Evaluation of antimutagenic and cytotoxic activity of skin secretion extract of *Rhinella marina* and *Rhaebo guttatus* (Anura, Bufonidae)

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ABSTRACT

The skin secretion from toads of the Bufonidae family has great potential in the search for new active compounds to be used as drug candidates in treating some diseases, among them cancer. In this context, this study aimed to evaluate the cytotoxic and antimutagenic activity of the parotoid gland secretion extracts of *Rhinella marina* and *Rhaebo guttatus*, as well as biochemically analyze transaminases and serum creatinine for liver and renal damage, respectively. Cytotoxicity was performed by the colorimetric method based on MTT (3- [4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide) with different concentrations of the extracts in Walker or splenic tumor cell cultures from rats and mice. The micronucleus test was performed with male Swiss mice treated orally with the extracts for 15 days, and then intraperitoneally with N-ethyl-N-nitrosurea (50 mg kg⁻¹). Micronucleated polychromatic erythrocytes (MNPCE) were evaluated in bone marrow. The extracts showed cytotoxic activity in the evaluated cells. There was a significant reduction in the frequency of MNPCE (*R. marina* = 56% and *R. guttatus* = 75%, p < 0.001), indicating antimutagenic potential of the extracts. The groups treated only with extract showed an increase in MNPCE frequency, evidencing mutagenic potential. Biochemical analyzes showed no significant difference between treatments. Thus, under our experimental conditions, the extracts of *R. marina* and *R. guttatus* skin secretions presented chemopreventive potential for cancer.

KEYWORDS: genotoxicity, bioprospecting, micronuclei, chemoprevention

Avaliação da atividade antimutagênica e citotóxica da secreção cutânea de *Rhinella marina* e *Rhaebo guttatus* (Anura, Bufonidae)

RESUMO

A secreção cutânea de anuros da família Bufonidae tem grande potencial na busca de novos compostos ativos para utilização como fármacos candidatos no tratamento de algumas doenças, entre elas o câncer. Neste contexto, o estudo teve como objetivo avaliar a atividade citotóxica e antimutagênica dos extratos da secreção de glândula parótida de *Rhinella marina* e *Rhaebo guttatus*, bem como a análise bioquímica de transaminases e creatinina séricas, para avaliar dano hepático e renal, respectivamente. A avaliação de citotoxicidade foi realizada pelo método colorimétrico baseado no MTT (3- [4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide), com diferentes concentrações dos extratos em culturas de células do Tumor de Walker ou células esplênicas de rato e camundongo. O teste do micronúcleo foi realizado com camundongos *Swiss* machos que receberam tratamento oral com os extratos durante 15 dias, seguido de tratamento intraperitoneal com N-étil-N-nitrosureia (50 mg kg⁻¹). A frequência de eritrócitos policromáticos micronucleados (PECMN) foi determinada em medula óssea. Os extratos apresentaram ação citotóxica nas células avaliadas. Houve uma redução significativa na frequência de PCEMN (*R. marina* = 56% e *R. guttatus* = 75%, p < 0,001), observando-se um potencial antimutagênico dos extratos. Os grupos tratados somente com os extratos apresentaram um aumento na frequência de PCEMN, evidenciando um potencial mutagênico. As análises bioquímicas não apresentaram diferença significativa entre os tratamentos. Assim, nas condições experimentais testadas, as secreções cutâneas de *R. marina* e *R. guttatus* apresentaram potencial quimiopreventivo para câncer.

PALAVRAS-CHAVE: genotoxicidade, bioprospecção, micronúcleos, quimioprevenção

INTRODUCTION

The search for new cancer drugs is one of the most prominent research areas of natural products (Cragg and Newman 2012). Numerous active compounds of animals in different phyla, such as chordates and arthropods, have already been studied with promising results (Calvete 2009). Animal toxins represent a rich source of bioactive compounds such as peptides and proteins (Favreau et al. 2006), which have been used more and more as pharmacological tools and as prototypes for drug development (Calvete 2009).

Bufonidae is a family of toads composed of approximately 471 species in 33 genera (Pramuk 2006). These include the _cururu_ toads, with prominence for the genus _Rhinella_ (Frost 2015). _Rhinella marina_ (Linnaeus 1758) is distributed from North America to the Brazilian Amazon (Frost 2015). _Rhaebo guttatus_ (Schneider 1799) is commonly found in the southern Brazilian Amazon, and its geographical distribution extends from the Amazon region of Ecuador to the central Brazilian Amazon (Amphiabiweb 2016).

