Toxicon 72 (2013) 43–51

Contents lists available at SciVerse ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Antiproliferative activity of *Rhinella marina* and *Rhaebo guttatus* venom extracts from Southern Amazon

Paulo Michel Pinheiro Ferreira [a,b], Daisy Jereissati Barbosa Lima [c], Bryan Wender Debiasi [d], Bruno Marques Soares [c], Kátia da Conceição Machado [b], Janaina da Costa Noronha [f], Domingos de Jesus Rodrigues [e,f], Adilson Paulo Sinhorin [d,e], Cláudia Pessoa [c], Gerardo Magela Vieira Júnior [d,e,*]

[a] *Department of Biological Sciences, Campus Senador Helvídio Nunes de Barros, Federal University of Piauí, 64607-670 Picos, Piauí, Brazil*
[b] *Postgraduate Program in Pharmaceutical Sciences, Federal University of Piauí, 64.049-550 Teresina, Piauí, Brazil*
[c] *Department of Physiology and Pharmacology, Federal University of Ceará, 60430-270 Fortaleza, Ceará, Brazil*
[d] *Instituto de Ciências Naturais, Humanas e Sociais, Laboratórios Integrados de Pesquisa em Ciências Químicas (LIPEQui), Universidade Federal de Mato Grosso, 78577-267 Sinop, Mato Grosso, Brazil*
[e] *Programa de Pós-graduação em Ciências Ambientais, Universidade Federal de Mato Grosso, 78577-267 Sinop, Mato Grosso, Brazil*
[f] *Instituto de Ciências Naturais, Humanas e Sociais, Acervo Biológico da Amazônia Meridional (ABAM), Universidade Federal de Mato Grosso, 78577-267 Sinop, Mato Grosso, Brazil*

ARTICLE INFO

Article history:
Received 28 March 2013
Received in revised form 11 June 2013
Accepted 13 June 2013
Available online 22 June 2013

Keywords:
Antiproliferative activity
Bufadienolides
Rhinella marina
Rhaebo guttatus

ABSTRACT

The venom of amphibians is a fascinating source of active substances. In view of their medical importance and aiming to explore the amazing Brazilian biodiversity, we conducted bioprospecting of antiproliferative activity in extracts of *Rhinella marina* and *Rhaebo guttatus* toads occurring in the Southern Amazon of Mato Grosso, Brazil. LC–MS and HPLC analysis of the venom extracts of *R. marina* revealed four bufadienolides (telocinobufagin – **1**, marinobufagin – **2**, bufalin – **3** and resibufogenin – **4**). *R. guttatus* venom extracts contained only marinobufagin (**2**). First, *R. marina* and *R. guttatus* venom extracts were evaluated for cytotoxicity against tumor cell lines by the MTT assay. All extracts revealed cytotoxicity, where *R. marina* extracts were comparable to doxorubicin (IC_{50} values ranging from 0.01 to 0.23 μg/mL). Only extracts of *R. guttatus* toad venom caused membrane disruption of human erythrocytes. The extracts were investigated for selective activity by determining their effect on stimulated human peripheral blood mononuclear cells (PBMC) with the Alamar Blue™ assay. The extracts were up to 80-fold more selective against leukemia cells when compared to dividing leukocytes. Aiming to confirm these antiproliferative effects, BrdU incorporation into DNA was measured in HL-60 treated cells with *R. marina* venom extracts. These extracts decreased BrdU incorporation at both concentrations tested. In summary, nine extracts of *R. marina* and *R. guttatus* venom showed pronounced lethal and discriminating effects on tumor lines, especially those from *R. marina*, highlighting toad parotoid gland secretions as a promising source for novel lead anticancer chemicals.

1. Introduction

Bioprospecting of secondary metabolites can be an important contribution to economic growth in developing countries. Thousands of new compounds can arise from

* Corresponding author. Avenida Alexandre Ferronato, 1200, Reserva 35, 78557-267 Sinop, Mato Grosso, Brazil. Tel.: +55 66 81083319.
E-mail addresses: magela@ufmt.br, gerardovieira@yahoo.com.br (G.M. Vieira Júnior).

0041-0101/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.toxicon.2013.06.009

prospecting programs and indicate new bioactive and/or prototypes for pharmaceutical development. In this context, animals, plants, fungi and bacteria are important sources of biologically active substances with structural diversity and novel mechanisms of action, which can possibly provide patentable products (Rocha et al., 2001; Clardy and Walsh, 2004; Cunha-Filho et al., 2010; Ferreira et al., 2011a, 2011b; Vieira Júnior et al., 2011; Militão et al., 2012).

The family Bufonidae possesses 33 genera and 471 species (Pramuk, 2006). It has a cosmopolitan distribution, except in Madagascar and Antarctica areas. *Rhinella* (formerly *Bufo* in the New World), the main genus of the family, consists of about 258 species. In Latin America, they are found in the Amazon regions of Brazil, Bolivia, Colombia, Peru, Suriname, Guiana and Venezuela (Frost et al., 2006).

The skin secretions and venom of amphibians are fascinating sources of active compounds, such as peptides, alkaloids, bufadienolides, biogenic amines and proteins. These molecules play a crucial role in the physiological functions of these animals, especially for predation and protection against microorganisms. In toads, particularly, the key compounds are biogenic amines and digitalis-like aglycones called bufadienolides, an important group of polyhydroxy C-24 steroids related to cholesterol, which have a 2-pyrone group attached at the C-17 position of the perhydrophenanthrene nucleus (Toledo and Jared, 1995; Dmitrieva et al., 2000; Xu-Tao et al., 2009; Yang et al., 2010; Gao et al., 2011). Structure–activity relationship studies of these compounds have shown cardiotonic (Imai et al., 1965), antiviral (Kamano et al., 1988; Wang et al., 2011), cytotoxic (Cunha-Filho et al., 2010; Gao et al., 2011; Sciani et al., 2012), antibacterial (Cunha-Flho et al., 2005), antiparasitic (Tempone et al., 2008) and insecticidal (Supratman et al., 2000) properties.

Animals contain a large assortment of structurally unique secondary metabolites that can be useful as new chemical templates for drug discovery (Rocha et al., 2001; Cunha-Filho et al., 2010). Although amphibian skin secretions have proved to be a rich source of exclusive molecules, they remain largely underexplored or entirely unexplored and represent a great potential for the development of new molecular models for pharmacological and toxicological evaluations and even for synthesis and medicinal chemistry. Our objectives has been to explore the biodiversity of Brazil, a country with the largest number of species in the world, possessing more than a hundred thousand species of invertebrates and about 8200 vertebrates. Therefore, we conducted bioprospecting in extracts of *Rhinella marina* (synonymy *Bufo marinus*) and *Rhaebo guttatus* toads occurring in the Southern Amazon of Mato Grosso, Brazil, in search of venoms with cytotoxic activity against tumor and normal cells. Antiproliferative activity in extracts was assessed using the BrdU immunocytochemistry assay.

