Genetics and Molecular Biology, 37, 2, 428-438 (2014)

www.sbg.org.br

Research Article

Evaluation of the mutagenicity and antimutagenicity of *Ziziphus joazeiro* Mart. bark in the micronucleus assay

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Abstract

The aim of this study was to evaluate the mutagenicity (clastogenicity/aneugenicity) of a glycolic extract of *Ziziphus joazeiro* bark (GEZJ) by the micronucleus assay in mice bone marrow. Antimutagenic activity was also assessed using treatments associated with GEZJ and doxorubicin (DXR). Mice were evaluated 24-48 h after exposure to positive (N-nitroso-N-ethylurea, NEU - 50 mg.kg^{-1} and DXR - 5 mg.kg^{-1}) and negative (150 mM NaCl) controls, as well as treatment with GEZJ (0.5-2 g.kg^{-1}), GEZJ (2 g.kg^{-1}) + NEU and GEZJ (2 g.kg^{-1}) + DXR. There were no significant differences in the frequencies of micronucleated polychromatic erythrocytes in mice treated with GEJZ and GEJZ + DXR compared to the negative controls, indicating that GEZJ was not mutagenic. Analysis of the polychromatic:normochromatic erythrocyte ratio revealed significant differences in the responses to doses of 0.5 g.kg^{-1} and 1-2 g.kg^{-1} and the positive control (NEU). These results indicated no systemic toxicity and moderate toxicity at lower and higher doses of GEZJ. The lack of mutagenicity and systemic toxicity in the antimutagenic assays, especially for treatment with GEZJ + DXR, suggested that phytochemical compounds in *Z. joazeiro* bark attenuated DXR-induced mutagenicity and the moderate systemic toxicity of a high dose of *Z. joazeiro* bark (2 g.kg^{-1}). Further studies on the genotoxicity of *Z. joazeiro* extracts are necessary to establish the possible health risk in humans and to determine the potential as a chemopreventive agent for therapeutic use.

Keywords: antimutagenicity, bone marrow, doxorubicin, micronucleus assay, mutagenicity, *Zizyphus joazeiro* Mart. (raspa-de-Juá).

Received: October 3, 2013; Accepted: March 17, 2014.

Introduction

Many species of medicinal plants, such as *Amburana cearensis*, *Anadenanthera colubrina*, *Mentha x villosa*, *Myracrodruon urundeuva*, *Plectranthus amboinicus*, *Ruta graveolens*, *Ximenia americana* and *Ziziphus joazeiro*, are widely used by communities in the Brazilian Caatinga to treat a large spectrum of clinical conditions ranging from diseases requiring palliative care to general aches, *e.g.*, bronchitis, sinusitis, rhinitis, nasal congestion, headaches, flu, fever, expectorant, colic, hypertension, thrombosis, indigestion, intestinal dysfunction, liver and kidney problems, infectious and inflammatory processes and pain in general (Cartaxo *et al.*, 2010). *Ziziphus joazeiro* Mart. (Rhamnaceae) is a native Brazilian tree resistant to dry environments (Cartaxo *et al.*, 2010). This species is an important source of water and food for animals in arid habitats (Braga, 1960; Cruz, 1985; Nunes *et al.*, 1987).

A phytochemical analysis of *Z. joazeiro* Mart. has shown that the leaf epicuticular wax is rich in *n*-alkanes (78.6%), very efficient compounds for impermeabilizing the leaf surface, and triterpenoids (Oliveira *et al.*, 2003). A similar analysis of a dichloromethane extract of *Z. joazeiro* Mart. bark identified triterpenoids with weak antibacterial activity (*e.g.*, betulinic, alphitolic and ursolic acids) and re-

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markable activity against *Staphylococcus epidermidis* [*e.g.*, betulinic acid ester derivatives such as 7β-(4-hydroxy-benzoyloxy), 7β-(4-hydro-3-methoxy-benzoyloxy) and 27-(4-hydroxy-3-methoxy-benzoyloxy)] (Schuhly *et al.*, 1999). *Ziziphus joazeiro* Mart. bark also contains an abundance of saponins that have been used as toothpastes, with aqueous extracts showing antimicrobial action against bacteria (planktonic cells and artificial biofilms) related to dental caries and periodontal diseases (Alviano *et al.*, 2008). Other popular therapeutic applications of *Z. joazeiro* Mart. include the treatment of dandruff, rheumatism, flu, fever, chronic bronchitis, gastric ulcers, indigestion, heartburn and headaches (Schuhly *et al.*, 1999; Cartaxo *et al.*, 2010). In addition, experimental studies have identified potential antifungal (Cruz *et al.*, 2007), antibacterial (Schuhly *et al*., 1999; Alviano *et al.*, 2008; Leal *et al.*, 2010), antioxidant (Alviano *et al.*, 2008) and antipyretic (Nunes *et al.*, 1987) activities, as well as low toxicity (Alviano *et al.*, 2008).

Biologically active compounds have been recognized for their pharmacological properties, but many of them are of limited therapeutic use because of their toxicological, carcinogenic and mutagenic properties (Ames, 1983; Konstantoupoulou *et al.*, 1992; Tavares, 1996). The analysis of genotoxicity is a major aspect of drug development since most pharmaceutical companies evaluate the potential of a new therapeutic agent based on its genotoxicity *in vitro* and *in vivo* (Purves *et al.*, 1995). In this context, the screening of popularly used plants and their isolated components for mutagenic activity is necessary and important for establishing adequate control measures. This screening can also provide insights into the mechanisms involved in the biological effects of plants used as therapeutic agents (Varanda, 2006).