Bufadienolides (cardiac glycosides) are the main active compounds originating from toads of the Bufonidae family (Bagrov et al. 2009). These compounds have cardiotoxic (Emam et al. 2017), antiviral (Wang et al. 2011), anti-bacterial (Cunha-filho et al. 2005), antiparasitic (Tempone et al. 2008; Banfi et al. 2016) and cytotoxic and/or antitumor activity (Cunha-Filho et al. 2010; Gao et al. 2011; Sciani et al. 2013; Ferreira et al. 2013; Zhang et al. 2013; Banfi et al. 2016).

Although bufadienolides are the main active components in bufonid gland secretions that have antitumor activity, their action mechanisms are still unclear (Chen et al. 2017). Four bufadienolides in the skin secretion of _Rhinella marina_ (telocinobufagin, marinobufagin, bufalin and resibufogenin) and one in the secretion of _Rhaebo guttatus_ (marinobufagin) have presented cytotoxic effects against different tumor lines, highlighting the secretions of toad parotid glands as a promising source for new anticancer compounds (Ferreira et al. 2013).

Cancer treatment is still considered one of the most challenging problems in medicine and is the object of worldwide research (Fukumatsu et al. 2008). Chemoprevention is a current strategy for cancer prevention by which natural and chemical products or their combinations are used to reduce the risk or delay the onset of this disease (Singh et al. 2014; Amereh et al. 2017). Considering that the identification of the biological potential of new compounds is relevant for identifying new agents with significant antineoplastic activity and toxicity to tumor cells, the present study evaluated the cytotoxic and chemoprotective potential of the crude extracts of the parotid gland secretion of _R. marina_ and _R. guttatus._

MATERIAL AND METHODS

Chemicals

In the _in vitro_ assay, RPMI 1640 19% SBF (Cultilab, Campinas – SP Brazil) was used as the culture medium. For the _in vivo_ assay, the chemical agent _N_-nitroso- _N_ -ethylurea (ENU, CAS 759-73-9, Sigma Aldrich, Saint Louis, USA) was diluted with phosphate buffer (pH 6.0) and intraperitoneally (i.p.) administered to test animals at the concentration of 50 mg/kg b.w.

Crude extracts

_Rhinella marina_ and _Rhaebo guttatus_ individuals were collected in a locality (9°13’46.71”S, 60°17’41.75”W) in Mato Grosso state, Brazil (collection license IBAMA/SISBIO # 30034-1 issued to D.J. Rodrigues). The secretions were obtained by manual compression of the parotid macrogland and the animals were returned to nature after this procedure. Voucher specimens (_R. marina_ - ABAM-H 1262 and _R. guttatus_ - ABAM-H 1538) were deposited in the zoological collection (Acervo Biológico da Amazônia Meridional) of the Federal University of Mato Grosso (Universidade Federal do Mato Grosso - UFMT) at Sinop, Mato Grosso state (Brazil). Secretion samples were dried and extracted three times (5 ml) with CHCl₃/MeOH (3:1) (chloroform/methanol) using ultrasound for 10 min at room temperature (Ferreira et al. 2013).

Biological material

Walker 256 Tumor cells, as well as rat and mouse splenic cells were used for the _in vitro_ assay. Tumor cells were kindly provided by Eveline Aparecida Isquierdo Fonseca de Queiroz, of Universidade Federal do Mato Grosso (Sinop, Brazil). Tumor cells were obtained through the inoculation of these cells into the peritoneal cavity of rats in the laboratory, where the tumor developed and was later removed for seeding on microplates.

_In vivo_ experiment

For the _in vivo_ assay, 6-week old male _Swiss_ mice (mean weight 30 g) were used. The mice were obtained from the central breeding colony of Universidade Federal do Mato Grosso - UFMT at Cuiabá (Brazil). During the experimental period, seven mice per group were kept in collective cages for 15 days at the breeding colony (CAIC) of UFMT at Sinop (Brazil), under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%), and 12-hour light/dark cycle, receiving pelleted commercial feed and filtered water _ad libitum_. All procedures were performed with the approval of the Committee on Ethics in the Use of Animals (CEUA) of UFMT/Cuiabá under protocol # 23108.720739/2016-12.

The following experimental protocol was used:

Group 1: Negative control group. The animals were treated with water via gavage throughout the experimental period. On the 15th day the animals were treated intraperitoneally with 0.9% NaCl (0.1 mL 10 g⁻¹ b.w.) and sacrificed 24 hours after treatment to obtain bone marrow cells.
Group 2: Positive control group. The animals were treated with water via gavage throughout the experimental period. On the 15th day the animals were intraperitoneally treated with ENU (50 mg kg⁻¹ b.w.) and sacrificed 24 hours after treatment to obtain bone marrow cells.

