A crude methanolic extract from the parotoid gland secretion of *Rhaebo guttatus* stimulates the production of reactive species and pro-inflammatory cytokines by peritoneal macrophages

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Received December 1, 2022; Accepted March 30, 2023

DOI: 10.3892/wasj.2023.192

Abstract. The parotoid gland secretion of toads of the family Bufonidae contain numerous pharmacologically active biocompounds. *Rhaebo guttatus* (*R. guttatus*) is a common species of this family in Brazil; however, this species has been poorly studied. Thus, the present study aimed to assess the immunomodulatory activity of a methanol extract from the parotoid gland secretion of *R. guttatus* in Swiss mice. Non-isogenic Swiss mice were administered (100 μl/mouse/day, orally) of the *R. guttatus* extract (8, 16, or 32 μg/ml), the vehicle (0.5% Tween-20, vehicle group), or distilled water (control group) for 7 or 30 days. After the treatment period, the mice were sacrificed, and the hearts, liver, lungs and kidneys were removed for histopathological analysis. Cells from peritoneal lavage and spleen were used for immunological analyses. The results revealed that the treatment did not affect water or feed consumption or body weight of the mice. The lungs of mice treated with 8 μg/ml for 30 days exhibited a reduction in relative weight compared to those of the control group. Histopathological analysis revealed that the extract induced edema, blood clots and leukocyte infiltrates, mainly after 30 days at doses of 8 and 32 μg/ml. The administration of the extract promoted the production of the pro-inflammatory cytokines, IL-12 and TNF-α, and reduced the production of IL-10 and IL-4. The spontaneous release of hydrogen peroxide by peritoneal macrophages was also induced in the mice treated with 16 μg/ml extract for 30 days. On the whole, the present study demonstrates that a crude methanolic extract from the parotoid gland secretion of *R. guttatus* has immunomodulatory activity and low toxicity, and may have potential as a stimulator of the immune system for immunosuppression conditions.

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Key words: biodiversity, Amazon, immunomodulation, poisonous toad, *Rhaebo guttatus*

Introduction

The importance and biotechnological potential of natural products can be demonstrated by the investment in research of approximately $480 million in 2020 by the National Institutes of Health (NIH), and by the approval of >680 new chemical compounds with pharmacological activities of natural origin or those derived from natural products by the US Food and Drug Administration between 1981 and 2010 (1,2).

Natural products are chemically and structurally diverse and possess various biological activities, often with low toxicity and few side-effects (3). They can be obtained from a wide variety of sources, such as plants, microorganisms, algae, fungi, lichens and animals (3,4). Toxins and poisons from animals, mainly amphibians, are also used as alternative medicines (2), and ~47 species of amphibians are recognized for their medicinal use (5). Their poisons or skin secretions, which are used by the animal in defense against predators and infection, contain bioactive compounds, such as steroids, alkaloids, biogenic amines, guanidine derivatives, proteins and peptides (2,5), and various biological properties have been reported, such as trypanocidal, leishmanicidal, antibacterial, antifungal, antiproliferative, insecticide, antiviral, antitumor and cardiotonic activities (2,5).

Toads of the family Bufonidae have a cosmopolitan distribution, and several Bufonidae species in the genera *Rhinella* and *Rhaebo* are found in Brazil (2). *Rhaebo guttatus* (*R. guttatus*), a poisonous toad species, is found in tropical forests and open areas, mainly in the Amazon Basin (2,5,6). The chemical composition of its parotoid gland secretion and its biological effects are poorly understood (7). Previous studies have identified alkaloids (N-methyl-5-hidroxytryptamine, bufotenine and dehydrobufotenin) and steroids (3β,16β-dihydroxybufa-8(14),

20,22-trienolide, marinobufagin and bufadienolides) in extracts obtained from the parotoid gland secretion of *R. guttatus* (7,8). Ferreira *et al* (8) demonstrated that extracts from the secretion of the parotoid gland of *R. guttatus* exhibit hemolytic and cytotoxic activity against tumor and non-tumor cells. Toxic activity against *Plasmodium falciparum* and phytopathogenic fungi has also been reported (9,10). Oliveira *et al* (11) observed that a methanolic extract from the parotoid gland secretion of *R. guttatus* exhibited both mutagenic and antimutagenic activities, indicating the need for further studies.

To enhance the current understanding of the biological effects of the compounds in the parotoid gland secretion of *R. guttatus*, the present study evaluated the activity of its crude methanolic extract (CME) on the immune system by analyzing its effects on cytokine production, lymphoproliferative activity and the production of reactive species by macrophages *in vitro*. In this manner, the present study aimed to contribute to bioprospecting studies and to the development of the bioeconomy.

Materials and methods

Poison collection and extract preparation. Adult (males and females, from 5 to 10 animals) *R. guttatus* were captured and identified by D.D.J.R. (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 secretion from the parotoid gland was obtained through the manual compression of the glands. The secretion was dried, crushed and extracted by maceration with 99% methanol in an ultrasonic bath (Ultronique, Indaiatuba, Brazil) for 2 h to obtain a CME of parotoid gland secretion. The extract was filtered through filter paper (Unifil, Curitiba, Brazil), and then macerated twice more as described above. Finally, the extracts were pooled, and the solvent was rotary evaporated (IKA-Werke GmbH & Co. KG) 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 were as previously described by Kerkhoff *et al* (5) and Sousa *et al* (2). The description of the chemical profile of CME was presented by Sousa *et al* (2).

Animals and experimental design. Male Swiss mice (mean weight, 35 g, 45 days old) obtained from the Central Bioterium of the Federal University of Mato Grosso, Cuiabá Campus were used in the experiment. The mice were housed in polyethylene boxes with a stainless-steel grid during the acclimatization (15 days) and experimental periods. They were divided into five groups of 6 mice in each and maintained under a 12-h light/dark cycle in a temperature-controlled room (24±1˚C, 55±2% relative humidity), with *ad libitum* access to food (Nuvilab) and filtered water. During the experimental period, the mice were administered water (control), 0.5% Tween-20 (vehicle), or various doses of the CME (8, 16 and 32 μg/ml) in a 100 μl volume per mouse per day via oral gavage for 7 or 30 days. The doses were selected based on the study by Oliveira *et al* (11). Aliquots were prepared in microtubes, diluted in 0.5% Tween-20 and stored at 4˚C. During the treatment period, the mice were observed daily for water and feed consumption. The body weight of the mice was measured at the beginning and end of the treatment period to assess body weight gain. At the end of the treatment period (24 h later), the mice were euthanized by cervical dislocation, and the lungs, heart, kidneys and liver were excised to evaluate their relative and absolute weight, and for use in histopathological analysis. Immunological analyses were performed using cells obtained from the spleen and peritoneal washes. The present study was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under no. A313DC9, and approved by the UFMT Ethics Committee on the Use of Animals (CEUA), under no. 23108.918243/2017-50.

