodulatory, and anti-inflammatory properties (Cunha Filho et al., 2005; Ferreira et al., 2013;Filho et al., 2021; Ibarra-Vega et al., 2023; Kerkhoff et al., 2016;Medeiros et al., 2019;Souza et al., 2020; Wang et al., 2011).

Toads of the family Bufonidae have a cosmopolitan distribution, comprising 52 genera and 644 species (Ibarra-Vega et al., 2023). This study evaluated *Rhinella marina* and *Rhaebo guttatus*,two species typically found in the Brazilian Amazon region (Oliveira et al., 2019). Previous research by our group has shown that methanolic extracts from *Rhinella marina* and *Rhaebo guttatus* exhibit immunomodulatory activity on murine macrophages, stimulating the production of pro-inflammatory cytokines, such as TNF-α and IL-12p70, as well as hydrogen peroxide (H_2O_2) (Pelissari et al., 2021; Pelissari et al., 2023). These findings have been corroborated by other authors, who have observed that compounds isolated from the secretions of Chinese toads (*Bufo melanostictus* Schneider and *Bufo bufogargarzinas* Cantor) can stimulate the immune system by enhancing macrophage activity and cytokine production (Cao et al., 2009;Deng et al., 2020; Shih et al., 2018; Xie et al., 2016;Yu et al., 2015).

Based on these findings, we considered it important to evaluate the activity of the extracts in phagocytes from another species, specifically human monocytes, to corroborate the effects observed in murine cells. Through this approach, we aim to further the understanding of the biological action of compounds in venom extracts on immune cells and their potential application for modulating the inflammatory response in chronic diseases. It is also noteworthy that the human peripheral blood monocytes in this study were isolated using the Henson et al., (1972) technique, as modified by Lucisano and Mantovani (1984), a method that, to our knowledge, has not been previously described in the literature for this purpose. Therefore, the objective of this study was to assess the effect of *Rhinella marina* and*Rhaebo guttatus* extracts on the function of human peripheral blood monocytes by analyzing cellular cytotoxicity and the production of nitric oxide (NO) and H_2O_2 by the cultured cells.

Material and methods

Sample collection

This study involved 16 healthy individuals of both sexes, aged 30 to 45 years, who presented a stable immunogenic profile and were not taking any class of medication. The participants were students and staff from the State Technical School of the State Secretariat of Science and Technology and the Federal University of Mato Grosso, Sinop Campus. Sample collection was performed after obtaining informed consent from all participants, in accordance with the guidelines of the Human Research Ethics Committee. A trained healthcare professional carried out the collection. Peripheral blood samples were obtained via antecubital venipuncture using a sterile, disposable vacuum system and collected into tubes containing an anticoagulant. A total of 20 mL of blood was drawn from each participant for this study. Participants were recruited during their regular work shifts, and blood was collected only once from each person; therefore, they did not visit the facility solely for the purpose of the research. This procedure was conducted under the supervision of Professor Lucinéia Reuse Albiero, MSc, as part of the project titled “Evaluation of the biological activity of venom extracts from the toads *Rhinella marina* and *Rhaebo guttatus* on peripheral blood monocytes from healthy human subjects,” which was approved by the Human Research Ethics Committee (CAAE: 64734122.3.1001.8097, approval no. 5.822.240).

Isolation of human monocytes

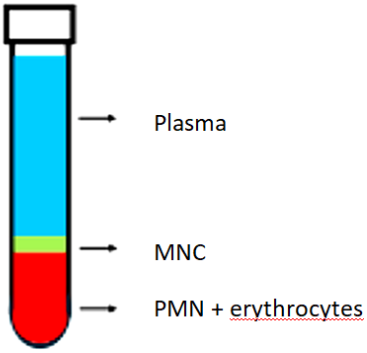
Following the collection of peripheral blood samples via antecubital venipuncture using an anticoagulant (Alsever’s solution, pH 6.1), monocytes were isolated using the Henson et al. (1972) technique, as modified by Lucisano and Mantovani (1984). This method also yields a suspension of mononuclear cells, which includes monocytes, as depicted in Figure 1.