As far as genotoxicity studies are concerned, the *in vivo* micronucleus (MN) assay in rodent bone marrow is a crucial part of the battery of tests used to identify hazardous mutagens (Mateuca *et al.*, 2006). This assay is especially suited for assessing mutagenic hazards because it contemplates various factors, such as *in vivo* metabolism, pharmacokinetics and DNA repair mechanisms, even though these processes vary among species and tissues and have different genetic endpoints (OECD, 1997a,b; Ribeiro *et al.*, 2003). Since bone marrow erythroblasts develop into polychromatic erythrocytes (PCEs), *i.e.*, cells generated by extrusion of the main nucleus, micronuclei may remain in an otherwise anucleated cytoplasm. Consequently, the frequency of micronucleated polychromatic erythrocytes (MNPCEs) has been the principal endpoint for MN assays. The measurement of MNPCEs in peripheral blood is possible in any species in which the spleen does not remove micronucleated erythrocytes, or that is sufficiently sensitive to agents that cause structural or numerical chromosomal aberrations. An increase in the frequency of MNPCEs in treated animals, *i.e.*, a positive result, indicates that a substance can cause the formation of micronuclei through chromosomal damage or damage to the mitotic apparatus of erythroblasts. On the other hand, a negative result implies that the test substance does not cause micronucleus formation in immature erythrocytes. The number of normochromatic erythrocytes (NCEs) in peripheral blood that contain micronuclei for a given number of mature erythrocytes can also be used as the endpoint of this assay (OECD, 1997c; Ribeiro *et al.*, 2003). Several studies have used the mammalian *in vivo* MN assay to understand the mutagenic effects induced by phytotherapeutics and foods (Indart *et al.*, 2007; Venkatesh *et al.*, 2007; Chandrasekaran *et al.*, 2011; Silva *et al*., 2011; Alves *et al.*, 2012).

Although several studies have examined the potential therapeutic effectiveness of *Z. joazeiro* Mart., there has been no systematic investigation of the genotoxic and mutagenic effects of this plant. In this work, we examined the mutagenic effects of a glycolic extract of *Z. joazeiro* Mart. bark as part of a wider study on the genotoxic potential of herbal extracts. The effect of the maximum permissible concentration of *Z. joazeiro* Mart. on the mutagenicity of doxorubicin (DXR) in mouse bone marrow, *i.e.*, its antimutagenic activity, was also examined.

Material and Methods

Raw material and sample preparation

A glycolic extract of *Z. joazeiro* bark (GEZJ) was purchased commercially and stored according to the manufacturer's recommendations (AKSY Comercial Ltda., São Bernardo do Campo, SP, Brazil). Aliquots (1.5 L) of this extract were submitted to solvent removal proceedings by rotary evaporation (40 rpm) (Rotavapor model R-215) coupled to a bath heating system maintained at 50-60 °C (Bath Heating model B-491), a vacuum pump (vacuum of 500 mm Hg; Vacuum Pump V-700 with Automatic Vacuum Controller V-855), a water recirculator (Recirculator Chiller F-100) and an evaporation bottle (Büchi Labortechnik AG, Switzerland). The final product was transferred to a 1 L reaction bottle (SCHOTT® DURAN®) and kept at -20 °C for 24 h in order to evaluate the freezing of the final product and the efficacy of solvent evaporation (Agência Nacional de Vigilância Sanitária (ANVISA) (2010)). Aliquots (40 mL) of this final product were transferred to penicillin-type glass vials (50 mL) and lyophilized (Lyophilizer model Alpha 1-2 LDPlus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) and the dry mass were measured (Electronic Analytical Balance AUW-220D, Shimadzu Corp., Kyoto, Japan). Aqueous solutions of the lyophilized product were prepared in type 1 water at twice the final concentration, sterilized by filtration (Millipore Corporation, hydrophilic Durapore® PVDF, 0.22 μm, ± 47 mm, cat. no. GVWP 047 00) and stored in sterile polypropylene tubes (50 mL) at -70 °C until used.

In vivo assays

Healthy, heterogeneous, young adult male and female Swiss mice (Unib:SW) 7-12 weeks old (pubescent period) weighing 30-40 g (weight variation among mice of each sex was < 20% of the mean weight) were provided by CEMIB (Centro Multidisciplinar para Investigação Biológica - UNICAMP) and erythrocytes from the bone marrow of these mice were used in the MN assay (Collaborative Study Group for the Micronucleus Test (CSGMT), 1986; Chorilli *et al.*, 2007).

Animals of the same sex were housed in polypropylene boxes in an air-conditioned environment to 22 ± 3 °C, with a relative air humidity of 50% ± 20% and a 12 h light/dark cycle. The mice were fed commercial rodent chow (Purina® Labina, Nestlé Purina Pet Care Company) and water *ad libitum*, and were acclimated to laboratory conditions for seven days prior to use in the experiments. At the end of this period, each mouse was weighed and then received 2 mL of liquid (containing the desired test agent) per 100 g body weight.

All animals were properly identified by numerical markings on their tails to ensure continuity of the records and reliable interpretation of the results throughout the study (OECD, 1997c). After the period of treatment, the mice were euthanized by inhalation of carbon dioxide in adapted acrylic chambers as described in the Report of the American Veterinary Medical Association panel on euthanasia (Beaver *et al.*, 2000). This study was done in accordance with the Universal Declaration of Animal Rights (UNESCO, 1978), the ethical principles for animal experimentation established by the Brazilian Society of Laboratory Animal Science (SBCAL - *Sociedade Brasileira de Ciência em Animais de Laboratório*), the Brazilian Environmental Crimes Law (Law no. 9.605, February 12, 1998), the Brazilian standards for Didactic-Scientific Practice of Vivisection of Animals (Law no. 6.638, May 8, 1979), and was approved by the Committee for Ethics in Research Involving Animals at UNIFENAS (CEPEAU Protocol no. 04A/2008).