Histopathological analysis. Fragments of the heart, liver, lungs and kidneys were harvested, and immediately fixed with 10% formaldehyde in sodium phosphate buffer (Synth, Diadema, Brazil), pH 7.4, at 4˚C for 24 h. Following dehydration with alcohol and clearing with xylol, the tissue fragments were embedded in paraffin, sectioned (5-μm-thick) using a HYRAX M60 microtome (Zeiss GmbH), deparaffinized and stained at room temperature (25˚C) with hematoxylin (1 min) and eosin (2 min) (MilliporeSigma) . The sections were examined under a AxioScope.A1 microscope (Zeiss GmbH). For the histopathological analysis, a score was generated (0-4) based on tissue edema, blood clots, leukocyte infiltrates and renal tubule damage, where 0 indicates no alterations and 4 indicates a high level of alteration (12).

Analysis of total splenic cell lymphoproliferation. The lymphoproliferation of total splenic cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) colorimetric assay (Cell Groth Determination kit, MTT based, MilliporeSigma, cat. no. CGD1) according to the manufacturer's recommendations. Briefly, the spleen was removed and transferred to a Petri dish containing RPMI-1640 medium (Cultilab) and macerated with a sieve and pistil. The cell suspension was then transferred to a falcon tube and centrifuged at 402 x g for 10 min at room temperature (25˚C). The cell pellet was resuspended in RPMI-1640 medium (500 μl) containing 20% fetal bovine serum (FBS; Cultilab), and the cell concentration was adjusted to $2x10^4$ cells/ml as determined using a Newbauer chamber (Global New Optics) and the Trypan blue (Qhemis) exclusion method. The cells were transferred to a 96-well microplate, and the mitogen, concanavalin A (Con A; MilliporeSigma), or RPMI 20% FBS (basal) was added in triplicate. The plates were incubated for 36 h at 37˚C and 5% CO_2. MTT reagent (100 μl) was then added to each well, and the plate was incubated at 37˚C and 5% CO_2 for 4 h. Solubilizing solution (0.1 N HCl in anhydrous isopropanol) was added, and the absorbance was measured on an ELISA plate reader (BioClin Medical Electronics Co.) at 630 nm.

Analysis of cytokine levels. The levels of selected cytokines in the supernatant of total splenic cell cultures were evaluated using commercial ELISA kits (eBioscience; IL-12p70 cat. no. 88 7121 88, IFN-g cat. no. 88 7314 88, IL-4 cat. no. 88 7044 88, IL-10 cat. no. 88 7104 88, TNF-α cat. no. 88 7324 88, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For the measurement of interleukin (IL)-4

and IL-10 levels, the splenic cell culture was stimulated with the mitogen Con A (3.5 μg/ml) for 24 h. For the assessment of IL12p70 and tumor necrosis factor (TNF)-α levels, the culture was stimulated with a formalinized aqueous suspension of *Staphylococcus aureus* Cowan strain 1 (SAC, 1:5,000; MilliporeSigma) for 48 h. The absorbance of the samples was measured on an ELISA reader at 450 nm. The concentrations were estimated based on a standard curve of cytokine standards in the kit.

Harvesting of peritoneal macrophages. Following sacrifice, peritoneal macrophages were obtained in a Class II Safety Cabinet by the addition of 10 ml sterile, cold phosphate-buffered saline (PBS) to the peritoneal cavity. The abdomen was massaged for 30 sec, and the collected peritoneal fluid was transferred to 50 ml Falcon tubes. This procedure was performed twice, to obtain a total of 20 ml fluid, which was placed in an ice bath and then centrifuged (Novatecnica) at 402 x g and 4˚C for 10 min. The supernatant was discarded, and the pellet was resuspended in 1 ml RPMI-1640 medium containing 10% FBS. The cells in the peritoneal wash were counted in a Neubauer Chamber and adjusted to $2x10^6$ cells/ml. The macrophages were plated in triplicate into 96-well microplates (100 μl/well) and incubated for 2 h at 37˚C and 5% CO_2. Following incubation, the supernatant was removed, and the wells were washed with 100 μl RPMI. Subsequently, 200 μl RPMI containing 10% FBS were added to each well, and the cells were incubated at 37˚C and 5% CO_2 for 36 h.

Assessment of spontaneous hydrogen peroxide (H_2O_2) release by peritoneal macrophages. The spontaneous production of H_2O_2 by peritoneal macrophages was assessed using the method developed by Pick and Mizel (13). The quantification of H_2O_2 is based on the horseradish peroxidase-dependent oxidation of phenol red by H_2O_2 into a compound that is assayed for its increased absorbance (13). Following incubation (37˚C and 5% CO_2 for 36 h), the supernatant of the macrophage culture was collected and stored for nitric oxide (NO^-) evaluation. To measure H_2O_2, 100 μl phenol red solution containing 140 mM NaCl, 10 mM K_2HPO_4, 5.5 mM dextrose and 5.5 mM peroxidase were added to the cell monolayer in the 96-well microplates. The plates were incubated at room temperature under a light for 60 min. The reaction was terminated by the addition of 10 μl of 1 M NaOH, and the absorbance of the solution at 630 nm was determined using an ELISA microplate reader. The blank was phenol red and 1 M NaOH. The amount of H_2O_2 produced by the macrophages was determined based on a standard curve of known H_2O_2 concentrations. The mean value of triplicate samples was calculated.

Assessment of NO^- production by peritoneal macrophages. The NO^- levels in the macrophage culture supernatant were assessed using the colorimetric method based on the Griess reaction, as previously described (14). Briefly, 100 μl Griess reagent [1% N-(1-Naphthyl) ethylenediamine dihydrochloride] in distilled water and 1% sulfanilamide diluted in 5% H_3PO_4 were added to the supernatants. The reagents were mixed in equal volumes at the time of the reaction. The absorbance of the samples at 492 nm was read on an ELISA reader. The blank was Griess reagent. The NO^- levels were calculated based on a standard curve of known concentrations of $NaNO_2$. The mean value of triplicate samples was calculated.

Statistical analysis. The present study was carried out in two independent experimental stages (A and B). The data were analyzed either separately or together when the data obtained in experiments A and B were homogeneous. The normal distribution of the data was assessed using the Kolmogorov and Smirnov test. One-way ANOVA followed by the Tukey-Kramer multiple comparisons test was used to assess the significance of differences between experimental groups. NO^- production in experiment B at 30 days was analyzed using an unpaired t-test. All results are expressed as the mean ± standard deviation. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of CME from the parotoid gland secretion of R. guttatus on body weight, food intake and organ weights. Body weight, food intake and organ weight were analyzed to assess the toxicity of the extract. These evaluations are important as toxic products usually induce changes in animal behavior, including a lack of appetite and a consequent reduction in body weight (15). As also previously reported by Oliveira *et al* (11), there were no significant differences between the groups as regards water and feed consumption (data not shown) or body weight gain (Table I). Although the relative weight of the lungs was reduced in the mice treated with 8 μg/ml CME for 30 days (8 μg/ml, 0.49±0.05; control, 0.62±0.09), the absolute and relative weights of the other organs did not differ significantly when compared to the control groups (control and vehicle; Table I).