The anticoagulated blood was centrifuged (1250 ×*g*, 10 min, 20 °C), and the plasma was removed. Using a Pasteur pipette, the mononuclear cell ring was collected, resuspended in a 2.5% gelatin solution in 0.15 mol L^{-1} NaCl, and incubated for 15 min at 37°C.Subsequently, the monocyte-containing supernatant was diluted with 0.15 mol L^{-1} NaCl and centrifuged (974 ×*g*, 10 min, 20 °C). Residual red blood cells were lysed with 0.83% NH_4Cl (pH 7.2) for 5 min. The cells were then centrifuged (974 ×*g*, 10 min, 20 °C), washed with 0.15 mol L^{-1} NaCl, and finally resuspended in RPMI 1640 culture medium (Sigma Aldrich Co, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cultilab, Campinas, Brazil).

Since the previously described technique is not commonly used, the number and viability of the monocytes obtained were compared with those from the conventional method. In this standard technique (Dias-Melicio et al., 2005; Gomes et al., 2020; Martins et al., 2017; Matias et al., 2019; Peraçoli et al., 2011), a 5 mL heparinized peripheral blood sample, collected via antecubital puncture, was diluted with 5 mL RPMI culture medium containing 10% FBS. This mixture was then layered over a Histopaque density gradient (*d* = 1.077; 5 mL;

Sigma Aldrich Co, St Louis, MO, USA) and centrifuged at 400 ×*g* for 30 min at room temperature. Following centrifugation, the mononuclear cell layer at the interface was gently collected with a Pasteur pipette and washed twice with PBS-EDTA and once with RPMI 1640 with 10% FBS (centrifuging at 300 ×*g* for 10 min for each wash).

The viability of the mononuclear cell suspension was assessed using the 0.2% Trypan Blue exclusion method, and monocytes were counted with 5% Turk's stain, with both procedures performed in a Neubauer chamber.



MNC: mononuclear cells
PMN: polymorphonuclear cells (neutrophils)

Figure 1. Schematic diagram of the Henson et al., (1972) technique, as modified by Lucisano and Mantovani (1984), for the isolation of human neutrophils from peripheral blood.

Human peripheral blood monocyte culture

Cell viability was determined using the 0.2% Trypan Blue exclusion method, and only samples with at least 70% viable cells were used in the experiments. Monocytes within the mononuclear cell suspension were identified by their morphology and counted using Turk's stain and a Neubauer chamber. The concentration was then adjusted to 1.0×10^6 monocytes/mL in RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS). Following this adjustment, the cell suspension containing 1.0×10^6 monocytes/mL was plated into 96-well flat-bottom microplates (100 µL/well) and incubated for 2 h at 37°C in a 5% CO_2 atmosphere. At the end of the incubation period, non-adherent cells were removed by aspiration, and each well was washed twice with RPMI 1640 medium containing 10% FBS.

Assessment of the cytotoxic potential of the extracts

The monocyte monolayer adhered to the microplate (as described in the previous section) was treated with either RPMI 1640 containing 10% FBS (100 mL/well, control group) or various concentrations of the toad venom extracts (diluted in RPMI with 10% FBS and 0.01% DMSO, 100 mL/well, test group). The plates were then incubated for 18 h at 37°C in a 5% CO_2 atmosphere. A group of cells cultured with Triton X-100 diluted in RPMI with 10% FBS was used as the positive control for cytotoxicity (cell lysis). The DMSO group, consisting of cells cultured with RPMI containing 10% FBS and 0.01% DMSO, served as the vehicle control for the extract diluent. Following this incubation period, the cells were treated with 0.3% trypsin (3 min, Cultilab, Campinas, Brazil), and the enzymatic action was neutralized with RPMI containing 50% FBS. Cell viability was assessed using the 0.2% Trypan Blue exclusion assay to determine the cytotoxic potential of the extracts and to establish the working concentration for subsequent cell activity analyses.

Measurement of spontaneous H_2O_2 release by monocytes

Spontaneous H_2O_2 production by monocytes was measured using the method developed by Pick and Mizel (1981). Following the incubation period, the supernatant from the adherent monocyte culture was collected and set aside for the nitric oxide (NO) assay. To determine H_2O_2 levels, 100 µL of a phenol red solution, containing 140 mM sodium chloride (NaCl), 10 mM dibasic potassium phosphate (K_2HPO_4), 5.5 mM dextrose, and 5.5 mM peroxidase, was added to the adherent cell monolayer in each well. The plate was then incubated for 60 min at room temperature, protected from light. After incubation, the reaction was stopped by adding 10 µL 1 M sodium hydroxide (NaOH), and the absorbance of the solution was measured at 630 nm using a microplate reader. The blank consisted of the phenol red solution with 1 M NaOH. The concentration of H_2O_2 produced by the monocytes was calculated from a standard curve generated with known H_2O_2 concentrations, with the

final value representing the mean of triplicate samples.