2. Material and methods

2.1. Qualitative HPLC and LC–ESIMS analysis

Analytical HPLC was performed on a Varian HPLC system Pro Star 325 LC plus UV detector, Pro Star 325 dual wavelength system. Electrospray ionization (ESI) mass spectra were acquired in the positive ion mode on a LC–MS system LCQ FLEET instrument (Thermo scientific) equipped with an ion-trap mass analyzer. Chromatographic separation was carried out in a Phenomenex Luna C18 column (250.0 mm × 4.6 mm, 5 μm). The mobile phase consisted of MeCN and water. A multistep gradient program was used as follows: 8% MeCN (0 min), 54% MeCN (45 min), 54% MeCN (55 min) and 95% MeCN (70 min). The flow rate was 0.8 mL/min, injection volume was 20 μL (4 mg/mL), and UV detection was at 296 nm (Gao et al., 2010).

2.2. Sample collection

Toad venom was collected from the secretion of *R. marina* and *R. guttatus* in Mato Grosso State, Brazil. The animals were identified by one of the authors (D. J. Rodrigues – IBAMA, SISBIO: number 30034-1). Voucher specimens (*R. marina* – ABAM-H 1262 and *R. guttatus* – ABAM-H 1538) were deposited in the Acervo Biológico da Amazônia Meridional (Sinop, Mato Grosso, Brazil).

2.3. Extraction of toad venom samples and standards

Nine samples (10.0 mg each) of toad venom of *R. marina* and *R. guttatus* were separated by gender (male/female), dried, powdered and extracted three times (5 mL) with $CHCl_3$/MeOH (8:2) by ultrasonication for 10 min at room temperature. The extracts were qualitatively analyzed by HPLC and LC–MS, and they were identified by the following codes: RMF – *R. marina* female, RMM – *R. marina* male, RGF – *R. guttatus* female and RGM – *R. guttatus* male (Gao et al., 2010). Reference standards of two authentic bufadienolides, namely telocinobufagin and marinobufagin, were supplied by Dr. Geraldino A. Cunha-Filho (University of Brasilia, Brazil).

2.4. Culture of normal and tumor cells

Heparinized human blood samples (from healthy, non-smoker donors who had not taken any drug for at least 15 days prior to sampling, aged 18–35 years old) were collected, and peripheral blood mononuclear cells (PBMC) were isolated by the standard method of density-gradient centrifugation over Ficoll–Hypaque. All studies were performed in accordance with Brazilian research guidelines (Law 196/96, National Council of Health) and with the Declaration of Helsinki.

Leukemia (HL-60), colon (HCT-116), glioblastoma (SF-295) and ovarian (OVCAR-8) tumor cells and PBMC were grown in RPMI-1640 medium supplemented with 20% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in a 5% CO_2 atmosphere.

2.5. Cytotoxicity assays

The cytotoxic properties of the extracts were assessed by colorimetric assays after 72 h exposure using HL-60, SF-295, HCT-116, OVCAR-8 and PMBC. Cell proliferation was determined spectrophotometrically using a multiplate

reader (DTX 880 Multimode Detector, Beckman Coulter). Control groups (negative and positive) received the same amount of dimethylsulfoxide solvent (0.1% DMSO) as test groups. Doxorubicin (Dox, 0.005–5.0 μg/mL) was used as positive control.

2.5.1. MTT assay

The cytotoxicity against HL-60, SF-295, HCT-116 and OVCAR-8 human cancer cells was determined by the MTT assay (Mosmann, 1983), which analyzes the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a purple formazan product. Briefly, cells were plated in 96-well plates (0.3–0.7 $\times$ 10^5 cells/well) and incubated to allow cell adhesion or equilibration (suspension cultures). Twenty-four hours later, extracts were added to each well (0.004–50 μg/mL). After 69 h of incubation, the supernatant was replaced with fresh medium containing 10% MTT, and the cells incubated for an additional 3 h. The plates were then centrifuged and the formazan product was dissolved in DMSO; absorbance was read at 595 nm.

2.5.2. Alamar Blue assay

The selectivity of the extracts was investigated in human PBMC using the Alamar Blue™ assay. PBMC were washed and resuspended (3×10^5 cells/mL) in supplemented RPMI-1640 medium plus 4% phytohemagglutinin for growth stimulation. PBMC were then plated in 96-well plates (3×10^5 cells/well in 100 μL of medium). After 24 h, extracts dissolved in DMSO were added to each well (0.004–50 μg/mL) and the cells were incubated for 72 h. Twenty-four hours before the end of the incubation, 10 μL of Alamar Blue™ stock solution (0.312 mg/mL) (Resazurin; Sigma Aldrich Co., USA) were added to each well. The absorbance was read at 570 and 595 nm and the drug effect was expressed as the percentage of the control (Ferreira et al., 2011b).

2.6. Membrane analysis assay

The extracts were assayed for hemolytic activity according to the method of Santos et al. (2010), with some modifications. Extracts (1.56–200 μg/mL) were incubated in 96-well plates for 60 min at room temperature (25 °C) in a suspension of human erythrocytes (2%) in 0.85% NaCl containing 10 mM $CaCl_2$. After centrifugation, hemoglobin levels in the supernatants were spectrophotometrically determined at 540 nm.

2.7. Inhibition of DNA synthesis

The BrdU assay is a reliable *in vitro* non-radioactive method, which is very often used to directly quantify cell proliferation (Costa et al., 2008; Ferreira et al., 2010). Accordingly, HL-60 cells were plated in 24-well tissue culture plates (1 mL/well) and treated with *R. marina* extracts (RMF-1, RMF-2, RMF-3, RMF-4 and RMM-5) at concentrations of 0.1 and 1 μg/mL for 24 h. Before the end of drug exposure, 10 μL of 10 mM 5-bromo-2′-deoxyuridine (BrdU) were added to each well and the cells incubated for an additional 3 h at 37 °C. To determine the amount of BrdU incorporated into DNA, cells were first harvested, transferred to cytospin slides, and allowed to dry for 2 h at room temperature (Pera et al., 1977). Cells that incorporated BrdU were labeled by direct peroxidase immunocytochemistry, using the chromogen diaminobenzidine (DAB). Slides were counterstained with hematoxylin. Cells were scored for BrdU positivity by light microscopy (Olympus, Tokyo, Japan), where 200 cells were counted per slide to determine the percentage of BrdU-positive cells.