Histopathological changes induced by CME from the parotoid gland secretion of R. guttatus. The results of the histopathological analysis are presented in Table II and Fig. 1 (that illustrates the histopathological analysis of the lungs and kidneys as a representative example of tissue damage considered in the study). Histopathological analysis of the heart, liver, kidneys and lungs revealed that treatment with CME from the parotoid gland secretion of *R. guttatus* induced changes in these tissues, including edema, intravascular clots, damage to the architecture of the renal tubules and leukocyte infiltrates. After 7 days of treatment, changes in all evaluated organs were observed, with predominant edema and mild leukocyte infiltrates (score 1). At this time point, the kidneys and lungs were the most affected, as they had a greater number of alterations; the most intense alterations were in the lungs, with scores of 2 and 3. After 30 days of treatment, the tissue changes increased both in number and severity, with the lungs and kidneys being the most affected organs. Tissue damage was more noticeable after 30 days of treatment, particularly at doses of 8 and 32 μg/ml.

Immunomodulatory activity of CME from the parotoid gland secretion of R. guttatus. The immunomodulatory activity of the CME from the parotoid gland secretion of *R. guttatus* was evaluated based on the lymphoproliferative response and

Table I. Body weight gain and organ weights (absolute and relative) of Swiss mice treated with a crude methanolic extract of *R. guttatus* toad poison via daily gavage for 7 or 30 days.

7 days

		Absolute weight of organs				Relative weight of organs			
Group (n=6/group)	Weight gain	Liver	Kidney	Lung	Heart	Liver	Kidney	Lung	Heart
Control	1.32±5.14	2.28±0.47	0.63±0.10	0.27±0.06	0.22±0.04	5.50±0.60	1.47±0.32	0.63±0.17	0.50±0.07
Vehicle	-0.74±8.97	2.18±0.45	0.53±0.09	0.28±0.10	0.20±0.04	5.13±0.84	1.24±0.16	0.68±0.27	0.46±0.04
8 μg/ml	2.53±3.99	2.16±0.29	0.48±0.07	0.23±0.03	0.18±0.03	5.26±0.39	1.18±0.16	0.55±0.05	0.45±0.04
16 μg/ml	2.19±8.50	2.19±0.36	0.54±0.14	0.23±0.06	0.19±0.04	5.44±0.75	1.36±0.33	0.58±0.14	0.48±0.09
32 μg/ml	-1.48±8.86	2.30±0.27	0.60±0.04	0.29±0.02	0.25±0.03	4.53±0.28	1.18±0.05	0.58±0.07	0.50±0.03

30 days

		Absolute weight of organs				Relative weight of organs			
Group (n=6/group)	Weight gain	Liver	Kidney	Lung	Heart	Liver	Kidney	Lung	Heart
Control	1.67±6.65	2.21±0.44	0.52±0.06	0.27±0.04	0.20±0.03	4.99±0.57	1.18±0.15	0.62±0.09	0.45±0.07
Vehicle	2.57±5.91	2.34±0.22	0.53±0.15	0.27±0.04	0.24±0.09	5.10±0.38	1.14±0.26	0.59±0.05	0.53±0.21
8 μg/ml	1.76±4.74	2.68±0.34	0.66±0.09	0.25±0.04	0.23±0.04	5.33±0.67	1.31±0.18	0.49±0.05[a]	0.47±0.05
16 μg/ml	4.83±1.90	2.27±0.20	0.63±0.08	0.27±0.03	0.24±0.03	4.57±0.27	1.26±0.14	0.53±0.06	0.48±0.05
32 μg/ml	1.33±2.77	2.50±0.16	0.59±0.06	0.29±0.02	0.22±0.01	5.05±0.25	1.19±0.16	0.58±0.04	0.45±0.03

The data, shown in grams, are the mean ± standard deviation. The relative weights of the organs refers to the absolute weight of the organ relative to the body weight of the mouse. One-way ANOVA was performed, followed by the Tukey-Kramer multiple comparisons test. [a]P<0.05 vs. the control group.

Table II. Histopathological analysis of organs from Swiss mice treated with a CME of *R. guttatus* toad poison via daily gavage for 7 or 30 days.

7 days

	Organ analyzed												
	Heart			Liver			Lungs			Kidneys			
Group	Edema	Blood clots	Leukocyte infiltrates	Edema	Blood clots	Leukocyte infiltrates	Edema	Blood clots	Leukocyte infiltrates	Edema	Blood clots	Tubular damage	Total score
Control	0	0	0	0	0	0	0	0	0	0	0	0	0
Vehicle	0	0	0	0	0	0	0	0	0	0	0	0	0
8 μg/ml	1 (5/6)	0	0	1 (4/6)	0	0	1 (4/6) 2 (2/6)	0	1 (4/6) 2 (2/6)	1 (6/6)	1 (3/6)	0	5.8
16 μg/ml	1 (5/6)	0	0	1 (2/6)	0	0	1 (6/6)	0	1 (2/6) 2 (4/6)	1 (6/6)	1 (3/6)	0	5.2
32 μg/ml	1 (6/6)	0	1 (2/6)	1 (5/6)	0	1 (2/6)	1 (4/6) 2 (2/6)	1 (2/6)	1 (1/6) 2 (3/6) 3 (2/6)	1 (6/6)	1 (6/6)	1 (1/6)	8.5

30 days

	Organ analyzed												
	Heart			Liver			Lungs			Kidneys			
Group	Edema	Blood clots	Leukocyte infiltrates	Edema	Blood clots	Leukocyte infiltrates	Edema	Blood clots	Leukocyte infiltrates	Edema	Blood clots	Tubular damage	Total score
Control	0	0	0	0	0	0	0	0	0	0	0	0	0
Vehicle	0	0	0	0	0	0	0	0	0	0	0	0	0
8 μg/ml	1 (6/6)	1 (3/6)	1 (2/6)	1 (5/6)	0	1 (4/6)	1 (5/6) 2 (1/6)	1 (4/6)	1 (1/6) 2 (4/6) 3 (1/6)	1 (6/6)	1 (6/6)	1 (3/6)	9.6
16 μg/ml	1 (6/6)	0	0	1 (4/6)	0	0	1 (6/6)	1 (3/6)	1 (1/6) 2 (5/6)	1 (6/6)	1 (6/6)	0	7.1
32 μg/ml	1 (6/6)	1 (4/6)	1 (3/6) 2 (3/6)	1 (5/6) 2 (1/6)	0	1 (4/6) 2 (2/6)	1 (3/6) 2 (3/6)	1 (6/6)	2 (3/6) 3 (3/6)	1 (6/6)	1 (4/6) 2 (2/6)	1 (5/6) 2 (1/6)	14.2

Histopathological analysis was performed, and the results are reported as a score of 0-4, with 0 indicating the absence of alterations and 4 indicating the presence of high rates of alterations. The numbers of mice that presented the indicated score in relation to the total number of mice in the group are presented in parentheses. The total score is the sum of the scores of each mouse in the group in relation to the total number of mice.