Measurement of NO production by monocytes

The previously collected supernatant from the monocyte cultures was used to quantify NO using the colorimetric method based on the Griess reaction Green et al., 1981). To each supernatant sample, 100 µL of Griess reagent was added. This reagent consisted of 1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NEED) in distilled water and 1% sulfanilamide in 5% phosphoric acid (H_3PO_4). These two components were mixed in equal volumes immediately before the assay. The absorbance of the samples was measured at 492 nm using an ELISA plate reader. The Griess reagent alone served as the blank. The concentration of NO produced by the monocytes was calculated from a standard curve generated with known concentrations of sodium nitrite solution. The final values represent the average of samples assayed in triplicate.

Substance under study

Five to ten adult toads (males and females) were collected and identified by Prof. Dr. Domingos de Jesus Rodrigues (a permanent license for the collection of zoological material has been obtained from IBAMA, SISBIO: 30034-1) in Nova Ubiratã, Mato Grosso, Brazil (13°6′16.20″ S 54°25′51.01″ W). The extracts were prepared by Prof. Dr. Adilson Paulo Sinhorin at the Phytochemistry Laboratory of the Federal University of Mato Grosso, Sinop Campus. The methods for extract preparation and chemical profiling were as described by Kerkhoff et al. (2016) and Sousa et al. (2020).

Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA), followed by the Tukey–Kramer post-hoc test. All data are expressed as mean ± standard deviation, and differences between groups were considered significant at a 5% level ($P < 0.05$).

Ethical principles and good practices in experimentation

This research was submitted to and approved by the Ethics Committee (Human Research Ethics Committee, CAAE: 64734122.3.1001.8097, approval no. 5.822.240) in accordance with ethical principles and current legislation.

Results and discussion

Analysis of the cytotoxic potential of Rhinella marina and Rhaebo guttatus venom on human peripheral blood monocytes

Figures 2 and 3 show the results of the cytotoxicity analysis for the *Rhinela marina* and *Rhaebo guttatus* extracts, respectively.

As shown in Figure 2, the 1000 µL mL^{-1}, 500 µL mL^{-1}, 250 µL mL^{-1}, 125 µL mL^{-1}, and 31.25 µL mL^{-1} concentrations of the *Rhinella marina* extract were found to be toxic. Their effects were similar to the Triton positive control and differed from the control and DMSO groups, with the 1000 µL mL^{-1} concentration exhibiting the highest toxicity. Conversely, the 62.5 µL mL^{-1}, 15.63 µL mL^{-1}, and 7.8 µL mL^{-1} concentrations were non-toxic, yielding results similar to those of the basal and DMSO controls and different from those of the Triton control. The comparable results of the basal and DMSO controls indicate that the vehicle used was not harmful to the cells.