Groups 3 and 4: The animals were treated with extract of *Rhinella marina* and *Rhaebo guttatus*, respectively, via gavage throughout the experimental period. On the 15th day the animals received intraperitoneal treatment with ENU (50 mg kg⁻¹ b.w.) and were sacrificed 24 hours after treatment to obtain bone marrow cells.

Groups 5 and 6: The animals were treated with extract of *Rhinella marina* and *Rhaebo guttatus*, respectively, via gavage throughout the experimental period. On the 15th day the animals received intraperitoneal treatment with 0.9% NaCl (0.1 mL 10 g⁻¹ b.w.) and were sacrificed 24 hours after treatment to obtain bone marrow cells.

The amount of feed provided to mice in each group was weighed every day to verify possible toxicity of the extract throughout the experimental period in order to control for potentially toxic effects of the extracts that may affect feed intake and weight gain of the mice.

**In vitro cytotoxicity assay**

For the cytotoxic activity analysis, we used the colorimetric method based on MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium, Cell Growth Determination Kit MTT Based, Sigma Aldrich, Saint Louis, USA) according to the manufacturer’s recommendations. MTT is tetrazolium salt, which becomes insoluble in water by forming formazan crystals and soluble in organic solvents after cleavage by mitochondrial dehydrogenases of cells, where only the enzymes of living cells cause this conversion (Mosmann 1983). In cell culture medium, MTT converted to formazan is solubilized and can be read by spectrophotometry (Mosmann 1983). This reaction can be expressed as a percentage of live cells according to the absorbancy, thereby obtaining the cell viability percentage. Absorbance values (abs) were measured on the Thermo Plate Spectrophotometer, microplate reader, TP READER. The cellular inhibition percentage was calculated by the equation: CI% = [(abs control) - (abs sample)] / (abs control) x 100.

Walker 256 Tumor cells (1x10⁶ cells mL⁻¹) (Colquhoun and Schumacher 2001), splenic cells from rats (Spinardi-Barbisan et al. 2004) and mice (4x10⁶ cells mL⁻¹) (Albieto et al. 2016) were seeded in triplicate in 96-well plates with 100 μL of RPMI 1640 medium with 19% FBS (Cultilab, Campinas – SP, Brazil) (Spinardi-Barbisan et al. 2004; Samarghandian et al. 2016) together with 100 μL of the compounds tested at concentrations of 7.81; 15.62; 31.25; 62.5 and 125 μg mL⁻¹, diluted in RPMI 1640 with 19% FBS. Next, 100 μL of RPMI 1640 with 19% FBS was used as a control of cell viability with the corresponding cellular concentrations. The microplates were cultured for 24 hours in an incubator with humidified atmosphere at 5% CO₂, 37°C. Independent triplicates were performed for each cell type, representative of the cell inhibition percentage and of the presented CI₅₀ (concentration of compound capable of causing 50% death of the cells relative to the control culture).

**In vivo micronucleus assay**

The micronucleus test in rodent bone marrow erythrocytes is used to detect and quantify the mutagenic effect of certain compounds on the cell life cycle. The technique for obtaining and preparing the bone marrow erythrocyte slides to evaluate the micronucleus (MN) frequency followed MacGregor et al. (1987), where 1000 cells per animal were analyzed under a light microscope with a 1000x (immersion). The material was analyzed blindly and the slides were decoded at the end of analyzes.

The reduction percentage of the frequency of micronucleated polychromatic erythrocytes (MNPCs) was calculated by the following equation (Manoharan and Banerjee 1985; Waters et al. 1990):

\[
\% \text{ reduction } = \frac{(\text{Frequency of MN in A}) - (\text{Frequency of MN in B}) \times 100}{(\text{Frequency of MN in A}) - (\text{Frequency of MN in C})}
\]

Where A corresponds to the group treated with ENU (positive control), B corresponds to the group treated with *R. marina* or *R. guttatus* extract plus ENU and C corresponds to the group treated with NaCl 0.9% (negative control).

**Biochemical analysis**

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) liver enzyme levels, as well as creatinine (CRT), were used as indicators of hepatic and renal damage, respectively. Whole blood samples from each animal were collected in anticoagulant free microtubes and centrifuged at 3500 rpm for 10 minutes. The supernatant was separated and stored at -20 °C for further determinations of serum biomarkers. The samples were measured through the COBAS INTEGRA® 400 Plus Roche® analyzer by the chemiluminescence method.