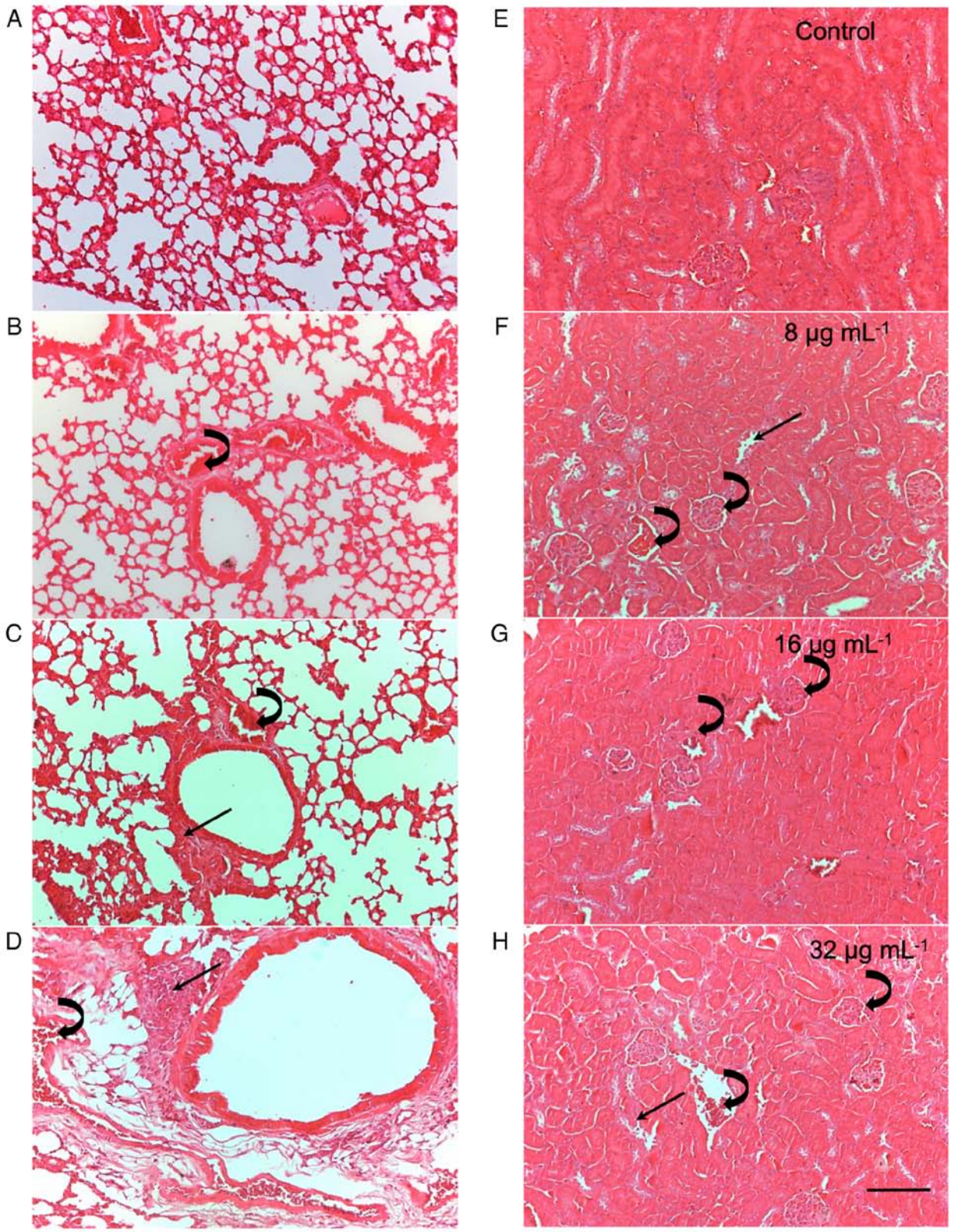

Figure 1. Histopathological analysis (hematoxylin and eosin staining) of (A-D) lungs and (E-H) kidneys from Swiss mice following treatment with various doses of a methanol extract of parotoid gland secretion of *Rhaebo guttatus* via daily gavage for 30 days. Mice treated with water (control group, A and E) showed no damage to the tissue architecture. Mice treated with (B and F) 8 μg/ml, (C and G) 16 μg/ml and (D and H) 32 μg/ml of *R. guttatus* extract presented tissue edema (curved arrows) and leukocyte infiltrates (arrows). Scale bar, 100 μm.

cytokine production of splenocytes from mice treated with the CME from the parotoid gland secretion of *R. guttatus* for 7 and 30 days. In addition, the capacity of peritoneal macrophages to generate $NO^{\cdot}$ and H_2O_2 *in vitro* was also evaluated.

Treatment with various doses of the extract did not markedly alter the lymphoproliferative capacity of the total splenocytes compared to the control groups at the two evaluated time points (7 and 30 days; Fig. 2).

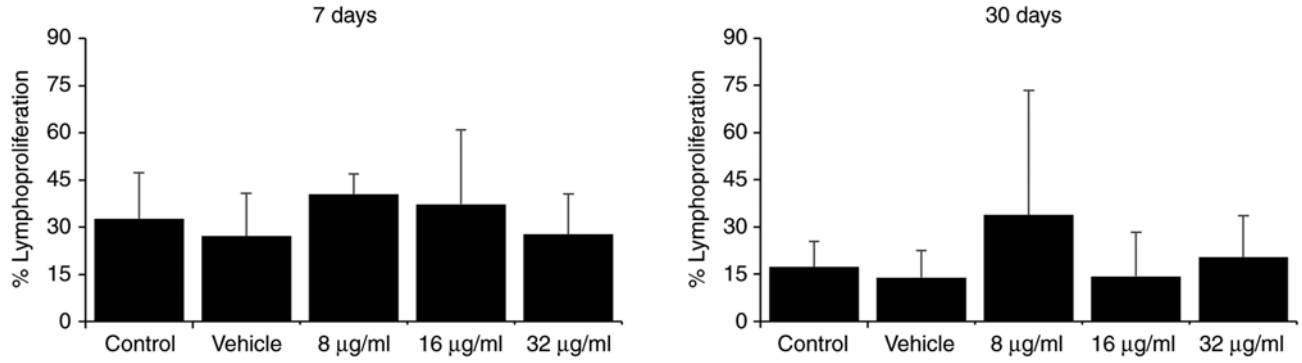

Figure 2. Percentage of total splenic cell lymphoproliferation from Swiss mice treated with different doses of a methanol extract of parotoid gland secretion of *Rhaebo guttatus* toad via daily gavage for 7 or 30 days. Values are the mean ± standard deviation.