Experimental groups

The experimental groups of mice (3 males and 3 females each) were assessed 24 h and 48 h after a single treatment administered by gavage (Figure 1). The mutagenic activity of GEZJ was assessed in mice that received doses of 0.5-2 g.kg^{-1} (groups 7-14) and the antimutagenic activity was assessed in mice that received NEU (50 mg.kg^{-1}) + GEZJ (2 g.kg^{-1}) (groups 15 and 16) and DXR (5 mg.kg^{-1}) + GEZJ (2 g.kg^{-1}) (groups 17 and 18). The doses of GEZJ were chosen based on previous acute toxicity experiments in mice that yielded LD_{50} values of 2.0-3.5 g/kg for several plant extracts, including *Z. joazeiro* (Alviano *et al.*, 2008). Negative controls (groups 1 and 2: 150 mM NaCl in type 1 water) and positive controls (groups 3 and 4: 50 mg.kg^{-1} of NEU; groups 5 and 6: 5 mg.kg^{-1} of DXR) were also included as single treatments administered by gavage (NaCl) and intraperitoneally (NEU and DXR) (OECD, 1997c).

Processing of bone marrow

MN assays using bone marrow erythrocytes were done 24 h and 48 h after treatment, using previously described methodology (Schmid, 1976; Zambrano *et al.*,

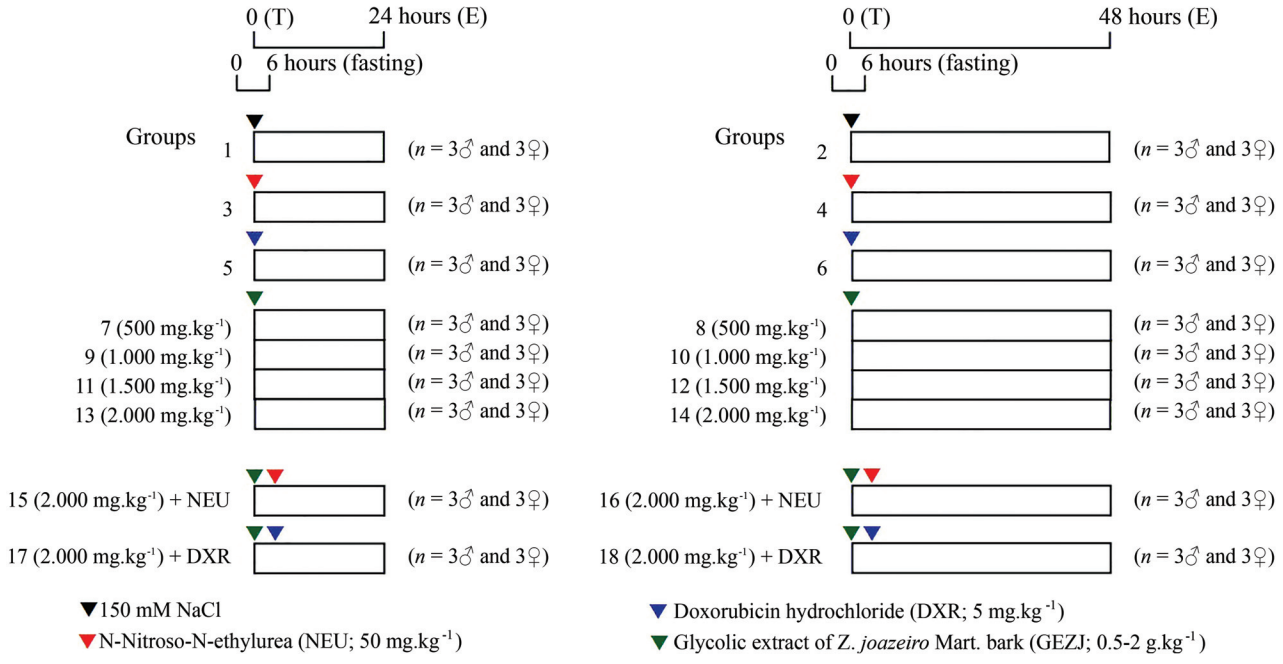

Figure 1 - Experimental protocol for assessing the mutagenic and antimutagenic activity of a glycolic extract of *Z. joazeiro* bark. T - treatment, E - euthanasia and *n* - number of mice.

1982). Shortly after euthanasia, the femora were surgically and aseptically removed and the mice were appropriately discarded. Each femur was sectioned at the proximal end and the contents of the spinal canal were washed with 1.5 mL of 150 mM NaCl and transferred to a 15 mL centrifuge tube. This material was resuspended with a Pasteur pipette to ensure a homogenous distribution of bone marrow cells. The suspension was then centrifuged at 1,000 rpm (Bench centrifuge, model NT 810, Nova Técnica Ind. e Com. de Equip. para Laboratório Ltda., Piracicaba, SP, Brazil) for 5 min. The supernatant was discarded and the resulting pellet was resuspended in 500 μL of 150 mM NaCI solution added 4% formaldehyde. The slides (two per animal) were prepared by smearing, dried at room temperature for 24 h and stained with Leishman's eosin methylene blue dye [pure dye for 3 min followed by diluted dye in distilled water (1:6) for 15 min] to differentiate polychromatic erythrocytes (PCEs) from monochromatic erythrocytes (NCEs).