2.8. Statistical analysis

The IC_{50} and EC_{50} values and their 95% confidence intervals were obtained by nonlinear regression using the GraphPad program (Intuitive Software for Science, San Diego, CA). Differences were evaluated by comparing data using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test ($p < 0.05$). All studies were carried out in triplicate and represented independent biological evaluations.

3. Results

As shown in Fig. 1, the chromatographic profile revealed four bufadienolides in *R. marina* extracts (RMF-1 and RMM-5), namely telocinobufagin (**1**), marinobufagin (**2**), bufalin (**3**) and resibufogenin (**4**) (Figs. 1 and 2), whereas in *R. guttatus* venom (RGF-6 and RGM-9), only one bufadienolide was identified (marinobufagin – **2**). The compounds were identified by comparison of retention times with standards and on the basis of UV and mass spectra. These findings are in agreement with previous data for *B. marinus* (Gao et al., 2010).

Regarding the biological assessments, the cytotoxicity of *R. marina* and *R. guttatus* venom extracts was first evaluated in a variety of tumor cell lines after 72 h exposure using the colorimetric MTT assay. All extracts of *R. marina* male/female venoms revealed higher cytotoxic activity, with IC_{50} values ranging from 0.01 μg/mL [RMF-1, RMF-3 and RMF-4 (HL-60); RMF-3 and RMF-4 (SF-295) and RMF-3 (HCT-116)] to 0.23 μg/mL (OVCAR-8) (Table 1). Meanwhile, *R. guttatus* venom extracts exhibited a lower cytotoxic effect when compared to those of *R. marina*, with their IC_{50} values being around 2.9–6.6 μg/mL. Second, the cytotoxicity of the extracts was determined against normal cells, using human PBMC for this purpose. Herein, higher IC_{50} values were found for proliferating leukocytes (0.8, 0.5, 0.4, 0.3, 1.1, 0.8, 16, 13.1 and 13.9 μg/mL for RMF-1, RMF-2, RMF-3, RMF-4, RMM-5, RGF-6, RGF-7, RGF-8 and RGM-9, respectively) (Table 2). Statistically, there were no differences in the cytotoxicity outcomes between samples obtained from female and male animals belonging to the same species ($p > 0.05$).

To better understand this potent cytotoxic activity, *in vitro* cytolytic analyses were performed with human erythrocytes. Interestingly, the most promising extracts obtained from *R. marina* (RMF-1, RMF-2, RMF-3, RMF-4 and RMM-5) were not able to cause hemolysis even at the highest concentration tested (200 μg/mL) (Table 2). On the other hand, all *R. guttatus* venom extracts led to hemolysis,

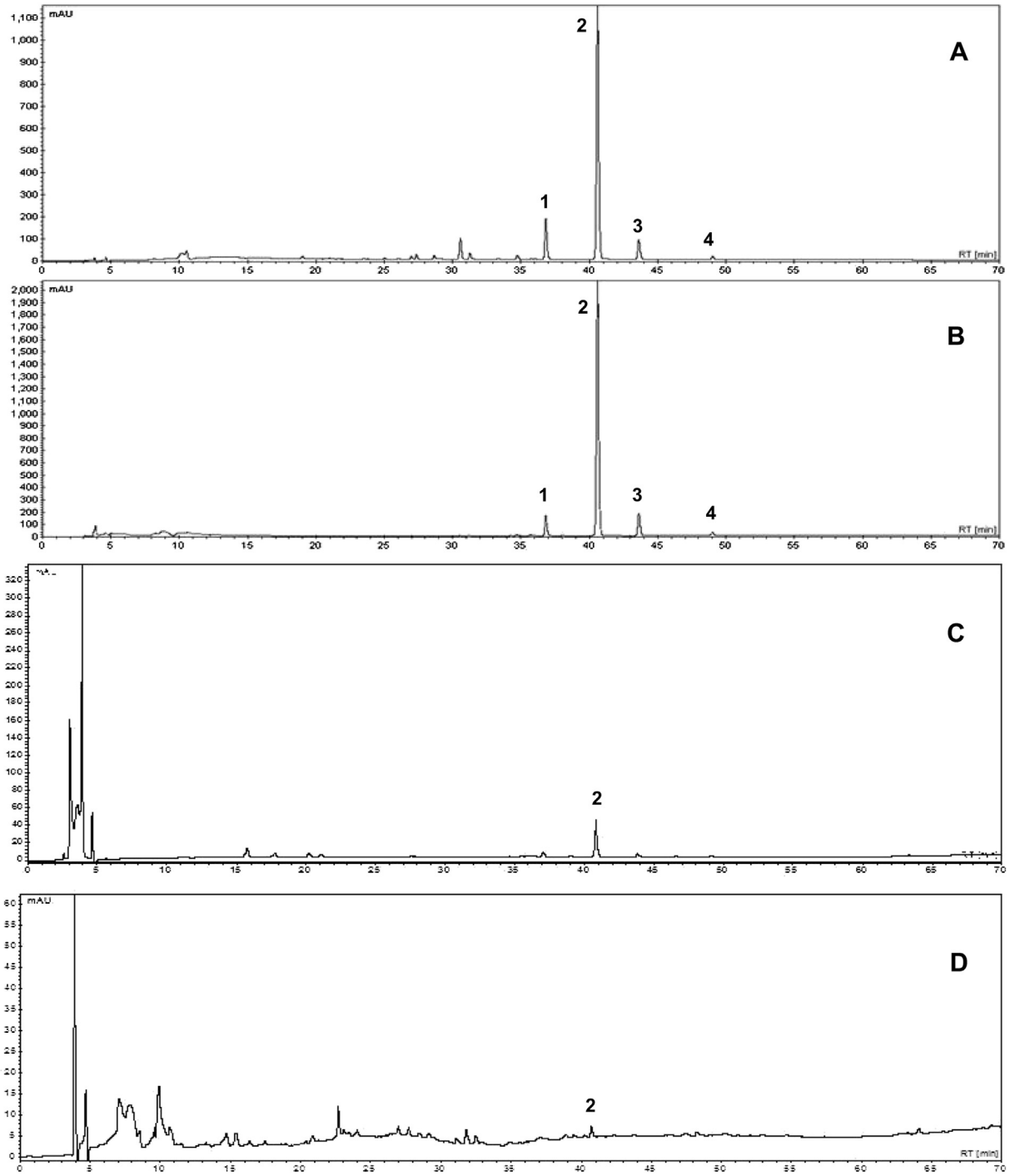

Fig. 1. HPLC chromatograms of the extracts of toad venom from *R. marina* RMF-1 (A), *R. marina* RMM-5 (B), *R. guttatus* RGF-6 (C) and *R. guttatus* RGM-9 (D). Telocinobufagin (**1**), marinobufagin (**2**), bufalin (**3**), and resibufogenin (**4**).

with EC_{50} values ranging from 20.8 (RGF-8) to 33.7 μg/mL (RGF-6).