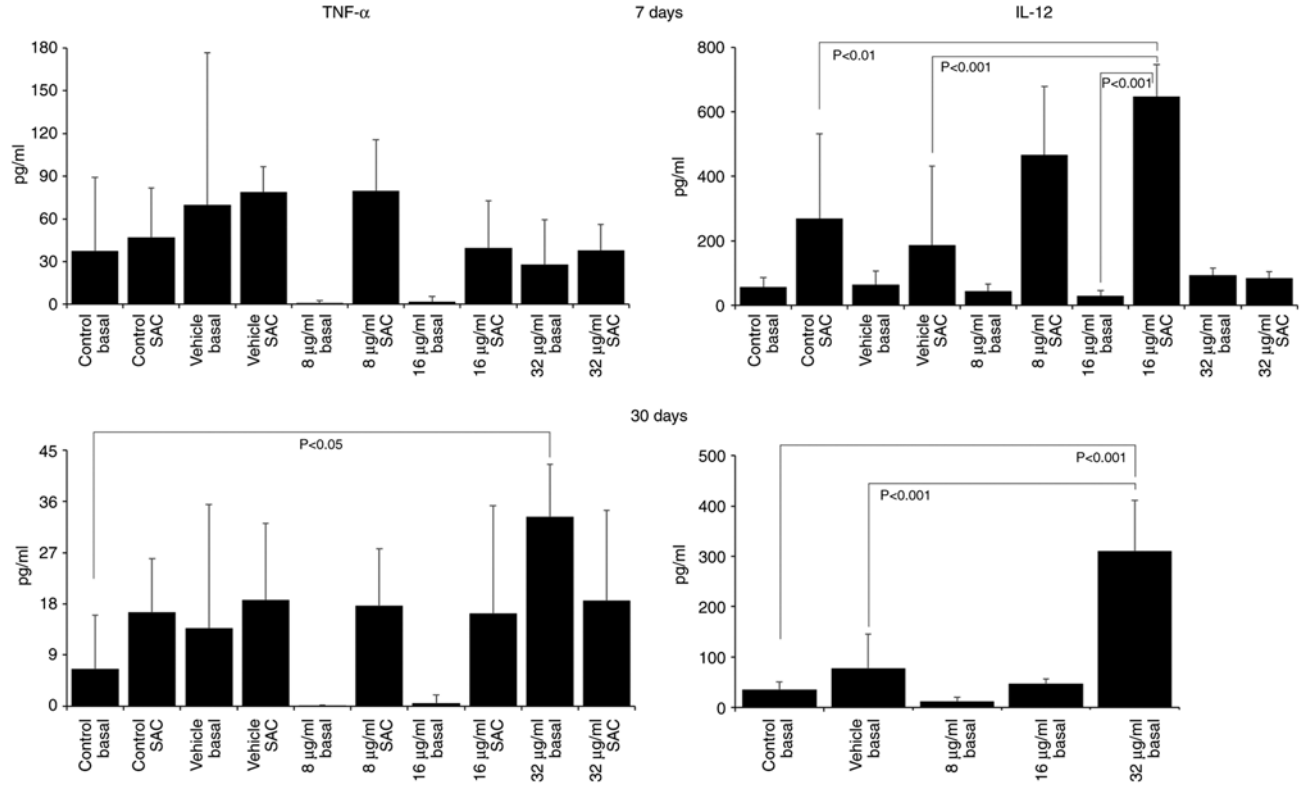

Figure 3. Production of TNF-α and IL-12 by total splenic cells from Swiss mice treated with various doses of a methanol extract of parotoid gland secretion of *Rhaebo guttatus* toad via daily gavage for 7 or 30 days. Values are the mean ± standard deviation. Cytokines were quantified in the supernatant of cells stimulated with SAC (a formalinized aqueous suspension of *Staphylococcus aureus*, 1:5,000) or not (basal) for 48 h. The results were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

The assessment of cytokine production revealed that treatment with the extract induced a pro-inflammatory profile, with increases in the IL-12 and TNF-α levels, and decreases in the IL-4 and IL-10 levels. After 7 days of treatment with 16 μg/ml CME, IL-12 production increased (SAC control, 268.10±263.47; vehicle SAC, 186.27±245.10; 16 μg/ml SAC, 647.78±98.73; Fig. 3) and IL-10 production decreased (Con A control, 2532.50±1863.00; 16 μg/ml Con A, 935.00±816.93; Fig. 4). IL-10 production was also decreased in the group treated with 8 μg/ml CME (8 μg/ml Con A, 720.00±79.06; Fig. 4). Treatment of the mice with 32 μg/ml CME for 30 days increased the production of IL-12 (basal control, 34.65±15.41; vehicle, 77.27±67.34; 32 μg/ml basal, 310.69±100.72; Fig. 3), TNF-α (basal control, 6.52±9.50; 32 μg/ml basal, 33.32±9.21; Fig. 3) and IL-4 (Con A control, 19.01±6.82; vehicle Con A, 29.83±9.69; 32 μg/ml Con A, 49.37±14.90; Fig. 5). However, the administration of 8 and 16 μg/ml CME reduced IL-4 production at the same time point (Con A control, 127.22±66.02; 8 μg/ml Con A, 48.76±40.93; 16 μg/ml Con A, 36.82±24.41; Fig. 5).

The assessment of H_2O_2 and $NO^{\cdot}$ production by macrophages is a method of evaluating their effector function. Macrophages are essential components of the innate immune response, as they respond rapidly to pathogenic stimulation and signs of tissue damage (16,17). The production of reactive oxygen and nitrogen species is a part of their antimicrobial mechanism (18). Herein, the effect of the CME from the