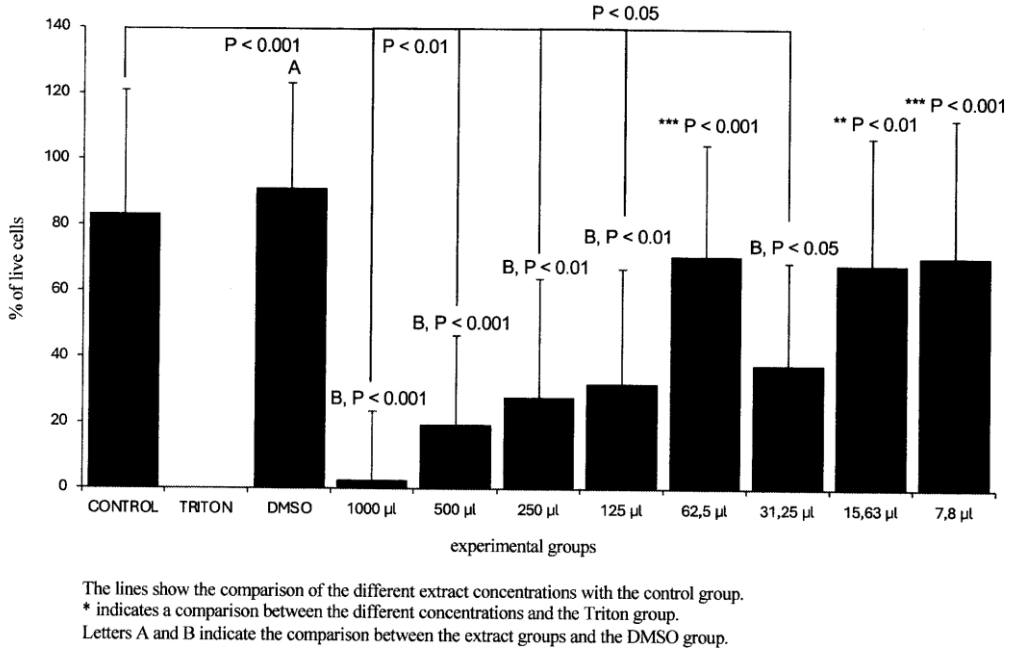
Figure 3 presents the results for the *Rhaebo guttatus* extracts. Consistent with the observations for the *Rhinela marina* extracts, concentrations ranging from 1000 µL mL^{-1} to 31.25 µL mL^{-1} were toxic, yielding results similar to those of the Triton group and distinct from those of the control and DMSO groups. In contrast, the 15.63 µL mL^{-1} and 7.8 µL mL^{-1} concentrations were comparable to those of the basal and DMSO groups and different from those of the Triton group, indicating a lack of cytotoxic activity. The vehicle used for extract preparation was not toxic to the cells, as its results were similar to those of the control group.

Based on the results from the cytotoxicity assessment of the extracts, the 7.8 µL mL^{-1} concentration was selected to study the effects of the extract on NO and H_2O_2 production by monocytes.

NO and H_2O_2 production by monocytes

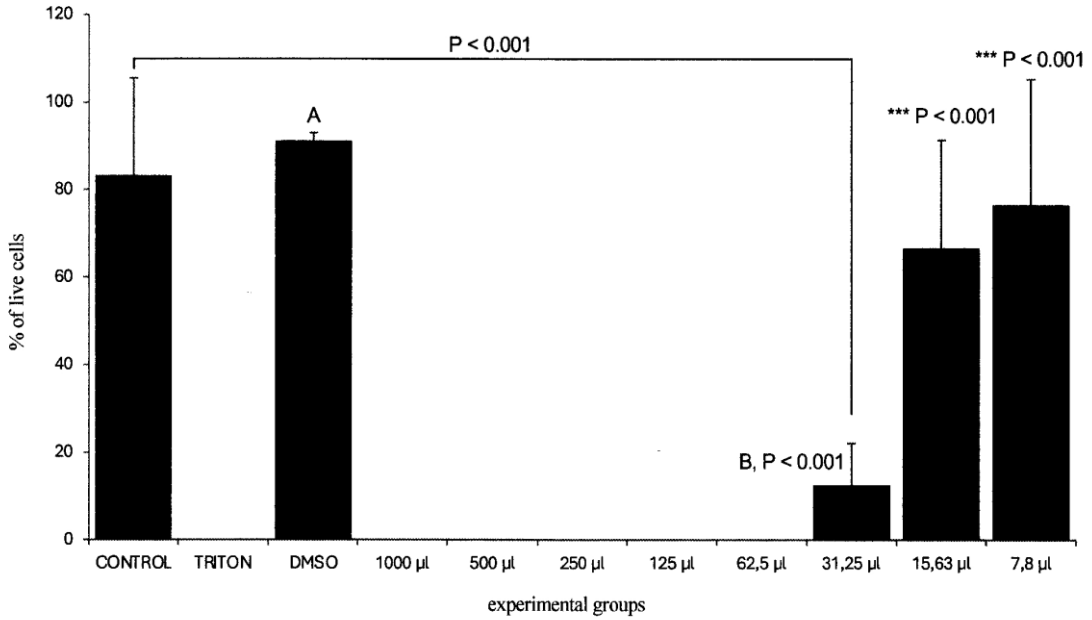
The extract from both toads failed to stimulate the production of NO and H_2O_2 by the monocytes (Figures 4 and 5, respectively), indicating that these cells were not modulated in response to stimulation with the extract.