BrdU incorporation into DNA was measured in HL-60-treated cells with *R. marina* venom extracts after 24 h exposure. As seen in Fig. 3, all extracts (RMF-1, RMF-2, RMF-3, RMF-4 and RMM-5) decreased BrdU incorporation, showing labeling of 35.4 ± 3.4, 30.7 ± 1.0, 25.1 ± 1.8, 28.0 ± 1.7 and 38.3 ± 2.6% at 0.1 μg/mL and 19.7 ± 1.3, 19.6 ± 1.2, 15.8 ± 1.8, 16.5 ± 0.8 and 29.5 ± 1.6% at 1 μg/mL, respectively, when compared to untreated cells (73.0 ± 3.2%) ($p < 0.05$). Dox (0.1 and 1 μg/mL) treatment resulted in 22.6 ± 1.9 and 12.7 ± 0.9% BrdU incorporation ($p < 0.05$).

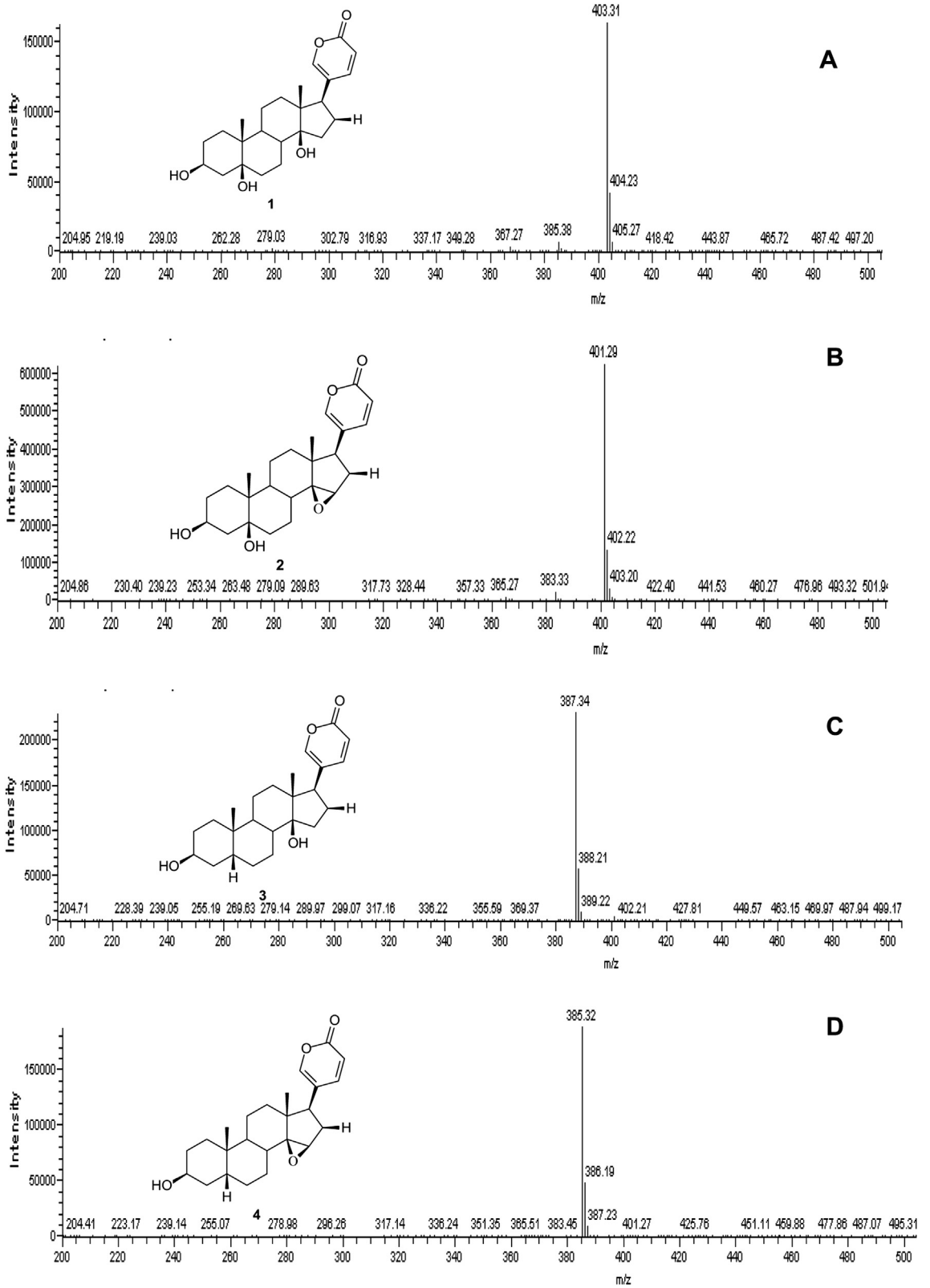

Fig. 2. ESI spectra of the quasi-molecular ion $[M+H]^+$ of the identified compounds. (A) telocinobufagin (1) $[M+H]^+$ 403.3; (B) marinobufagin (2) $[M+H]^+$ 401.2; (C) bufalin (3) $[M+H]^+$ 387.3; (D) resibufogenin (4) $[M+H]^+$ 385.3.

Table 1
Cytotoxic potential of *Rhinella marina* and *Rhaebo guttatus* venom extracts on human cancer cell lines after 72 h of exposure evaluated by MTT assay.