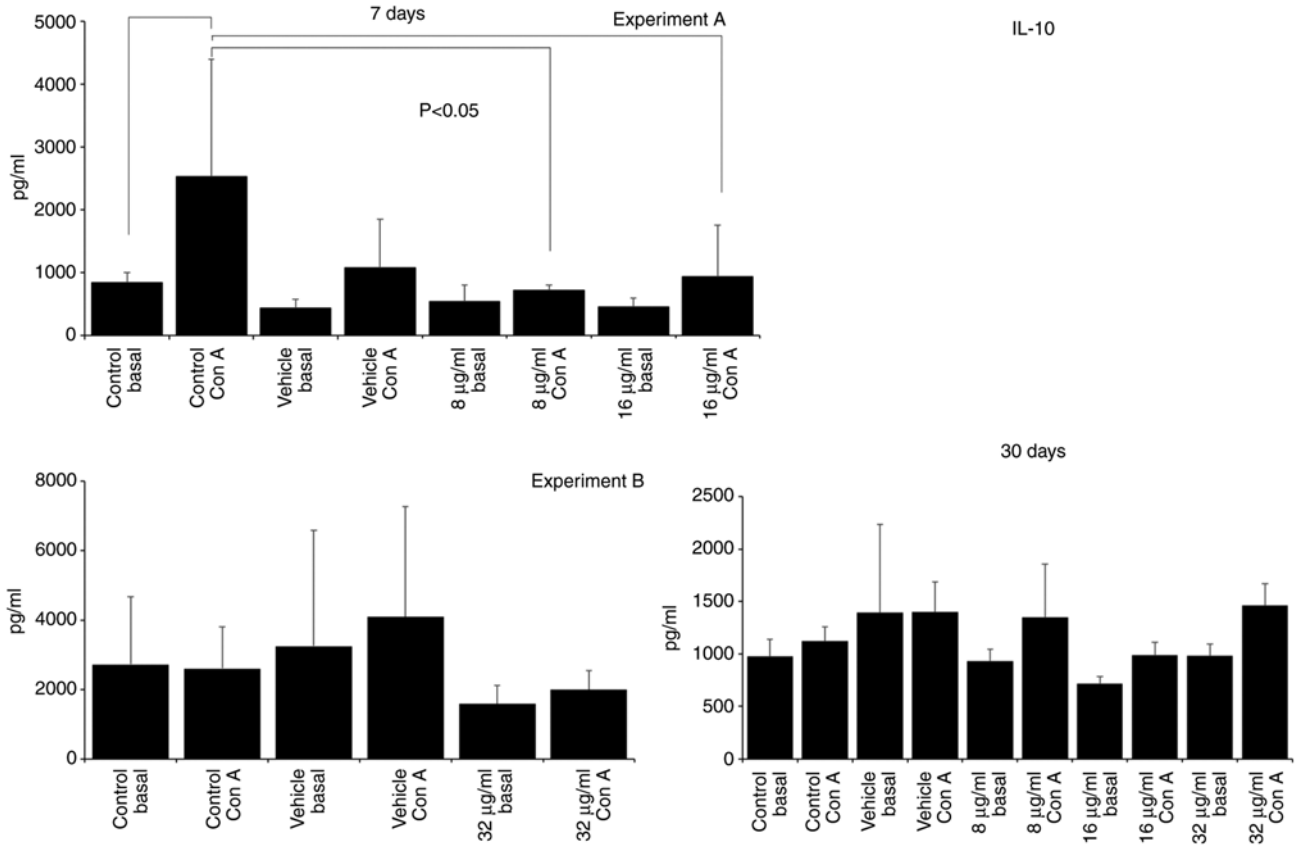

Figure 4. Production of IL-10 by total splenic cells from Swiss mice treated with various doses of a methanol extract of parotoid gland secretion of *Rhaebo guttatus* toad via daily gavage for 7 or 30 days. Values are the mean ± standard deviation. Cytokine levels were quantified in the supernatant of a cell culture stimulated with the mitogen concanavalin A (Con A, 3.5 μg/ml) or not (basal) for 24 h. Two independent 7-day treatment experiments (Experiments A and B) were performed, and the results were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

parotoid gland secretion of *R. guttatus* on the production of H_2O_2 and $NO^{\cdot}$ by macrophages was more evident with a longer treatment duration; there were no marked differences between the groups at 7 days (Figs. 6 and 7), whereas at 30 days, an increased H_2O_2 production was observed in the groups treated with 16 μg/ml extract (control, 0.18±0.07; 16 μg/ml, 0.47±0.14; Fig. 6) and reduced production of H_2O_2 (vehicle, 0.41±0.24, 8 μg/ml, 0.11±0.08; Fig. 6) and $NO^{\cdot}$ (vehicle, 81.82±5.28; 16 μg/ml, 71.06±7.38; Fig. 7) in the group treated with 8 and 16 μg/ml CME, respectively.

Discussion

The present study demonstrated that a CME from the parotoid gland secretion of *R. guttatus* stimulated peritoneal macrophages to produce H_2O_2 and the pro-inflammatory cytokines, IL-12 and TNF-α. The extract also reduced the production of the anti-inflammatory cytokines, IL-10 and IL-4. To the best of our knowledge, this is the first study to explore the immunomodulatory potential of an extract obtained from the poison of this toad species.

The pharmacological effects of the chemical compounds isolated from the glandular secretions of frogs have been studied for a long time (2). For thousands of years, Chansu, an aqueous extract obtained from the post-auricular and skin glands of *Bufo chansa gargarizans* Cantor, has been used as a Traditional Chinese Medicine for the treatment of various conditions, such as swelling, pain, and heart failure and, more recently, for cancers of the liver, lung, colon, pancreas and stomach (19-21). In Brazil, the most extensively studied amphibian-derived skin secretions are from *Rhinella marina* (*R. marina*; Cururu toad), which contains compounds, such as marinobufagin, telecinobufagin, bufalin, marinobufotoxin, cholesterol, dehydrobufotenine, and suberoyl arginine (2,22,23). A variety of biological effects have been attributed to the compounds in *R. marina* skin secretions extracts, including cytotoxic, immunomodulatory, antifungal, antimutagenic, and anti-plasmodial activities (9-11,22-25).

In addition to *R. marina*, another common frog species in Brazil is *R. guttatus*, particularly in the Amazon Basin; however the compounds in their skin secretions are poorly characterized (2,5). Some compounds were identified, such as N-methyl-5-hidroxytryptamine, bufotenine, dehydrobufotenine, 3β,16 β-dihydroxybufa-8 (14),20,22-trienolide, marinobufagin, and bufatrienolides (2,7,8), and biological effects have been reported in the literature, including cytotoxic, anti-plasmodial, fungicidal, hemolytic and antimutagenic activities (8-11).

In the present study, it was observed that the administration of a CME of *R. guttatus* to mice did not alter food and water consumption or body weight. This was also observed in the study by Oliveira *et al* (11), as well as in a study using extracts from other species, such as *R. marina* (23). Although the absolute and relative organ weights were similar among the test