1972) technique, as modified by Lucisano and Mantovani (1984) and described in the Materials and Methods section. While this technique is typically used to separate human neutrophils from peripheral blood samples, it also yields a monocyte suspension (Figure 1). This fraction, which would otherwise be discarded, was repurposed for the present study, thereby optimizing the use of patient samples. When this adapted Henson technique using a gelatin solution (Alsever) was compared to the conventional method using Histopaque, no significant difference was observed in the mean number of monocytes obtained (Figure 6: Alsever: 5.56 ± 1.86; Ficoll: 4.67 ± 2.13).



The lines show the comparison of the different extract concentrations with the control group.
* indicates a comparison between the different concentrations and the Triton group.
Letters A and B indicate the comparison between the extract groups and the DMSO group.

Figure 2: Cytotoxicity of *Rhinella marina* extracts on peripheral blood monocytes from healthy humans (n = 7). Percentage of live cells in relation to the total number of cells, both live and dead.



The lines show the comparison of the different extract concentrations with the control group.
* indicates a comparison between the different concentrations and the Triton group.
Letters A and B indicate the comparison between the extract groups and the DMSO group.

Figure 3: Cytotoxicity of *Rhaebo guttatus* extracts on peripheral blood monocytes from healthy humans (n = 5). Percentage of live cells in relation to the total number of cells, both live and dead.

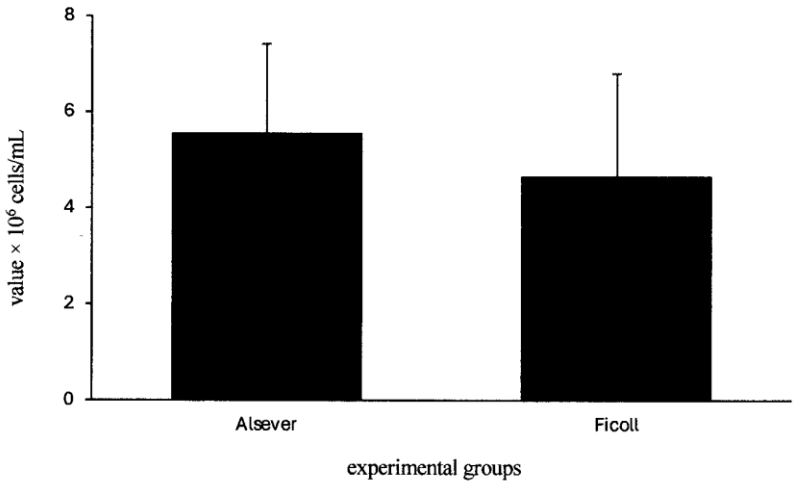


Figure 4. *In vitro* NO production by peripheral blood monocytes from healthy individuals, stimulated with extracts of *Rhinella marina* (RM) and *Rhaebo guttatus* (RG) for 24 h at a concentration of 7.8 µL mL^{-1}. Values expressed in mM.

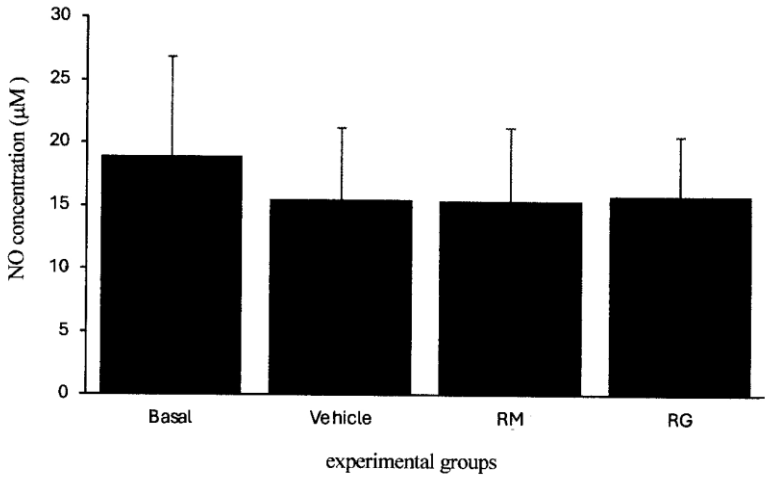


Figure 5. *In vitro* H_2O_2 production by peripheral blood monocytes from healthy individuals, stimulated with extracts of *Rhinella marina* (RM) and *Rhaebo guttatus* (RG) for 24 h at a concentration of 7.8 µL mL^{-1}. Values expressed in nM.