Extract	CI_{50} (μg/mL)[a]			
	SF-295	OVCAR-8	HL-60	HCT-116
RMF-1	0.03	0.06	0.01	0.02
	0.03–0.04	0.05–0.09	0.008–0.01	0.01–0.02
RMF-2	0.08	0.09	0.05	0.06
	0.06–0.09	0.07–1.0	0.04–0.06	0.04–0.07
RMF-3	0.01	0.03	0.01	0.01
	0.01–0.02	0.02–0.03	0.01–0.01	0.01–0.01
RMF-4	0.01	0.03	0.01	0.02
	0.01–0.02	0.02–0.03	0.01–0.02	0.01–0.02
RMM-5	0.09	0.23	0.07	0.06
	0.08–0.11	0.18–0.28	0.05–0.09	0.04–0.08
RGF-6	4.0	2.9	3.2	4.0
	3.2–5.0	2.1–4.0	2.8–3.6	2.6–5.9
RGF-7	3.2	3.8	4.6	3.1
	2.7–3.8	3.1–4.9	2.2–7.5	2.0–4.7
RGF-8	4.8	5.2	3.6	4.9
	4.1–5.6	4.4–6.2	2.9–4.4	4.4–5.4
RGM-9	6.6	4.5	4.9	5.9
	5.2–8.4	3.2–6.5	4.1–6.1	3.8–9.1
Doxorubicin	0.2	1.3	0.02	0.01
	0.2–0.3	1.0–1.9	0.01–0.02	0.01–0.02

[a] Data are presented as IC_{50} values and 95% confidence intervals for leukemia (HL-60), colon carcinoma (HCT-116), ovarian carcinoma (OVCAR-8) and glioblastoma (SF-295). Doxorubicin was used as positive control. Experiments were performed in triplicate. *Rhinella marina* female/male (RMF/RMM); *Rhaebo guttatus* female/male (RGF/RGM).

4. Discussion

Drug discovery and development have established a respectable armamentarium of useful chemotherapeutic agents as well as a number of important successes in the treatment and management of human cancer. Nevertheless, the most common tumors are resistant to current antineoplastic drugs and the majority of these agents have only limited activity against solid tumors (Harris, 2002; Srivastava et al., 2005). However, research on antiproliferative compounds has still demonstrated the great pharmacological importance of biological extracts (Clardy and Walsh, 2004; Cragg and Newman, 2005; Ferreira et al., 2011b).

In the last decades, toads have received special attention, with many publications describing the biological activities of molecules and aqueous and organic extracts obtained from skin glands, whose secretions exhibit bufadienolides, compounds that may act as endogenous steroidal hormones (Schoner and Scheiner-Bobis, 2005) and display antiangiogenic (Lee et al., 1997), antihypertensive (Vu et al., 2006), immunosuppressive (Terness et al., 2001), anti-endometrial (Nasu et al., 2005) and positive inotropic (Cruz and Matsuda, 1993) actions. Herein, we investigated the chemical composition of extracts of *R. marina* and *R. guttatus* venoms and their antiproliferative activity in transformed and normal cells.

Chemical investigations showed significant differences in composition between *R. marina* and *R. guttatus* venoms, in terms of the number and type of constituents. *R. marina* venom contained four bufadienolides, namely telocinobufagin (**1**), marinobufagin (**2**), bufalin (**3**) and resibufogenin (**4**) (Figs. 1 and 2), whereas only one bufadienolide (marinobufagin – **2**) was identified in *R. guttatus* venom. No obvious chemical differences were observed between male and female toads. These compounds have also been identified in other toad species such as *Rhinella schneideri*, *Bufo bufo gargarizans*, *Bufo melanosticus*, *Bufo viridis* and *Bufo rubescens* (Gao et al., 2010; Cunha-Filho et al., 2010, 2005). There are a number of potential reasons for this variation in venom composition such as species-specific differences, the diet of each species, and environmental factors (Gao et al., 2010). The chemical profile of the toad venoms (*R. marina* and *R. guttatus*) in terms of the number and type of compounds present is mainly determined by the species of origin.

Venom extracts from *R. marina* and *R. guttatus* (male and female) showed cytotoxic activity against cancer lines after 72 h exposure, mainly *R. marina* extracts, whose IC_{50} values were comparable to that of the positive control Dox. According to the American National Cancer Institute (NCI), an $IC_{50} \leq 30$ μg/mL is needed to consider a crude extract promising for further purification and biological analyses (Suffness and Pezzuto, 1990; Ferreira et al., 2011b). Previous *in vitro* analyses have already demonstrated a multiplicity of bufadienolides with cytotoxic potential. These compounds include gamabufotalin rhamnoside, bufotalin, hellebrin, epoxy-marinobufagin, bufalin, bufalin 3-acetate, hellebregenin, hellebregenin 3-acetate, resibufogenin 3-acetate, marinobufagin, marinobufagin 3-acetate, cinobufagin, telocinobufagin, 3β-acetoxy-marinobufagin, 3β-acetoxy-bufalin, 3β-acetoxy-telocinobufagin and 20S,21R-epoxy-marinobufagin, isolated from skin secretions of *Rhinella*, *Bufo* and *Rhaebo* species (*B. melanosticus*, *R. schneideri*, *R. margaritifer*, *R. hypocondrialis*, *R. major*, *R. margaritifera*, *R. crucifer* and *R. jimi*), bufadienolides extracted from the Chinese traditional drug *Ch'an Su* and from plants (*Urginea maritima*, *U. aphylla*, *U. maritima* and *U. hesperia*), displaying activity against tumor lines, such as

Table 2
Hemolytic study of *Rhinella marina* and *Rhaebo guttatus* venom extracts spectrophotometrically determined at 540 nm and cytotoxic activity on peripheral blood mononuclear cells (PBMC) quantified by Alamar Blue assay.

Extract	EC_{50} (μg/mL)[a]	IC_{50} (μg/mL)[b]	Selectivity[c]
	Human erythrocytes	PBMC	
RMF-1	>200	0.8 (0.6–0.9)	80
RMF-2	>200	0.5 (0.3–0.7)	10
RMF-3	>200	0.4 (0.2–0.6)	40
RMF-4	>200	0.3 (0.2–0.4)	30
RMM-5	>200	1.1 (0.5–2.4)	15.6
RGF-6	33.7 (28.1–40.5)	0.8 (0.6–0.9)	0.3
RGF-7	30.8 (27.7–34.2)	16.0 (10.6–24.3)	3.5
RGF-8	20.8 (15.8–27.4)	13.1 (11.1–15.4)	3.6
RGM-9	27.9 (22.8–34.3)	13.9 (11.0–17.6)	2.8
Doxorubicin	Nd[d]	0.9 (0.5–1.8)	45

[a] Data are presented as EC_{50} values and 95% confidence interval for human erythrocytes after 1 h of incubation. Triton x-100 (1%) was used as positive control.

[b] Data are presented as IC_{50} values and 95% confidence interval for PBMC after 72 h exposure. Doxorubicin was used as positive control.

[c] Selectivity coefficient determined by IC_{50} in PBMC/IC_{50} in HL-60 cells (see Table 1). *Rhinella marina* female/male (RMF/RMM); *Rhaebo guttatus* female/male (RGF/RGM). Experiments were performed in triplicate.

[d] Not determined.