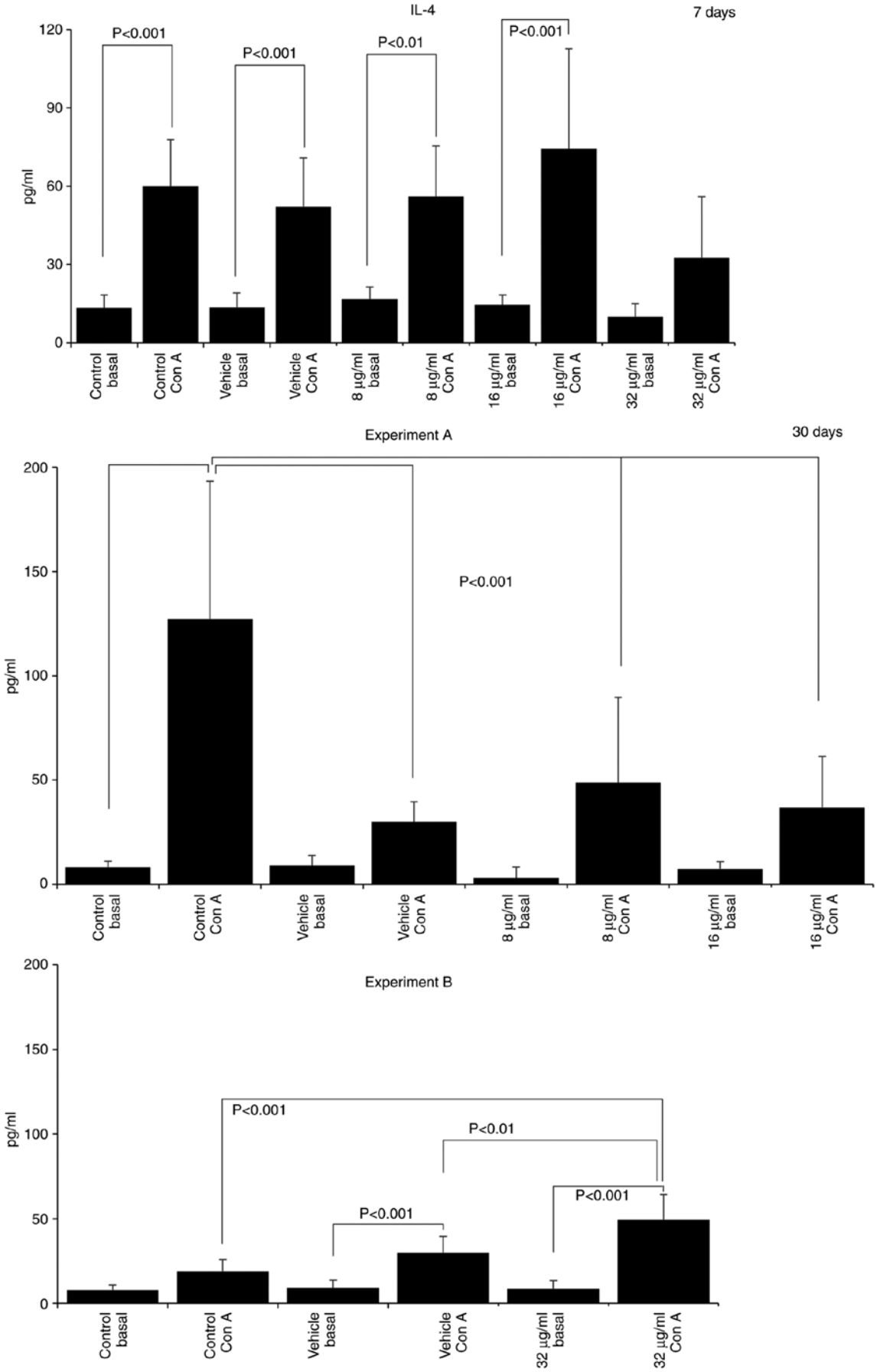

Figure 5. IL-4 production by total splenic cells from Swiss mice treated daily with various doses of a methanol extract of parotoid gland secretion of *Rhaebo guttatus* toad via daily gavage for 7 or 30 days. Values are the mean ± standard deviation. Cytokine levels were quantified in the supernatant of cells stimulated with the mitogen concanavalin A (Con A, 3.5 μg/ml) or not (basal) for 24 h. Two independent 30-day treatment experiments (Experiments A and B) were performed. The results were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

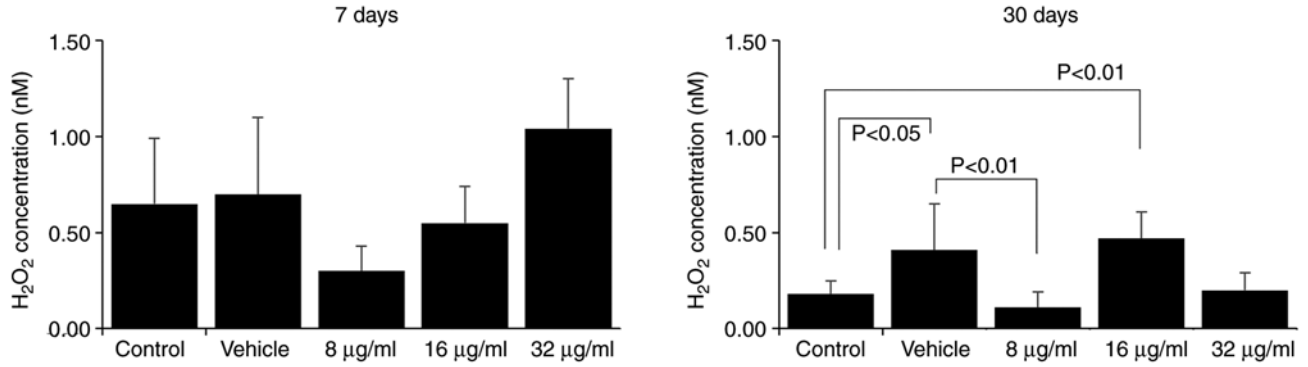

Figure 6. H_2O_2 produced by peritoneal macrophages from Swiss mice treated with various doses of a methanol extract of parotoid gland secretion of *Rhaebo guttatus* toad via daily gavage for 7 or 30 days. Values are the mean ± standard deviation. H_2O_2 was quantified using the method developed by Pick and Mizel (13), and the results were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple comparisons test. H_2O_2, hydrogen peroxide.

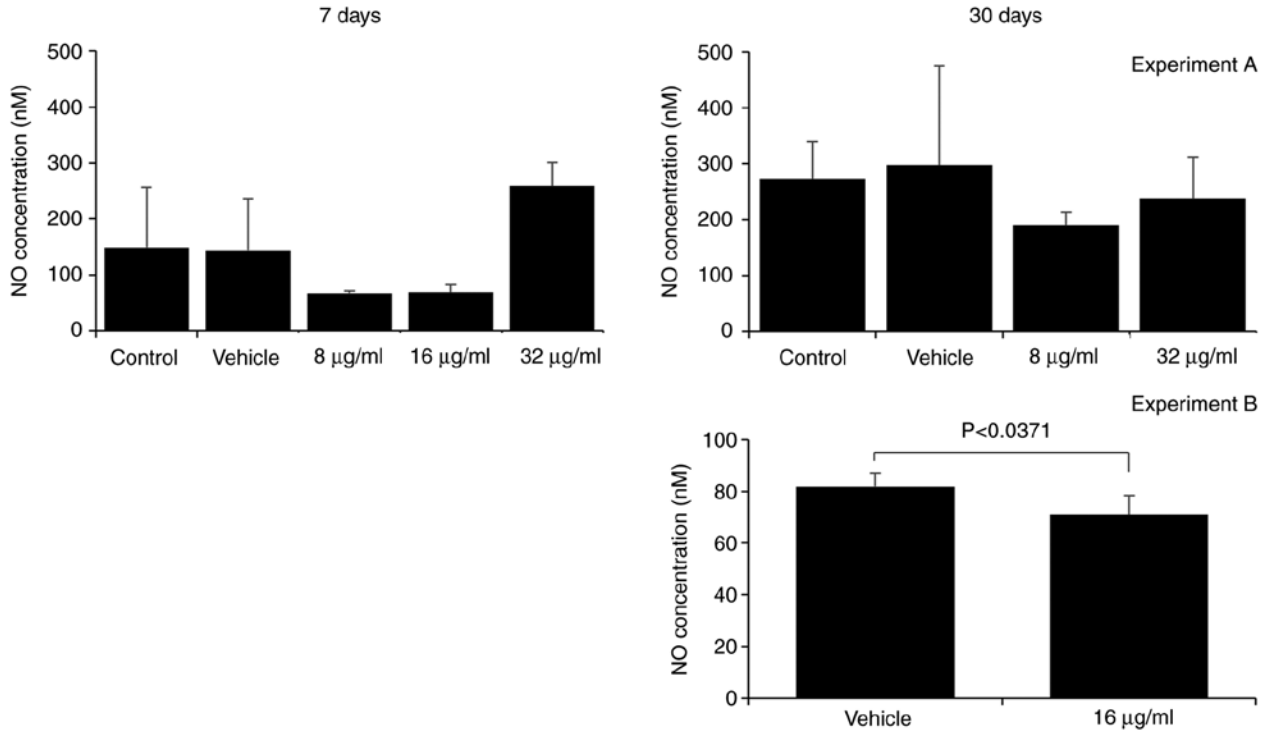

Figure 7. (NO^-) produced by peritoneal macrophages from Swiss mice treated with various doses of methanol extract of parotoid gland secretion of *Rhaebo guttatus* toad via daily gavage for 7 or 30 days. Values are the mean ± standard deviation. The NO^- concentration in the culture supernatant was quantified using the colorimetric method based on the Griess reaction. Two independent 30-day treatment experiments (Experiments A and B) were performed, and the results of experiment B with 30 day-treated macrophages were analyzed using an unpaired t-test. NO^-, nitric oxide.

groups, the relative weight of the lungs was reduced in mice treated with 8 μg/ml extract for 30 days. Histopathological analysis indicated that the lungs and kidneys were the organs most affected by treatment with the CME, with edema, leukocyte infiltrates and blood clots. These effects were more prominent at 30 days and in the groups treated with 8 and 32 μg/ml CME. These results indicated that this CME of *R. guttatus* had low toxicity in the mice, since the tissue alteration scores were low (1 and 2) and were not accompanied by macroscopic or behavioral alterations. However, treatments >30 days may aggravate tissue damage and may impair lung and kidney function.