**Statistical analysis**

The frequency of micronucleated cells was compared among experimental groups using a chi-square test (Pereira 1991). Biochemical parameters, feed consumption and body weight were compared among treatments through analysis of variance (ANOVA), complemented by the Tukey and Scott-Knott test, using the SISVAR 5.6 program (p < 0.05). The CI₅₀ was calculated through a linear regression analysis (GraphPadInStat 3.06).
RESULTS

The extracts presented cytotoxicity for the studied cells in all evaluated concentrations. The toxic effect was lower on the rat (R. marina: 10.98%) and mouse (R. marina: 1.96%; R. guttatus: 0.33%) splenic cells at the concentration of 7.81 μg mL⁻¹. The 125 μg mL⁻¹ concentration had the highest inhibition rates for all cells tested. It is important to note that the inhibitory effect of R. guttatus extract on splenic cells was more pronounced than the effect of R. marina extract (Table 1). Thus, the concentration of 7 μg mL⁻¹ was chosen for the in vivo experiment.

The groups treated with the R. marina + ENU and R. guttatus + ENU extracts at a concentration of 7 μg mL⁻¹ showed a significant reduction in the frequency of MNPCES in relation to the positive control group (p < 0.001) of 56% and 75%, respectively. The groups treated with extracts + NaCl had significantly higher MNPCE frequency relative to the negative control (p < 0.01), indicating mutagenic potential of the extracts. Both venom extracts showed antimutagenic and mutagenic activities under the tested conditions (Table 2).

The groups treated with R. marina and R. guttatus extracts did not differ significantly from the positive and negative controls in plasma concentrations of AST, ALT and CRT. The results indicate that there was no toxic effect of extracts on the liver or on the kidneys of treated animals during the experimental period (Table 3).

The average feed intake of the mice during the experimental period differed significantly among groups (p < 0.05). Highest feed consumption was recorded for the group treated with R. guttatus extract + ENU, followed by the R. marina + NaCl and R. guttatus + NaCl groups (Table 4).

| Table 1. Frequency of splenic (rat and mouse) cell inhibition (CI) and Walker’s Tumor 256 cells cultured with Rhinella marina and Rhaebo guttatus skin secretion extracts for 24 hours at 37 °C and 5% CO₂
<table>
<thead>
<tr>
<th>Concentration (μg mL⁻¹)</th>
<th>Rhinella marina</th>
<th>Rhaebo guttatus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen mouse</td>
<td>Spleen rat</td>
</tr>
<tr>
<td></td>
<td>Spleen mouse</td>
<td>Spleen rat</td>
</tr>
<tr>
<td>125</td>
<td>21.47</td>
<td>29.02</td>
</tr>
<tr>
<td></td>
<td>66.40</td>
<td>62.67</td>
</tr>
<tr>
<td>62.50</td>
<td>2.88</td>
<td>20.26</td>
</tr>
<tr>
<td></td>
<td>56.53</td>
<td>41.59</td>
</tr>
<tr>
<td>31.25</td>
<td>-1.37</td>
<td>18.04</td>
</tr>
<tr>
<td></td>
<td>43.07</td>
<td>43.10</td>
</tr>
<tr>
<td>15.62</td>
<td>1.70</td>
<td>24.51</td>
</tr>
<tr>
<td></td>
<td>17.16</td>
<td>37.33</td>
</tr>
<tr>
<td>7.81</td>
<td>1.96</td>
<td>10.98</td>
</tr>
<tr>
<td></td>
<td>0.33</td>
<td>30.04</td>
</tr>
<tr>
<td>CI₅₀ (μg mL⁻¹)</td>
<td>306.46</td>
<td>330.95</td>
</tr>
<tr>
<td></td>
<td>74.92</td>
<td>77.60</td>
</tr>
</tbody>
</table>

*Concentration required to cause 50% cell death relative to the control culture.