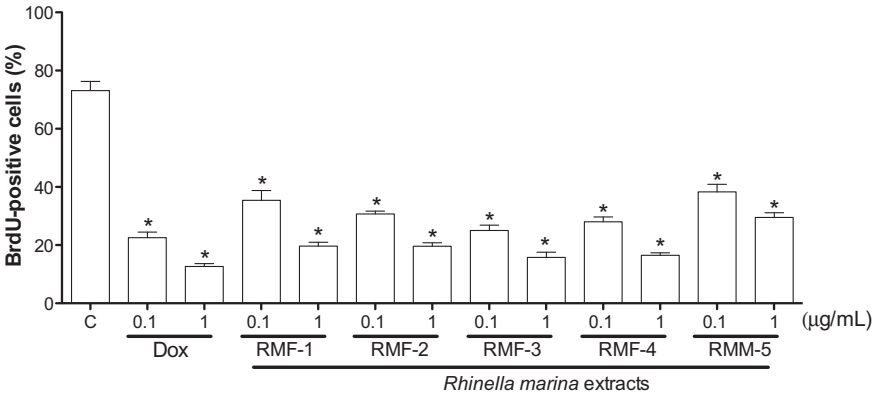

Fig. 3. Effects of *Rhinella marina* female/male (RMF/RMM) venom extracts on BrdU (5-bromo-2'deoxyuridine) incorporation by leukemia cells (HL-60) after 24 h of incubation in the concentrations of 0.1 and 1 μg/mL. Negative control (C) was treated with the vehicle used for diluting the tested substance. Doxorubicin was used as positive control (D). Results are expressed as mean ± standard error of measurement (S.E.M) from three independent experiments. $^{*}p < 0.05$ compared to control by ANOVA followed by Student Newman–Keuls test.

colon (26-L5, CT26.WT), leukemia (K562, U937, ML1), melanoma (MDA/MB-435, B16/F10, SKMEL-28), breast (MCF-7, MDA/MB-231), prostate (DU-145, PC-3, LNCaP), nervous system (Hs683, U373) and primary liver carcinoma (PLC/PRF/5) (Zhang et al., 1992; Nogawa et al., 2001; Ogasawara et al., 2001; Kamano et al., 2002; Yeh et al., 2003; Cunha-Filho et al., 2010; Sciani et al., 2012; Banuls et al., 2013). Hellebregenin, for example, is highly cytotoxic to HL-60 cells without causing DNA damage but inducing morphological changes characteristic of cell death by apoptosis (Cunha-Filho et al., 2010).

Previous studies have reported the cytotoxicity of the compounds identified in *R. marina* (**1**, **2**, **3**, and **4**) and *R. guttatus* (**2**) venoms. Bufalin (**3**) showed the most potent cytotoxic activity, followed by telocinobufagin (**1**), resibufogenin (**4**), and marinobufagin (**2**) against the following cancer cell lines: leukemia (HL-60), colon (HCT-116), glioblastoma (SF-295), ovarian (OVCAR-8), melanoma (MDA-MB435), human gastric (BGC-823), hepatoma (Bel-7402), cervical carcinoma (HeLa), and primary liver carcinoma (PLC/PRF/5) (Kamano et al., 1998; Ye et al., 2006; Cunha-Filho et al., 2010). The higher cytotoxic activity of venom extracts from *R. marina* in comparison with *R. guttatus* can be attributed to the presence of three other bufadienolides (**1**, **3**, and **4**) as well as marinobufagin (**2**), a bufadienolide identified only in *R. guttatus* venom. The above findings suggest synergistic effects due to the presence of different active principles contributing to the same activity (Wattenberg, 1985). Thus, it is proposed that compounds present in the extracts act together to kill neoplastic cells.

Regarding chemotherapeutic potential, it is important to determine if the antineoplastic substance shows harmful effects on normal cells (Anazetti et al., 2003; Santos et al., 2010). Accordingly, primary cultures of PBMC were prepared to assess this injurious potential of the extracts. Surprisingly, most of them were not cytotoxic to PBMC as seen as with transformed cells, where the extract RMF-1 was up to 80-fold more selective against leukemia cells when compared to dividing leukocytes, a very desired advantage in new anticancer leads to overcome adverse effects due to a narrow therapeutic window, multiple drug resistance and morphological and physiological similarities between transformed and normal cells. Meanwhile, Dox showed a selectivity coefficient of 45 determined by IC_{50} in PBMC/IC_{50} in HL-60.

R. marina extracts did not cause hemolysis even at the highest concentration tested, suggesting that the mechanism of cytotoxicity is probably related to a more specific pathway and is not associated with direct membrane damages. Corroborating these findings, Cunha-Filho et al. (2010) and Sciani et al. (2012) did not find hemolytic activity in amphibian skin secretions from *R. crucifer*, *R. marina*, *R. schneideri* and *R. major* at a concentration of 50 μg/mL, though secretions of *R. jimi*, *R. margaritifer* and *Phyllomedusa hypochondrialis* showed membrane disruption after 1 h incubation. Divergent results were seen with *R. guttatus* venom extracts, whereas all exhibited hemolytic potentiality, a contradictory finding when compared to that described by Sciani et al. (2012), who reported no membrane damage. It is likely that this difference should be correlated with range of concentrations used.

The antiproliferative effects of the extracts were investigated on the basis of the incorporation of BrdU, a thymidine analog, into DNA, which occurs during the S phase of the cell cycle. *R. marina* extracts caused inhibition of DNA synthesis in HL-60 leukemia as evidenced by the decrease in BrdU incorporation, corroborating outcomes achieved with MTT and Alamar Blue™ assays. In fact, investigations have demonstrated that some toad skin secretions possess compounds able to induce cell cycle arrest in G_2/M phase, decrease cell viability, activate initiator and effector caspases and provoke morphological alterations (chromatin condensation, nuclear fragmentation, cytoplasm retraction, cell detachment, membrane blebs and apoptotic bodies) in prostate and breast carcinomas (Yeh et al., 2003; Sciani et al., 2012). Since cardiotonic steroids of two chemical classes, cardenolides (ouabain, for example) and bufadienolides, bind specifically to the subunits of the sodium/potassium pump (Na^+/K^+-ATPase) (Newman et al., 2008; Gao et al., 2011), it is possible that the stimulation of apoptosis by bufadienolides is associated with this bioactivity.

In summary, nine extracts of *R. marina* and *R. guttatus* venoms showed pronounced lethal and discriminating effects in tumor lines, especially those from *R. marina*, highlighting toad parotoid gland secretions as a promising source of novel lead anticancer compounds. HPLC and LC–MS analysis of the extracts of *R. marina* and *R. guttatus* venom showed significant differences between them, where four bufadienolides (**1**, **2**, **3**, and **4**) were identified in different extracts from *R. marina* and only one (**2**) in *R. guttatus*.