The authors have previously found that the administration of a *R. guttatus* CME increased the levels of thiobarbituric acid reactive substances in the livers of mice, which was accompanied by the increased activity of glutathione-S-transferase (GST) and decreased levels of reduced glutathione (GSH) (unpublished data). These data indicate that substances present in the parotoid gland secretion activate lipid peroxidation and induce an imbalance in the protective antioxidants GSH and GST, corroborating the histopathological changes observed in the present study. The livers of animals treated with various doses of *R. guttatus* CME also exhibited histological alterations indicative of local inflammation, such as the presence of edema and leukocyte infiltrates. The metabolites of hepatotoxic drugs can trigger the production of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, resulting in liver injury (26,28). Thus, the histopathological findings are in

accordance with the effects of CME on the production of pro-inflammatory cytokines and the spontaneous release of H_2O_2. Treatment of mice with 16 μg/ml of the extract increased IL-12 production by splenocytes stimulated with SAC (at 7 days), stimulated the spontaneous release of H_2O_2 by peritoneal macrophages (at 30 days), and inhibited the production of IL-10 and IL-4 by splenocytes stimulated with Con A (at 7 and 30 days, respectively), as well as $NO^{\cdot}$ release from macrophages. At 30 days, the basal production of TNF-α and IL-12 increased in the mice treated with 32 μg/ml of the extract. Treatment with 8 μg/ml of the extract inhibited the production of H_2O_2 (at 30 days), IL-10 (at 7 days) and IL-4 (at 30 days). Taken together, these results demonstrate that the CME of *R. guttatus* induced a pro-inflammatory state in treated mice, by promoting the production of IL-12 and TNF-α and inhibiting the production of IL-4 and IL-10.

TNF-α is a pro-inflammatory cytokine produced mainly by macrophages that regulates several cellular functions, such as leukocyte activation, cytokine and chemokine production, and the release of reactive oxygen and nitrogen species (29). It is one of the main inflammation-inducing cytokines and promotes vascular changes, such as the activation of endothelial cells, increased vascular permeability and vasodilation, and leukocyte influx, in addition to being a potent neutrophil activator (18,29,30). IL-12 is also produced by macrophages and is considered to function as a bridge between the innate and specific immune responses by promoting the cell-mediated response, including differentiation of T-helper (Th) 1 and cytotoxic T-lymphocytes, and the activation of microbicidal functions and the oxidative burst of macrophages via interferon-γ (IFN-γ)-induced feedback (18,30,31). The oxidative burst results from the formation of active NADPH oxidase within the macrophage phagolysosome, resulting in increased oxygen consumption (18). This generates superoxide anion radicals in the lumen of the phagolysosome, which are converted to H_2O_2 by superoxide dismutase (18). In the present study, the mitogenic stimulus (SAC) was commonly used to stimulate macrophages *in vitro* and measure the subsequent IL-12 and TNF-α production as it is rich in immunostimulant components, such as peptidoglycans and lipoteichoic acid (32). Thus, the results revealed that the compounds present in the CME of *R. guttatus* are able to modulate the function of macrophages to a pro-inflammatory profile, characteristic of M1-type cells.

Treatment with the extract also led to decreases in IL-10, IL-4 and $NO^{\cdot}$ production. IL-10 is an anti-inflammatory cytokine produced by diverse cells, including macrophages and T-lymphocytes (30). IL-10 inhibits the production of cytokines, such as IL-12, TNF-α and IFN-γ, by activated phagocytes and T-lymphocytes, and reduces lymphocyte activation by inhibiting antigen presentation by dendritic cells and macrophages (30,33). IL-4 is produced by T-lymphocytes, mast cells, basophils and eosinophils, and, in contrast to the effects of IL-12, IL-4 favors the differentiation of Th 2 lymphocytes, characteristic of the humoral response of allergic inflammation and parasitic infection (30,34). The Th 2 response-promoting effect of IL-4 results in inhibition of the Th 1 response and, consequently, in the decreased production of IL-12, TNF-α and IFN-γ (33). In addition, IL-4 suppresses macrophage activity, inhibiting the microbicidal activity of the cell, including the production of $NO^{\cdot}$ (18,33). Thus, these effects are in accordance with the results obtained in the present study and reinforce the pro-inflammatory effect of the compounds present in the extract.

However, the administration of 32 μg/ml extract promoted IL-4 production by splenocytes stimulated with Con A after 30 days of treatment. Despite the inhibitory effect of IL-4 on the Th 1 cellular response and its anti-inflammatory character, this cytokine is a key cofactor in the production of IL-12 and IFN-γ, which are characteristic of the Th 1 profile (35,36). Some studies have shown that a lack of IL-4 results in the development of a deficient Th 1 response, with impairs responses to tumors and infections caused by *Candida albicans* and *Leishmania major* (35,36). Thus, the increased production of IL-4, observed in the present study, may also have contributed to the development of the pro-inflammatory profile.

The results of the present study are reinforced by similar findings in previous studies on bufadienolides obtained from other amphibian species, including the promotion of the Th 1 response (37), the differentiation of M1 macrophages (38), and the stimulation of IL-12 and TNF-α (38) and reactive oxygen species (39,40).

Finally, it should be noted that the dose of 16 μg/ml CME induced the most prominent immunomodulatory response with the lowest histopathological damage score, corroborating the observation that intermediate doses are optimal as they induce immunological responses with maximum effector action (41).

In conclusion, the results of the present study demonstrated that a CME of *R. guttatus* has low toxicity in Swiss mice and an immunomodulatory effect; it promotes the development of a pro-inflammatory response. Further studies are required however, to identify the main compounds and intracellular pathways involved in this process. Thus, *R. guttatus* is a promising species for bioprospecting studies, particularly for conditions in which stimulation of the immune response is advantageous, such as neoplastic diseases.

Nature is a rich source of diverse bioactive compounds. As an example, the marine algal polysaccharides with several pharmaceutical activities have already been described, including antioxidant, anti-inflammatory, immunomodulatory and antidiabetic effects, being beneficial to human health and nutrition (42). Thus, studies with natural products become essential for the development of the bioeconomy.

Acknowledgements

The authors would like to thank Dr Amilcar Sabino Damazo, Department of Basic Science in Health, Faculty of Medical Sciences, Federal University of Mato Grosso, Cuiabá, Brazil, for his assistance in the histopathological analysis.

Funding

The authors wish to acknowledged the CAPES Foundation for the availability of a scholarship.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VDGS, DDJR, LC and APS conceived the study and designed the experiments. VDGS and LC participated in the design and interpretation of the data. SRDNP, EBRDS and LC performed the experiments and the data analysis. LC and VDGS wrote the manuscript and participated in the manuscript revisions. LC, SRNP and EBRS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under no. A313DC9, and approved by the UFMT Ethics Committee on the Use of Animals (CEUA), under no. 23108.918243/2017-50.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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