PCEs were observed by light microscopy (Nikon Eclipse E-200 microscope) at a magnification of 1000x, counted (at least 2000 anucleated polychromatic erythrocytes per animal) with the aid of a digital cell counter (Contador Diferencial CCS02, Kacil Indústria e Comércio Ltda., PE, Brazil) and photographed using an 8.1 Megapixel Digital Camera (DC FWL 150). The number of PCEs, the number and frequency of MNPCEs and the ratio of polychromatic to monochromatic erythrocytes (PCE/NCE) were determined.

Statistical analysis

The data from the MN assay were analyzed by one-way analysis of variance (ANOVA) using a 9 x 2 x 2 (treatment x gender x time) factorial scheme followed by multiple comparisons with the Tukey test ($\alpha = 0.05$). All analyses were done using SAS® version 9.2 computer software.

Results and Discussion

Ziziphus joazeiro Mart. has been popularly used to treat dandruff, rheumatism, flu, fever, chronic bronchitis, gastric ulcers, indigestion, heartburn and headaches and to clean teeth (Schuhly *et al.*, 1999; Cartaxo *et al.*, 2010). In addition, Z. joazeiro has potential antifungal (Cruz *et al.*, 2007), antibacterial (Schuhly *et al.*, 1999; Alviano *et al.*, 2008; Leal *et al.*, 2010), antioxidant (Alviano *et al.*, 2008) and antipyretic (Nunes *et al.*, 1987) activities, as well as low toxicity (Alviano *et al.*, 2008). This information partly supports the popular use of Z. joazeiro for certain treatments and agrees with ethnopharmacological studies designed to select plants for bioactivity screening (Cruz *et al.*, 2007). In contrast, few studies have examined the mutagenic and antimutagenic effects of *Z. joazeiro* Mart.

In the present study, the number and frequency of MNPCEs and the PCE/NCE ratios in mouse bone marrow were analyzed in mutagenic and antimutagenic assays of a glycolic extract of *Z. joazeiro* bark (Table 1 and Figure 2). Analysis of the MNPCEs revealed no significant differences between the 24 h and 48 h results for the negative (NaCl) and positive (DXR and NEU) controls. However, there were significant differences ($p < 0.05$) between the negative and positive controls at the two time intervals. There were no differences between the negative controls and the treatments with GEJZ (0.5-2 $g.kg^{-1}$) or with GEJZ (2 $g.kg^{-1}$) + DXR (5 $mg.kg^{-1}$): these responses showed no dose or time dependence, but varied between male and female mice. Mice treated with GEJZ (2 $g.kg^{-1}$) + NEU (50 $mg.kg^{-1}$) had intermediate values (*n* and %) that differed significantly from the negative and positive controls. These results suggest absence of mutagenicity (clastogenicity and/or aneugenicity) for GEZJ, regardless of the extract dose and time interval, although the responses varied between sexes. In contrast, GEJZ (2 $g.kg^{-1}$) showed antimutagenic activity (anticlastogeny and/or antianeugeny) towards the chemotherapeutic agent DXR (5 $mg.kg^{-1}$) or NEU (50 $mg.kg^{-1}$), regardless of the time interval, although once again intersex variation was observed. These findings indicate that compounds in GEZJ can act against DXR-induced mutagenic effects in mouse bone marrow. Such compounds could include *n*-alkanes, triterpenoids [*i.e.*, betulinic acid, alphitolic acid, ursolic acid, ester derivatives of betulinic acid such as 7β-(4-hydroxy-benzoyloxy)-betulinic acid, 7β-(4-hydro-3-methoxy-benzoyloxy)-betulinic acid and 27-(4-hydroxy-3-methoxy-benzoyloxy)-betulinic acid] (Oliveira *et al.*, 2003; Schuhly *et al.*, 1999) and saponins (Alviano *et al.*, 2008). DXR has been reported to induce micronuclei, chromatid and chromosomal aberrations, and DNA single- and double-strand breaks *in vitro* and *in vivo* (Bean *et al.*, 1992; Al-Harbi, 1993; Al-Shabanah, 1993; Delvaeye *et al.*, 1993; Jagetia and Nayak, 1996, 2000; Shan *et al.*, 1996; Dhawan *et al.*, 2003; Jagetia and Aruna, 2000). In addition, the major acute toxicity induced by DXR is bone marrow suppression, while the long-term clinical usefulness is limited by a cumulative, dose-dependent, irreversible, chronic cardiotoxicity that manifests itself as congestive heart failure or cardiomyopathy (Van Acker *et al.*, 1995, 2000).

For the PCE/NCE ratio, there were no significant differences between the negative controls (NaCl), the positive control DXR (5 $mg.kg^{-1}$), the GEZJ (0.5 $mg.kg^{-1}$) group, and mice treated with GEZJ (2 $g.kg^{-1}$) + NEU (50 $mg.kg^{-1}$) or with GEZJ (2 $g.kg^{-1}$) + DXR (5 $mg.kg^{-1}$) (Table 1 and Figure 1). For the treatments with GEZJ, there was a significant difference between the dose of 500 $mg.kg^{-1}$ and the doses of 1.5 $g.kg^{-1}$ and 2 $g.kg^{-1}$. Although there were no significant intersex differences, the responses did vary with time (24 h *vs.* 48 h). Lower doses of GEZJ (0.5-1 $g.kg^{-1}$) were not toxic to bone marrow compared to higher doses

Table 1 - MNPCE frequencies and PCE/NCE ratios in mouse bone marrow in mutagenic and antimutagenic assays of *Z. joazeiro* bark.