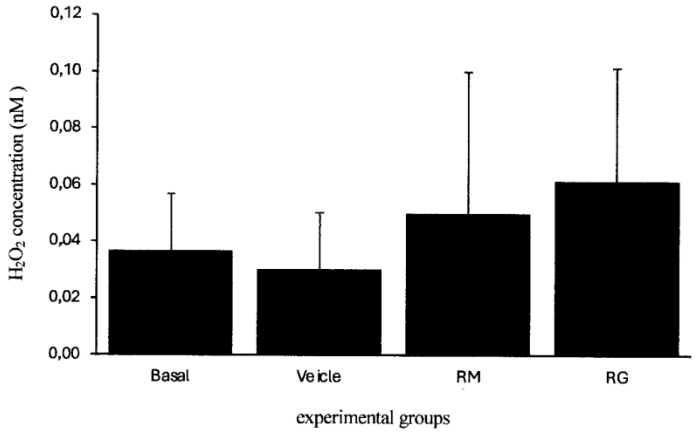


Figure 6. Mean and standard deviation of the monocyte count (× 10^6 cells/mL) obtained using the Henson (1972) technique, as modified by Lucisano and Mantovani (1984) (Alsever group), and the conventional technique with Histopaque (Ficoll group).

Monocytes were isolated using the Henson (

The crude venom extract of amphibians from the genera *Rhinella* and *Rhaebo* contains various biologically active compounds (Ibarra-Veja et al., 2023; Kerkhoff et al., 2016; Schmeda-Hirschmann et al., 2022; Souza et al., 2020). Studies have identified the presence of biogenic amines, steroids, alkaloids, proteins, and peptides capable of inducing antiproliferative, cytotoxic, vasoconstrictor, antimutagenic, antibacterial, antiparasitic, insecticidal, immunomodulatory, anticonvulsant, and antioxidant effects, as well as inhibiting the pathological progression of Alzheimer's disease (Baldo et al., 2019; Banfi et al., 2016; Ferreira et al., 2013; Filho et al., 2021; Ibarra- Vega et al., 2023; Kerkhoff et al., 2016; Medeiros et al., 2019; Oliveira et al., 2019;Pelissari et al., 2021; Pelissari et al., 2023;Rodriguez et al., 2024; Santos et al., 2022; Schmeda-Hirschmann et al., 2022;Sousa et al., 2017; Souza et al., 2020; Spinelli et al., 2022). This makes the venom a promising source of novel molecules for the pharmaceutical industry.

This study evaluated the effect of the crude methanolic extract from the venom of the toads *Rhinella marina* and *Rhaebo guttatus* on human peripheral blood monocytes, assessing its cytotoxic action and its modulatory effect on the cellular production of NO and H_2O_2.

The results demonstrated that the extracts exhibit a concentration-dependent cytotoxic effect; however, they did not alter the production of oxygen- and nitrogen-derived free radicals by the monocytes.

The cytotoxic action of compounds found in the venom of these animals has been well-documented across various concentrations and experimental models, involving diverse cell types of both tumor and non-tumor origin (Abdelfatah et al., 2019; Banfi et al., 2016; Ferreira et al., 2013; Filho et al., 2021; Garcia et al., 2019; Oliveira et al., 2019;Sousa et al., 2017). Generally, this cytotoxic effect is attributed to bufadienolides in the venom, such as marinobufagin, marinobufotoxin, bufatoxin, and telocinobufagin(Abdelfatah et al., 2019; Ferreira et al., 2013; Garcia et al., 2019; Sousa et al., 2017), compounds present in our extract as demonstrated by Kerkhoff et al. (2016) and Sousa et al. (2020). These compounds can induce cellular apoptosis by blocking the cell cycle at the G2/M phase, disrupting tubulin function in microtubule formation, activating caspases 3 and 9, promoting intracellular Ca^{2+} influx, and inhibiting Na/K-ATPase (Abdelfatah et al., 2019; Ferreira et al., 2013; Garcia et al., 2019; Sousa et al., 2017). Therefore, our findings are supported by other studies, strengthening the body of evidence on the cytotoxicity of compounds in these animal venoms and highlighting them as a promising avenue for the study of new anticancer treatment strategies.