Acknowledgments

We are grateful to the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Mato Grosso (FAPEMAT), Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) and Fundação de Amparo à Pesquisa do Estado do Piauí (FAPEPI) for financial support. The authors are indebted to Prof. Dr. M. L. dos Santos and Dr. G. A. Cunha-Filho (University of Brasília, Brazil) for providing the standards. Dr. A. Leyva (USA) helped with English editing of the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest and affirm that this paper consists of original and unpublished work.

References

Anazetti, M.C., Melo, P.S., Duran, N., Haun, M., 2003. Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leukemia cell line (HL-60) and human peripheral blood mononuclear cells. Toxicology 188, 261–274.

Banuls, L.M.Y., Urban, E., Gelbcke, M., Dufrasne, F., Kopp, B., Kiss, R., Zehl, M., 2013. Structure–activity relationship analysis of bufadienolide-induced in vitro growth inhibitory effects on mouse and human cancer cells. J. Nat. Prod. (in press).

Clardy, J., Walsh, C., 2004. Lessons from natural molecules. Nature 432, 829–837.

Costa, P.M., Ferreira, P.M.P., Bolzani, V.S., Furlan, M., Santos, V.A.F.F.M., Corsino, J., Moraes, M.O., Costa-Lotufo, L.V., Montenegro, R.C., Pessoa, C., 2008. Antiproliferative activity of pristimerin isolated from *Maytenus ilicifolia* (Celastraceae) in human HL-60 cells. Toxicol. In Vitro 22, 854–863.

Cragg, G.M., Newman, D.J., 2005. Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100, 72–79.

Cruz, J.S., Matsuda, H., 1993. Arenobufagin, a compound in toad venom, blocks Na^+–K^+ pump current in cardiac myocytes. Eur. J. Pharmacol. 239, 223–226.

Cunha-Filho, G.A., Resck, I.S., Cavalcanti, B.C., Pessoa, C.O., Moraes, M.O., Ferreira, J.R., Rodrigues, F.A., Dos Santos, M.L., 2010. Cytotoxic profile of natural and some modified bufadienolides from toad *Rhinella schneideri* parotoid gland secretion. Toxicon 56, 339–348.

Cunha-Flho, G.A., Schwartz, C.A., Resck, I.S., Murta, M.M., Lemos, S.S., Castro, M.S., Kyaw, C., Pires Jr., O.P., Leite, J.R.S., Bloch Jr., C., Schwartz, E.F., 2005. Antimicrobial activity of the bufadienolides marinobufagin and telocinobufagin isolated as major components from skin secretion of the *Bufo rubescens*. Toxicon 45, 777–782.

Dmitrieva, R.I., Bagrov, A.Y., Lalli, E., Sassone-Corsi, P., Stocco, D.M., Doris, P.A., 2000. Mammalian bufadienolides is synthesized from cholesterol in the adrenal cortex by a pathway that is independent of cholesterol side-chain cleavage. Hypertension 36, 442–448.

Ferreira, P.M.P., Santos, A.G., Tininis, A.G., Costa, P.M., Cavalheiro, A.J., Bolzani, V.S., Moraes, M.O., Costa-Lotufo, L.V., Montenegro, R.C., Pessoa, C., 2010. Casearin X exhibits cytotoxic effects in leukemia cells triggered by apoptosis. Chem. Biol. Interact. 188, 497–504.

Ferreira, P.M.P., Costa-Lotufo, L.V., Moraes, M.O., Barros, F.W.A., Martins, A.M.A., Cavalheiro, A.J., Bolzani, V.S., Santos, A.G., Pessoa, C., 2011a. Folk uses and pharmacological properties of *Casearia sylvestris*: a medicinal review. An. Acad. Bras. Cienc. 83, 1373–1384.

Ferreira, P.M.P., Farias, D.F., Viana, M.P., Souza, T.M., Vasconcelos, I.M., Soares, B.M., Pessoa, C., Costa-Lotufo, L.V., Moraes, M.O., Carvalho, A.F.F.U., 2011b. Study of the antiproliferative potential of seed extracts from Northeastern Brazilian plants. An. Acad. Bras. Cienc. 83, 1045–1058.

Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., de Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. Bull. Am. Mus. Nat. Hist. 297, 1–370.

Gao, H., Zehl, M., Leitner, A., Wu, X., Zhimin, W., Kopp, B., 2010. Comparison of toad venoms from different *Bufo* species by HPLC and LC-DAD-MS/MS. J. Ethnopharmacol. 131, 368–376.

Gao, H., Popescu, R., Kopp, B., Wang, Z., 2011. Bufadienolides and their antitumor activity. Nat. Prod. Rep. 28, 953–969.

Harris, A.L., 2002. Hypoxia – a key regulatory factor in tumour growth. Nat. Rev. Cancer 2, 38–47.

Imai, S., Murase, H., Katori, M., Okada, M., Shigei, T., 1965. A study on the structure–activity relationship of the cardiotonic steroids. Jpn. J. Pharmacol. 15, 62–71.

Kamano, Y., Satoh, N., Nakayoshi, H., Pettit, G.R., Smith, C.R., 1988. Rhinovirus inhibition by bufadienolides. Chem. Pharm. Bull. 36, 326–332.

Kamano, Y., Kotake, A., Hashima, H., Inoue, M., Morita, H., Takeya, K., Itokawa, H., Nandachi, N., Segawa, T., Yukita, A., Saitou, K., Katsuyama, M., Pettit, G.R., 1998. Structure-cytotoxic activity relationship for the toad poison bufadienolides. Bioorg. Med. Chem. 6, 1103–1115.

Kamano, Y., Yamashita, A., Nogawa, T., Morita, H., Takeya, K., Itokawa, H., Segawa, T., Yukita, A., Saito, K., Katsuyama, M., Pettit, G.R., 2002. QSAR evaluation of the Chan'Su and related bufadienolides against the colchicine-resistant primary liver carcinoma cell line PLC/PRF/5. J. Med. Chem. 45, 5440–5447.

Lee, D.Y., Yasuda, M., Yamamoto, T., Yoshida, T., Kuroiwa, Y., 1997. Bufalin inhibits endothelial cell proliferation and angiogenesis in vitro. Life Sci. 60, 127–134.