Treatment	Number of PCEs analyzed		MNPCEs *				PCE / (PCE + NCE) **		NCE (*n*)	
	24 h	48 h	24 h (*n*) ^{A}	48 h (*n*) ^{A}	24 h (%) $^{A'}$	48 h (%) $^{A'}$	24 h $^{A''}$	48 h $^{B''}$	24 h	48 h
150 mM NaCl										
F_1	2095	2097	7	10	0.33	0.48	1.00	1.00	5	3
F_2	2094	2095	9	10	0.43	0.48	1.00	1.00	6	5
F_3	2087	2089	11	8	0.53	0.38	0.99	0.99	13	11
Σ F	Σ 6276	Σ 6281	Σ 27	Σ 28	0.43 ± 0.10	0.45 ± 0.05	1.00 ± 0.00	1.00 ± 0.00	Σ 24	Σ 19
M_1	2095	2088	9	13	0.43	0.62	1.00	0.99	5	12
M_2	2055	2088	12	11	0.58	0.53	0.98	0.99	45	12
M_3	2058	2084	7	11	0.34	0.53	0.98	0.99	42	16
Σ M	Σ 6208	Σ 6260	Σ 28	Σ 35	0.45 ± 0.12	0.56 ± 0.06	0.99 ± 0.01	0.99 ± 0.00	Σ 92	Σ 40
Σ	Σ 12484	Σ 12541	Σ 55 ^{A}	Σ 63 ^{A}	0.44 ± 0.08 $^{A'}$	0.50 ± 0.06 $^{A'}$	0.99 ± 0.01 $^{A''}$	1.00 ± 0.00 $^{A''}$	Σ 116	Σ 59
N-Nitroso-N-ethylurea (NEU, 50 mg.kg^{-1})										
F_1	2148	2075	38	36	1.77	1.73	0.49	0.65	2252	1125
F_2	1884	2032	32	34	1.70	1.67	0.54	0.81	1616	468
F_3	2002	1948	15	31	0.75	1.59	0.61	0.93	1298	152
Σ F	Σ 6034	Σ 6055	Σ 85	Σ 101	1.41 ± 0.57	1.67 ± 0.07	0.54 ± 0.06	0.80 ± 0.14	Σ 5166	Σ 1745
M_1	2025	1999	64	31	3.16	1.55	0.41	0.36	2875	3501
M_2	2028	1916	105	40	5.18	2.09	0.51	0.55	1972	1584
M_3	2004	2069	25	38	1.25	1.84	0.67	0.65	996	1131
Σ M	Σ 6057	Σ 5984	Σ 194	Σ 109	3.20 ± 1.97	1.83 ± 0.27	0.53 ± 0.13	0.52 ± 0.14	Σ 5843	Σ 6216
Σ M and F	Σ 12091	Σ 12039	Σ 279 ^{C}	Σ 210 ^{C}	2.30 ± 1.66 $^{C'}$	1.75 ± 0.18 $^{C'}$	0.54 ± 0.06 $^{D''}$	0.66 ± 0.16 $^{D''}$	Σ 11009	Σ 7961
Doxorubicin hydrochloride (DXR, 5 mg.kg^{-1})										
F_1	2091	2017	49	36	2.34	1.78	0.72	0.96	809	83
F_2	2106	2077	73	63	3.47	3.03	0.98	0.99	44	23
F_3	2056	2092	57	50	2.77	2.39	0.84	0.95	394	108
Σ F	Σ 6253	Σ 6186	Σ 179	Σ 149	2.86 ± 0.57	2.40 ± 0.62	0.85 ± 0.13	0.97 ± 0.02	1247	214
M_1	2067	2086	53	61	2.56	2.92	0.98	0.95	33	114
M_2	2063	2042	56	70	2.71	3.43	0.98	0.97	37	58
M_3	2082	2075	46	50	2.21	2.41	0.99	0.99	18	25
Σ M	Σ 6212	Σ 6203	Σ 155	Σ 181	2.50 ± 0.26	2.92 ± 0.51	0.99 ± 0.00	0.97 ± 0.02	88	197
Σ M and F	Σ 12465	Σ 12389	Σ 334 ^{D}	330 ^{D}	2.68 ± 0.42 $^{D'}$	2.66 ± 0.43 $^{D'}$	0.92 ± 0.07 $^{AB''}$	0.97 ± 0.01 $^{AB''}$	1335	411
Glycolic extract of *Z. joazeiro* Mart. bark (0.5 mg.kg^{-1})										
F_1	2051	2085	8	8	0.39	0.38	0.77	0.95	620	115

Table 1 (cont.)