Another property attributed to amphibian venom is its effect on the effector function of immune cells (Cao et al., 2009; Deng et al., 2020; Pelissari et al., 2021; Pelissari et al., 2023; Shih et al., 2018; Xie et al., 2016; Yu et al., 2015). Cao et al. (2009) have demonstrated that telocinobufagin, isolated from Chinese toads of the genus *Bufo*, enhances the proliferative function and pro-inflammatory cytokine production of murine splenic Th lymphocytes, while also increasing the activity of peritoneal macrophages and the cytotoxicity of NK cells. These same immunomodulatory effects were observed by Wang et al. (2011) for cinobufagin. However, cinobufagin can also exhibit anti-inflammatory effects by inhibiting the maturation of human dendritic cells and their production of TNF-α and IL-12p40 in response to lipopolysaccharide (Xie et al., 2016). In human neutrophils, an opposite effect was observed, with cinobufagin promoting the cells' bactericidal function through the exocytosis of defensins and cathelicidins(Xie et al., 2016). Bufadienolides have also been shown to inhibit the expansion and function of regulatory T cells (Deng

et al., 2020). Bufalin, another bufadienolide, can also modulate immune cell function by stimulating the phagocytosis of murine peritoneal macrophages while reducing NK cell function (Shih et al., 2018).

These immunomodulatory effects are corroborated by our group's previous studies, in which we observed that the methanolic extract of *Rhinella marina* and *Rhaebo guttatus* stimulates the production of TNF-α, IL-12p70, and H_2O_2 by murine peritoneal macrophages (Pelissari et al., 2021; Pelissari et al., 2023). The compounds marinobufagin, telocinobufagin, and bufalin were identified in the extracts used in this study (Kerkhoff et al., 2016; Pelissari et al., 2021). However, contrary to expectations, in the present study, we observed that the extracts could not alter the ability of human monocytes to produce NO and H_2O_2.

Bufadienolides are cardiotonic steroids that act as specific inhibitors of and ligands for Na/K-ATPase (Liu et al., 2018; Prassas; Diamandis, 2008). They bind to the enzyme's α subunit, thereby modulating its function (Liu et al., 2018; Prassas; Diamandis, 2008). Na/K-ATPase is an ion transport pump that also functions as a receptor, signal transducer, and multiprotein interaction complex (Liu et al., 2018). It has been shown that the α subunit can vary in its sensitivity to ouabain (a type of cardiotonic steroid), with the rodent α subunit being two to three times less sensitive than the α subunit of human cells (Liu et al., 2018). In this context, studies have demonstrated that cardiotonic steroids induce a multireceptor complex in macrophages—composed of Na/K-ATPase, CD36, and TLR4—which activates the transcription factor NF-κβ, resulting in the production of pro-inflammatory cytokines and the generation of reactive oxygen species (Chen et al., 2017; Zhang et al., 2022).

In light of this information, we can postulate that the lack of effect of the extracts on NO and H_2O_2 production, as observed in this study, may be a consequence of using a crude extract. A crude extract contains a mixture of several compounds, such as marinobufagin, telocinobufagin, and bufalin, at varying concentrations. Depending on which type of cardiotonic steroid binds to the Na/K-ATPase, the resulting conformational change in the α subunit can trigger different intracellular activation pathways and lead to distinct biological effects within the cell (Carvalho et al., 2019; Zhang et al., 2022). Furthermore, the binding of these compounds can also vary depending on the sensitivity of the Na/K-ATPase α subunit, which is known to differ among species (Liu et al., 2018).

Furthermore, human peripheral blood monocytes can be divided into different subpopulations (classical, intermediate, and non-classical), which have distinct functional characteristics and whose distribution varies with age (Ozanska et al., 2020). The production of reactive species is primarily attributed to intermediate monocytes, and these cells represent the most mature subtype only in the early years of life and after the age of 50 (Ozanska et al., 2020). This could be another important factor influencing the outcome of the extract's modulatory action, as the cells used in the present study were from 30–45-year-old individuals. In this case, the difference in response could be due to the lower prevalence of the intermediate subtype.

Finally, to the best of our knowledge, this is the first study to use Alsever's solution in a technique for isolating human monocytes. Despite the limited sample size, the procedure yielded a quantity of blood monocytes comparable to that obtained with the conventional Histopaque method. This represents a viable strategy that can optimize the use of both participant samples and financial resources.

Conclusion

Thus, this study demonstrated that methanolic extracts from the venom of the toads *Rhinella marina* and *Rhaebo guttatus* are cytotoxic to human monocytes but do not modulate the production of NO and H_2O_2 by these cells. Further studies are needed to understand and identify which compounds are responsible for these biological actions, as well as their mechanism of action.

Acknowledgment

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