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ORIGINAL ARTICLE

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, este estudo teve como objetivo avaliar a atividade citotóxica e antimutagênica dos extratos da secreção da 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-etil-N-nitrosuréia (50 mg kg^{-1}). A frequência de eritrócitos policromáticos micronucleados (PCEMN) 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 PCEMNs, 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

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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 c*ururu* 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 (Amphibiaweb 2016).

Bufadienolides (cardiac glycosides) are the main active compounds originating from toads of the Bufonidae family (Bagrov *et al.* 2009). These compounds have cardiotonic (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 mechnisms 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 tumoral 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 (Fukumasu *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 parotoid 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 $CHCl_3$/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 ºC), 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 absorbance, 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)} x100.

Walker 256 Tumor cells (1x10^6 cells mL^{-1}) (Colquhoun and Schumacher 2001), splenic cells from rats (Spinardi-Barbisan *et al.* 2004) and mice (4x10^6 cells mL^{-1}) (Albiero *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% CO_2, 37 °C. Independent triplicates were performed for each cell type, representative of the cell inhibition percentage and of the presented CI_{50} (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 (MNPCEs) was calculated by the following equation (Manoharan and Banerjee 1985; Waters *et al.* 1990):

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

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 CI_{50} 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. gutttatus* + 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 ºC and 5% CO_2.

Concentration (μg mL^{-1})	Rhinella marina			Rhaebo guttatus		
	Spleen mouse	Spleen rat	Walker's tumor	Spleen mouse	Spleen rat	Walker's tumor
125	21.47	29.02	47.91	66.40	62.67	42.21
62.50	2.88	20.26	25.45	56.53	41.59	36.43
31.25	-1.37	18.04	30.82	43.07	43.10	40.70
15.62	1.70	24.51	32.80	17.16	37.33	40.70
7.81	1.96	10.98	31.81	0.33	30.04	33.42
CI_{50}*(μg mL^{-1})	306.46	330.95	179.24	74.92	77.60	340.78

*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.

Treatment	N analyzed PCEs	MNPCEs		% reduction
		N	%	
Water + 0.9% NaCl [a]	6000[c]	217	3.61	
Water + ENU[b]	6000[c]	398	6.63	
RM Extract + ENU	7000	296**	4.22	56
RG Extract + ENU	7000	262**	3.74	75
RM Extract + 0.9% NaCl	7000	323*	4.61	
RG Extract + 0.9% NaCl	7000	324*	4.62	

[a]Negative control; [b]Positive control; [c]One animal died; *$p < 0.01$; **$p < 0.001$ 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.

Treatment	N	AST*	ALT*	CRT*
Water + 0.9% NaCl [a]	6[c]	248.07 ± 70.29	60.80 ± 13.84	0.24 ± 0.08
Water + ENU[b]	6[c]	262.15 ± 59.75	58.08 ± 10.28	0.21 ± 0.03
RM Extract + ENU	7	260.73 ± 73.89	49.47 ± 7.35	0.30 ± 0.27
RG Extract + ENU	7	223.43 ± 61.80	60.53 ± 40.94	0.29 ± 0.22
RM Extract + 0.9% NaCl	7	195.90 ± 42.52	55.05 ± 10.04	0.28 ± 0.11
RG Extract + 0.9% NaCl	7	181.53 ± 4.42	58.49 ± 9.87	0.21 ± 0.02

[a]Negative control; [b]Positive control; [c]One animal died. *$p < 0.05$ 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.

Treatment	N	Feed intake* (g $week^{-1}$ $group^{-1}$)	Body weight*(g)
Water + 0.9% NaCl [a]	6[c]	70.43 ± 10.21	34.91 ± 2.24
Water + ENU [b]	6[c]	66.29 ± 7.87	35.58 ± 1.53
RM Extract + ENU	7	79.14 ± 5.08	35.14 ± 2.02
RG Extract + ENU	7	87.29 ± 7.63	37.07 ± 3.73
RM Extract + 0.9% NaCl	7	84.29 ± 6.78	35.57 ± 3.63
RG Extract + 0.9% NaCl	7	81.71 ± 8.83	36.78 ± 3.13

[a]Negative control; [b]Positive control; [c]One animal died. *$p < 0.05$ 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 CI_{50} 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 (CI_{50} 55000-66200 µg mL^{-1}) and K562 leukemic cells (CI_{50} 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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