Treatment	Number of PCEs analyzed		MNPCEs *				PCE / (PCE + NCE) **		NCE (*n*)	
	24 h	48 h	24 h (*n*) ^{A}	48 h (*n*) ^{A}	24 h (%) $^{A'}$	48 h (%) $^{A'}$	24 h $^{A''}$	48 h $^{B''}$	24 h	48 h
F_2	2082	2035	4	8	0.19	0.39	0.94	0.97	129	65
F_3	2083	2005	11	2	0.53	0.10	0.94	0.95	134	95
Σ F $^{A* A**}$	Σ 6216	Σ 6125	Σ 23	Σ 18	0.37 ± 0.17	0.29 ± 0.17	0.88 ± 0.10	0.96 ± 0.01	Σ 883	Σ 275
M_1	2076	2060	9	7	0.43	0.34	0.99	0.98	24	40
M_2	2002	2046	7	7	0.35	0.34	0.95	0.97	98	54
M_3	2038	2071	7	17	0.34	0.82	0.97	0.99	62	29
Σ M $^{B* A**}$	Σ 6116	Σ 6177	Σ 23	Σ 31	0.38 ± 0.05	0.50 ± 0.28	0.97 ± 0.02	0.98 ± 0.01	Σ 184	Σ 123
Σ M and F	Σ 12332	Σ 12302	Σ 46 ^{A}	Σ 49 ^{A}	0.37 ± 0.00 $^{A'}$	0.40 ± 0.15 $^{A'}$	0.92 ± 0.07 $^{AB''}$	0.97 ± 0.02 $^{AB''}$	Σ 1067	Σ 398
Glycolic extract of *Z. joazeiro* Mart. bark (1 g.kg^{-1})										
F_1	2062	2047	9	10	0.44	0.49	0.80	0.89	531	253
F_2	2044	2053	10	9	0.49	0.44	0.91	0.93	190	147
F_3	2023	2067	11	8	0.54	0.39	0.86	0.94	337	138
Σ F $^{A* A**}$	Σ 6129	Σ 6167	Σ 30	Σ 27	0.49 ± 0.05	0.44 ± 0.05	0.86 ± 0.06	0.92 ± 0.03	Σ 1058	Σ 538
M_1	2073	2014	15	12	0.72	0.60	0 81	0 92	489	175
M_2	2056	2032	3	13	0.15	0.64	0 98	0 95	37	110
M_3	2101	2075	20	16	0.95	0.77	0 84	0 97	389	68
Σ M $^{B* A**}$	Σ 6230	Σ 6121	Σ 38	Σ 41	0.61 ± 0.42	0.67 ± 0.09	0 88 ± 0.09	0 95 ± 0.02	Σ 915	Σ 353
Σ M and F	Σ 12359	Σ 12288	Σ 68 ^{A}	Σ 68 ^{A}	0.55 ± 0.09 $^{A'}$	0.55 ± 0.16 $^{A'}$	0.86 ± 0.01 $^{BC''}$	0.93 ± 0.02 $^{BC''}$	Σ 1973	Σ 891
Glycolic extract of *Z. joazeiro* Mart. bark (1.5 mg.kg^{-1})										
F_1	2082	2041	21	16	1.01	0.78	0.80	0.84	518	401
F_2	2134	2107	18	15	0.84	0.71	0.89	0.80	266	528
F_3	2095	2075	16	14	0.76	0.67	0.81	0.91	505	195
Σ F $^{A* A**}$	Σ 6311	Σ 6223	Σ 55	Σ 45	0.87 ± 0.12	0.72 ± 0.06	0.83 ± 0.05	0.85 ± 0.06	Σ 1289	Σ 1124
M_1	2045	2075	12	27	0.59	1.30	0.82	0.96	455	84
M_2	2125	2048	14	16	0.66	0.78	0.86	0.92	352	173
M_3	2108	2022	16	8	0.76	0.40	0.85	0.69	374	928
Σ M $^{B* A**}$	Σ 6278	Σ 6145	Σ 42	Σ 51	0.67 0.09	0.83 0.45	0.84 0.02	0.84 0.15	Σ 1181	Σ 1185
Σ M and F	Σ 12589	Σ 12368	Σ 97 ^{AB}	Σ 96 ^{AB}	0.77 ± 0.14 $^{AB'}$	0.78 ± 0.08 $^{AB'}$	0.84 ± 0.01 $^{C''}$	0.84 ± 0.01 $^{C''}$	Σ 2470	Σ 2309
Glycolic extract of *Z. joazeiro* Mart. bark (2 g.kg^{-1})										
F_1	2032	2162	21	14	1.03	0.65	0.69	0.85	922	388
F_2	2173	2037	17	23	0.78	1.13	0.93	0.79	176	532
F_3	2020	2070	18	18	0.89	0.87	0.84	0.85	387	378

Table 1 (cont.)