| Table 2. Frequency of micronuclei in polychromatic erythrocytes (MNPCES) of bone marrow of mice after pre-treatment with the Rhinella marina (RM) and Rhaebo guttatus (RG) skin secretion extracts. N = sample size.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>N analyzed PCEs</th>
<th>MNPCES</th>
<th>% reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water + 0.9% NaCl</td>
<td>6000¹</td>
<td>217</td>
<td>3.61</td>
</tr>
<tr>
<td>Water + ENU</td>
<td>6000</td>
<td>398</td>
<td>6.63</td>
</tr>
<tr>
<td>RM Extract + ENU</td>
<td>7000</td>
<td>296²</td>
<td>4.22</td>
</tr>
<tr>
<td>RG Extract + ENU</td>
<td>7000</td>
<td>262²</td>
<td>56</td>
</tr>
<tr>
<td>RM Extract + 0.9% NaCl</td>
<td>7000</td>
<td>323³</td>
<td>4.61</td>
</tr>
<tr>
<td>RG Extract + 0.9% NaCl</td>
<td>7000</td>
<td>324³</td>
<td>4.62</td>
</tr>
</tbody>
</table>

¹Negative control; ²Positive control; ³One animal died; ⁴p < 0.01; ⁵p < 0.001 according to the chi-square test.

| Table 3. Mean values ± standard deviation of AST, ALT and creatinine analytes of mice in different groups after 15 days pretreatment with Rhinella marina (RM) and Rhaebo guttatus (RG) skin secretion extracts. N = sample size.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>AST* (U/L)</th>
<th>ALT* (U/L)</th>
<th>CRT* (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water + 0.9% NaCl</td>
<td>6</td>
<td>248.07 ± 70.29</td>
<td>60.80 ± 13.84</td>
<td>0.24 ± 0.08</td>
</tr>
<tr>
<td>Water + ENU</td>
<td>6</td>
<td>262.15 ± 59.75</td>
<td>58.08 ± 10.28</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>RM Extract + ENU</td>
<td>7</td>
<td>260.73 ± 73.89</td>
<td>49.47 ± 7.35</td>
<td>0.30 ± 0.27</td>
</tr>
<tr>
<td>RG Extract + ENU</td>
<td>7</td>
<td>223.43 ± 61.80</td>
<td>60.53 ± 40.94</td>
<td>0.29 ± 0.22</td>
</tr>
<tr>
<td>RM Extract + 0.9% NaCl</td>
<td>7</td>
<td>195.90 ± 42.52</td>
<td>55.05 ± 10.04</td>
<td>0.28 ± 0.11</td>
</tr>
<tr>
<td>RG Extract + 0.9% NaCl</td>
<td>7</td>
<td>181.53 ± 4.42</td>
<td>58.49 ± 9.87</td>
<td>0.21 ± 0.02</td>
</tr>
</tbody>
</table>

*Negative control; ²Positive control; ³One animal died; ⁴p < 0.05 according to ANOVA.

| Table 4. Mean value ± standard deviation of feed intake and body weight of mice after pre-treatment for 15 days with Rhinella marina (RM) and Rhaebo guttatus (RG) skin secretion extracts. N = sample size.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Feed intake* (g week⁻¹ group⁻¹)</th>
<th>Body weight* (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water + 0.9% NaCl</td>
<td>6</td>
<td>70.43 ± 10.21</td>
<td>34.91 ± 2.24</td>
</tr>
<tr>
<td>Water + ENU</td>
<td>6</td>
<td>66.29 ± 7.87</td>
<td>35.58 ± 1.53</td>
</tr>
<tr>
<td>RM Extract + ENU</td>
<td>7</td>
<td>79.14 ± 5.08</td>
<td>35.14 ± 2.02</td>
</tr>
<tr>
<td>RG Extract + ENU</td>
<td>7</td>
<td>87.29 ± 7.63</td>
<td>37.07 ± 3.73</td>
</tr>
<tr>
<td>RM Extract + 0.9% NaCl</td>
<td>7</td>
<td>84.29 ± 6.78</td>
<td>35.57 ± 3.63</td>
</tr>
<tr>
<td>RG Extract + 0.9% NaCl</td>
<td>7</td>
<td>81.71 ± 8.83</td>
<td>36.78 ± 3.13</td>
</tr>
</tbody>
</table>

*Negative control; ²Positive control; ³One animal died; ⁴p < 0.05 according to ANOVA and the Scott-Knott test.
DISCUSSION