Militão, G.C.G., Dantas, I.N.F., Ferreira, P.M.P., Alves, A.P.N.N., Chaves, D.C., Monte, F.J.Q., Pessoa, C., Moraes, M.O., Costa-Lotufo, L.V., 2012. *In vitro* and *in vivo* anticancer properties of cucurbitacin isolated from *Cayaponia racemosa*. Pharm. Biol. 50, 1479–1487.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 16, 55–63.

Nasu, K., Nishida, M., Ueda, T., Takai, N., Bing, S., Narahara, H., Miyakawa, I., 2005. Bufalin induces apoptosis and the G_0/G_1 cell cycle arrest of endometriotic stromal cells: a promising agent for the treatment of endometriosis. Mol. Hum. Reprod. 11, 817–823.

Newman, R.A., Yang, P., Pawlus, A.D., Block, K.I., 2008. Cardiac glycosides as novel cancer therapeutic agents. Mol. Interv. 8, 36–49.

Nogawa, T., Kamano, Y., Yamashita, A., Pettit, G.R., 2001. Isolation and structure of five new cancer cell growth inhibitory bufadienolides from the Chinese traditional drug Ch'an Su. J. Nat. Prod. 64, 1148–1152.

Ogasawara, M., Matsubara, T., Suzuki, H., 2001. Screening of natural compounds for inhibitory activity on colon cancer cell migration. Biol. Pharm. Bull. 24, 720–723.

Pera, F., Mattias, P., Detzer, K., 1977. Methods for determining the proliferation kinetics of cells by means of 5-bromodeoxyuridine. Cell Tissue Kinet. 10, 255–264.

Pramuk, J.B., 2006. Phylogeny of South American Bufo (Anura: Bufonidae) inferred from combined evidence. Zool. J. Linn. Soc. 146, 407–452.

Rocha, A.B., Lopes, R.M., Schwartsmann, G., 2001. Natural products in anticancer therapy. Curr. Opin. Pharmacol. 1, 364–369.

Santos, A.G., Ferreira, P.M.P., Vieira-Júnior, G.M., Perez, C.C., Tininis, A.G., Silva, G.H., Bolzani, V.S., Costa-Lotufo, L.V., Pessoa, C., Cavalheiro, A.J., 2010. Casearin X, its degradation product and other clerodane diterpenes from leaves of *Casearia sylvestris*: evaluation of cytotoxicity against normal and tumour human cells. Chem. Biodivers. 7, 205–215.

Schoner, W., Scheiner-Bobis, G., 2005. Endogenous cardiac glycosides: hormones using the sodium pump as signal transducer. Semin Nephrol 25, 343–351.

Sciani, J.M., de-Sá-Júnior, P.L., Ferreira, A.K., Pereira, A., Antoniazzi, M.M., Jared, C., Pimenta, D.C., 2012. Cytotoxic and antiproliferative effects of crude amphibian skin secretions on breast tumor cells. Biomed. Prev. Nutr. 1, 10–18.

Srivastava, V., Negi, A.S., Kumar, J.K., Gupta, M., Khanuja, S.P.S., 2005. Plant-based anticancer molecules: a chemical and biological profile of some important leads. Bioorg. Med. Chem. 13, 5892–5908.

Suffness, M., Pezzuto, J.M., 1990. *Assays* related to cancer drug discovery. In: Hostettamann, K. (Ed.), Methods in Plant Biochemistry: Assays for Bioactivity. Academic Press, London, pp. 71–133.

Supratman, U., Fugita, T., Akiyama, K., Hayashi, H., 2000. New insecticidal bufadienolide, bryophyllin C, from *Kalanchoe pinnata*. Biosci. Biotechnol. Biochem. 64, 1310–1312.

Tempone, A.G., Pimenta, D.C., Lebrun, I., Sartorelli, P., Taniwaki, N.N., de Andrade Jr., H.F., Antoniazzi, M.M., Jared, C., 2008. Antileishmanial and antitrypanosomal activity of bufadienolides isolated from the toad *Rhinella jimi* parotoid macrogland secretion. Toxicon 52, 13–21.

Terness, P., Navolan, D., Dufter, C., Kopp, B., Opelz, G., 2001. The T-cell suppressive effect of bufadienolides: structural requirements for their immunoregulatory activity. Int. Immunopharmacol. 1, 119–134.

Toledo, R.R., Jared, R., 1995. Cutaneous granular glands and amphibian venoms. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 111, 1–29.

Vieira Júnior, G.M., Dutra, L.A., Ferreira, P.M.P., Moraes, M.O., Costa-Lotufo, L.V., Pessoa, C., Torres, R.B., Boralle, N., Bolzani, V.S., Cavalheiro, A.J., 2011. Cytotoxic clerodane diterpenes from *Casearia rupestris*. J. Nat. Prod. 74, 776–781.

Vu, H., Ianosi-Irimie, M., Danchuk, S., Rabon, E., Nogawa, T., Kamano, Y., Pettit, G.R., Wiese, T., Puschett, J.B., 2006. Resibufogenin corrects hypertension in a rat model of human preeclampsia. Exp. Biol. Med. 231, 215–220.

Wang, D.L., Qi, F.H., Tang, W., Wang, F.S., 2011. Chemical constituents and bioactivities of the skin of *Bufo bufo gargarizans* Cantor. Chem. Biodivers. 8, 559–567.

Wattenberg, L.W., 1985. Chemoprevention of cancer. Cancer Res. 45, 1–8.

Xu-Tao, C., Dong, W., Na, W., Zheng, C., 2009. Water-soluble constitutions from the skin of *Bufo bufo gargarizans* Cantor. Chin. J. Nat. Med. 7, 181–183.

Yang, J., Zhang, Y.H., Miao, F., Zhou, L., Sun, W., 2010. Two new bufadienolides from the rhizomes of *Helleborus thibetanus* Franch. Fitoterapia 81, 636–639.

Ye, M., Guo, H., Guo, H., Han, J., Guo, D., 2006. Simultaneous determination of cytotoxic bufadienolides in the Chinese medicine ChanSu by high-performance liquid chromatography coupled with photodiode array and mass spectrometry detections. J. Chromatogr. B 838, 86–95.

Yeh, J.Y., Huang, W.J., Kan, S.F., Wang, P.S., 2003. Effects of bufalin and cinobufagin on the proliferation of androgen dependent and independent prostate cancer cells. Prostate 54, 112–124.

Zhang, L.S., Nakaya, K., Yoshida, T., Kuroiwa, Y., 1992. Induction by bufalin of differentiation of human leukemia cells HL60, U937, and ML1 toward macrophage/monocyte-like cells and its potent synergistic effect on the differentiation of human leukemia cells in combination with other inducers. Cancer Res. 52, 463–4641.