Treatment	Number of PCEs analyzed		MNPCEs *				PCE / (PCE + NCE) **		NCE (*n*)	
	24 h	48 h	24 h (*n*) ^{A}	48 h (*n*) ^{A}	24 h (%) $^{A'}$	48 h (%) $^{A'}$	24 h $^{A''}$	48 h $^{B''}$	24 h	48 h
Σ F $^{A* A**}$	Σ 6225	Σ 6269	Σ 56	Σ 55	0.90 ± 0.13	0.88 ± 0.24	0.82 ± 0.12	0.83 ± 0.03	Σ 1485	Σ 1298
M_1	2033	2010	17	14	0.84	0.70	0.84	0.77	383	590
M_2	2058	2056	18	17	0.87	0.83	0.79	0.86	542	344
M_3	2020	2037	10	26	0.50	1.28	0.70	0.88	880	277
Σ M $^{B* A**}$	Σ 6111	Σ 6103	Σ 45	Σ 57	0.74 ± 0.21	0.93 ± 0.30	0.78 ± 0.07	0.83 ± 0.06	Σ 1805	Σ 1211
Σ M and F	Σ 12336	Σ 12372	Σ 101 ^{AB}	Σ 112 ^{AB}	0.82 ± 0.12 $^{AB'}$	0.91 ± 0.04 $^{AB'}$	0.79 ± 0.03 $^{C''}$	0.83 ± 0.00 $^{C''}$	Σ 3290	Σ 2509
Glycolic extract of *Z. joazeiro* Mart. bark (2 g.kg^{-1}) + NEU (50 mg.kg^{-1})										
F_1	2052	2079	31	24	1.51	1.15	0.93	0.99	148	21
F_2	2072	2055	26	16	1.25	0.78	0.99	0.98	28	45
F_3	2071	2167	23	16	1.11	0.74	0.99	0.99	29	33
Σ F $^{A* A**}$	Σ 6195	Σ 6301	Σ 80	Σ 56	1.29 ± 0.20	0.89 ± 0.23	0.97 ± 0.03	0.98 ± 0.01	Σ 205	Σ 99
M_1	2138	2241	32	43	1.50	1.92	0.97	0.90	62	259
M_2	2144	2103	29	28	1.35	1.33	0.97	0.73	56	797
M_3	2072	2076	32	27	1.54	1.30	0.94	0.99	128	24
Σ M $^{B* A**}$	Σ 6354	Σ 6420	Σ 93	Σ 98	1.46 ± 0.10	1.53 ± 0.35	0.96 ± 0.02	0.86 ± 0.13	Σ 246	Σ 1080
Σ M and F	Σ 12549	Σ 12721	Σ 173 ^{B}	Σ 154 ^{B}	1.38 $^{B'}$	1.21 $^{B'}$	0.97 ± 0.00 $^{AB''}$	0.92 ± 0.09 $^{AB''}$	Σ 451	Σ 1179
Glycolic extract of *Z. joazeiro* Mart. bark (2 g.kg^{-1}) + DXR (5 mg.kg^{-1})										
F_1	2086	2090	23	18	1.10	0.86	0.99	0.99	14	10
F_2	2080	2100	23	20	1.11	0.95	0.99	1.00	20	1
F_3	2080	2075	21	17	1.01	0.82	0.99	0.99	20	24
Σ F $^{A* A**}$	Σ 6246	Σ 6265	Σ 67	Σ 55	1.07 ± 0.05	0.88 ± 0.07	0.99 ± 0.00	0.99 ± 0.01	Σ 54	Σ 35
M_1	2086	2088	24	12	1.15	0.57	0.99	0.99	14	12
M_2	2083	2076	15	18	0.72	0.87	0.99	0.99	17	24
M_3	2065	2096	17	14	0.82	0.67	0.98	1.00	35	4
Σ M $^{B* A**}$	Σ 6234	Σ 6260	Σ 56	Σ 44	0.90 ± 0.22	0.70 ± 0.15	0.99 ± 0.01	0.99 ± 0.00	Σ 66	Σ 40
Σ M and F	Σ 12480	Σ 12525	Σ 123 ^{AB}	Σ 99 ^{AB}	0.99 ± 0.12 $^{AB'}$	0.79 ± 0.12 $^{AB'}$	0.99 ± 0.00 $^{A''}$	0.99 ± 0.00 $^{A''}$	Σ 120	Σ 75

Means with different letters are significantly different ($p < 0.05$). M: Male. F: Female.

(1.5-2 g.kg^{-1}), regardless of sex, but varied between time intervals. Thus, the PCE/NCE ratio at higher doses was significantly lower than observed in positive the controls treated with NEU. These results suggest the absence of systemic toxicity at GEZJ doses of 0.5-1 g.kg^{-1} and moderate toxicity at doses of 1.5-2 g.kg^{-1}, regardless of mouse gender, with variable responses over time (24-48 h). Whereas treatment with GEZJ (2 g.kg^{-1}) + DXR (5 mg.kg^{-1}) significantly reduced the MNPCEs (*n* and %), there was a significant increase in the PCE/NCE ratio with this same treatment, indicating that this combination was not toxic to mouse bone marrow. These results also suggest that the