Previous in vitro analyses have already demonstrated a multiplicity of bufadienolides of *R. marina* and *R. guttatus* with cytotoxic potential. The cytotoxicity of the skin secretion extracts of both species was observed in a variety of tumor cell lines, such as leukemia (HL-60), colon carcinoma (HCT-116), ovarian carcinoma (OVCAR-8), human breast cancer (MDA-MB-231, MCF-7) and normal cells (PBMC) using colorimetric MTT assay (Ferreira et al. 2013; Sciani et al. 2013). Our results corroborate these previous studies by indicating a potent cytotoxic activity of the skin secretion extracts of *R. marina* and *R. guttatus* in different tumor cell lines. This effect can be attributed to the presence of bufadienolides in the secretions (telocinobufagin, bufalin, marinobufagin and resibufogenin in *R. marina*, and marinobufagin in *R. guttatus* extract; Ferreira et al. 2013). The differences of our CI95 values in relation to those observed in other studies (Silva et al. 2004; Sciani et al. 2013; Ferreira et al. 2013; Schmeda-Hirschmann et al. 2014, 2016) likely reflects the morphological differences of the tumor types tested (Sciani et al. 2013; Ferreira et al. 2013).

Isolated bufalin had an antiangiogenic effect, potently inhibiting the proliferation and formation of vascular endothelial cells by inhibiting the G2/M phase of the cell cycle (Lee et al. 1997). Bufalin was also observed to induce apoptosis (Watabe et al. 1998; Han et al. 2007), mitochondrial dysfunction and increase radiosensitivity in glioblastoma cells (Zhang et al. 2017) and to reverse acquired resistance to antineoplastic agents (Sun et al. 2017). Marinobufagin showed cytotoxic activity in tests with four tumor cell lines (HL-60, HCT-116, OVCAR-8 and SF-295), as well as toxicity, cytotoxicity and genotoxicity in the root meristem of *Allium cepa*, through changes in root growth, inhibition of mitotic index, and chromosomal aberrations (Machado et al. 2018).

The action mechanism of the cytotoxic effect of bufadienolides on tumor cells, remain unknown in many cases (Gao et al. 2011). Bufadienolides of *Bufo melanostictus* showed cytotoxicity in U937 non-tumor cells (CI95 55000-66200 μg mL⁻¹) and K562 leukemic cells (CI95 8100-92200 μg mL⁻¹), antiproliferative action and apoptosis induction, with G1 cell cycle stoppage (Giri et al. 2006). These contrasting results point to different mechanisms for cytotoxicity induction by bufadienolides, via lytic activity in cell membrane or by carrying out apoptosis (Rodríguez et al. 2017). The lack of hemolytic activity of bufadienolides against mouse erythrocytes, even at doses as high as 50 μg mL⁻¹, suggests that these compounds cause apoptosis without disruption of the cell membrane (Cunha-Filho et al. 2010). However, the *Rhaebo guttatus* extract induced cell membrane disruption of human erythrocytes, indicating hemolytic potential (Ferreira et al. 2013). Further studies are needed to elucidate the action mechanisms of different bufadienolides in anticancer research.

The mutagenic potential of the skin secretion of *R. marina* and *R. guttatus* was indicated by the increase of MNPCes in the groups treated only with the extracts. Chan Su (an extract of skin secretion of *Bufo bufo gargarizans*) showed genotoxic effects in vitro on tumor cells (MCF-7, A-549 and Jurkat T) through an increase in chromosomal damage and formation of micronuclei, but not on peripheral blood mononuclear cells (PBMCs) (Lee et al. 2017). The antimutagenic and mutagenic effects of the extracts depends on their chemical profile, which varies according to species origin, diet and environmental factors (Gao et al. 2010; Ferreira et al. 2013).

In contrast to in vitro toxicity and genotoxic potential in vivo, biochemical analyzes of AST, ALT and CRT in serum did not demonstrate liver and kidney damage in treated animals. Also, no tendency of weight loss was observed in the test animals treated with *R. marina* and *R. guttatus* skin extracts during 15 days, suggesting that there was no direct toxic effect at the implemented doses. Overall, the animals showed no signs of toxicity effects of the skin secretion extracts during the whole treatment period. Future studies should focus on the analysis of compounds isolated from the extracts in order to identify which molecules act as possible chemoprotective agents in preventing carcinogenesis.

CONCLUSIONS

The crude extracts of parotoid gland secretion of *Rhinella marina* and *Rhaebo guttatus* showed pronounced toxic effects on Walker 256 Tumor cells and splenic mouse and rat cells, regardless of the dosage used. The micronucleus test revealed antimutagenic and mutagenic effects of the two extracts. There was no hepatic or renal damage in treated animals as observed via biochemical analysis of AST, ALT and CRT in serum. Due to the absence in the literature of antigenotoxicity studies involving these extracts, it is emphasized that our results are unprecedented in the mutagenesis area. Under the performed conditions, the results show a protective effect of *R. marina* and *R. guttatus* extracts against chemically induced damage to the DNA.

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