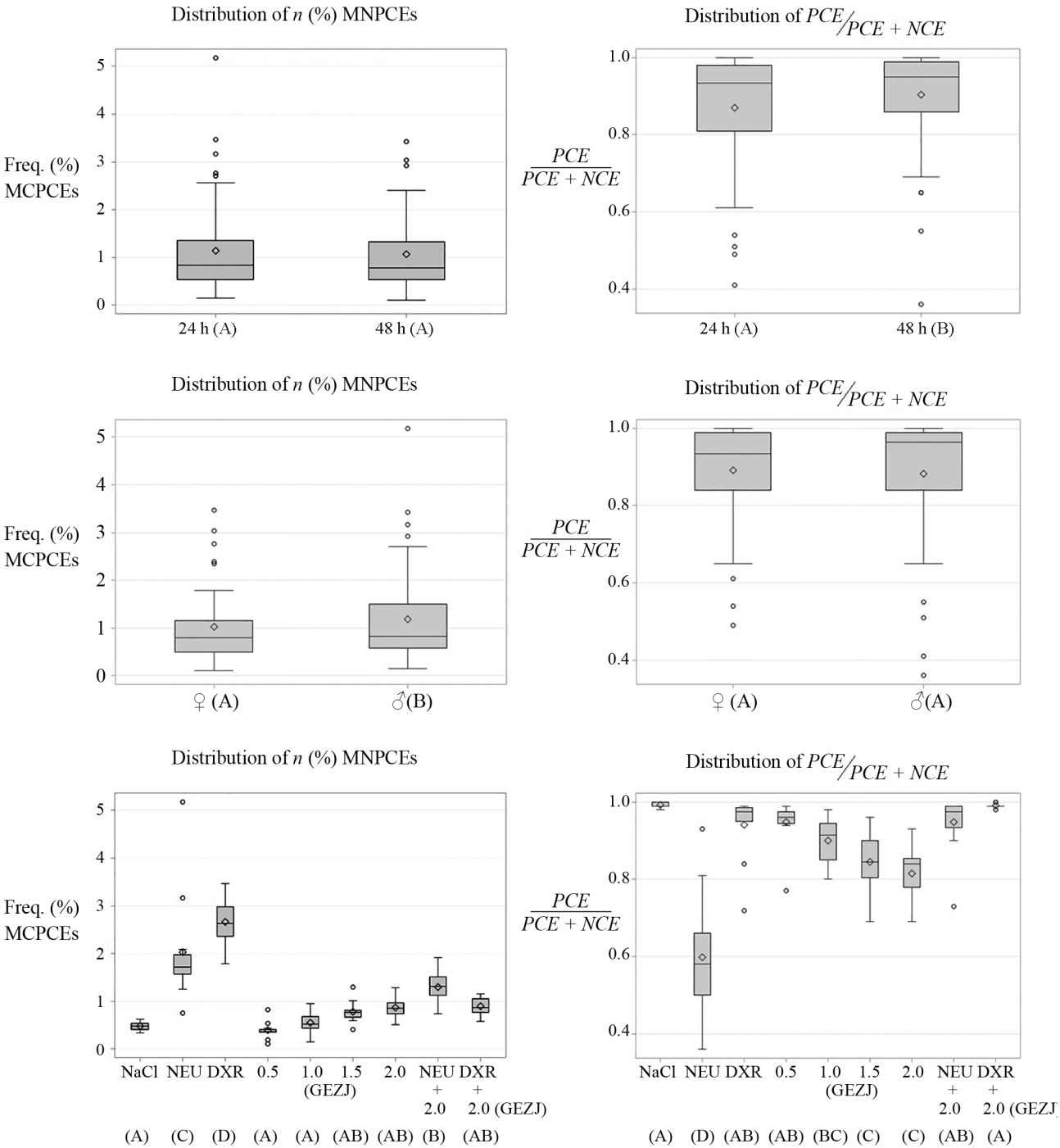

Figure 2 - Box-plots showing the MNPCE frequencies and PCE/NCE ratios in mouse bone marrow in mutagenic and antimutagenic assays of *Z. joazeiro* bark. Means with different letters are significantly different ($p < 0.05$). NaCl - control group treated with 150 mM NaCl, NEU - N-nitroso-N-ethylurea (50 mg.kg^{-1}), DXR - doxorubicin hydrochloride (5 mg.kg^{-1}), GEZJ - Glycolic extract of *Z. joazeiro* Mart. bark (0.5-2 g.kg^{-1}), GEZJ (2 g.kg^{-1}) + NEU (50 mg.kg^{-1}) and GEZJ (2 g.kg^{-1}) + DXR (5 mg.kg^{-1}).

phytochemical compounds responsible for the moderate toxicity (altered PCE/NCE ratio) of GEZJ (2 $g.kg^{-1}$) in bone marrow may also have an important role in attenuating the mutagenicity (*n* and % of MNPCE) of DRX (5 $mg.kg^{-1}$).

The acute toxicity of different plant extracts, including *Z. joazeiro*, has previously been based on doses (1 to 4 or 5 g/kg) administered orally to different groups of mice (one dose per mouse, with each group containing eight animals: four males and four females) (Alviano *et al.*, 2008). Behavioral parameters, including convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, and changes in food and water intake were also noted. These animals were observed and weighed over a period of 14 days; no weight loss was detected. Treated mice showed no behavioral alterations and the extract LD_{50} values ranged from 2.0-3.5 g/kg. None of the extracts was lethal to mice at the doses tested and the data from the *in vivo* assays indicated that the extracts had low toxicity (Alviano *et al.*, 2008). The data from the MN assays presented here provides additional information on the systemic toxicity of *Z. joazeiro* in mouse bone marrow based on the PCE/NCE ratio that suggested moderate toxicity of GEZJ at doses of 1.5-2 $g.kg^{-1}$ that was independent of mouse gender but varied with time (24-48 h).

The PCE/NCE ratio is an indicator of the acceleration or inhibition of erythropoiesis and varies with the scoring interval. A continuous decline in the PCE/NCE ratio may reflect the inhibition of cell division, the killing of erythroblasts, the removal of damaged cells, or dilution of the existing cell pool with newly formed cells (Venkatesh *et al.*, 2007). Several mechanisms may contribute to the cytotoxicity of DXR and MN induction (Gewirtz, 1999), including the intercalation of DXR in cellular DNA (Painter, 1978; Kiyomiya *et al.*, 2001), stabilization of the topoisomerase II-DNA complex (Pommier *et al.*, 1985; Guano *et al.*, 1999), free radical-mediated toxicity caused by redox cycling of the semiquinone radical (Bachur *et al.*, 1979), or the formation of reactive oxygen species by the DXR-iron complex (Eliot *et al.*, 1984; Myers, 1998; Konorev *et al.*, 1999). On the other hand, chemicals such as captopril and desferrioxamine (Al-Harbi, 1993; Al-Shabanah, 1993), β-carotene and vitamins A, C and E (Lu *et al.*, 1996; Gulkac *et al.*, 2004; Costa and Nepomuceno, 2006), thiol N-acetylcysteine, probucol, lovastatin and hydrophilic flavonoids such as rutin and luteolin (Al-Gharably, 1996; Sadzuka *et al.*, 1997; D'Agostini *et al.*, 1998; Bardeleben *et al.*, 2002) can also reduce DXR-induced MN formation, genotoxicity and cytotoxicity. However, proponents of herbal medicine always claim that mixtures are better than pure chemicals because the dozens of biologically active compounds in plants work together to produce a greater effect than any one chemical on its own (Mackenzie, 2001).

Screening for newer pharmacological agents that can protect normal cells against DXR-induced cumulative toxicity is essential. Many plants widely used in traditional medicine are less toxic than pharmaceutical agents and have recently attracted the attention of researchers around the world. Plants contain many compounds and it is likely that these can provide better protection than a single molecule (Vidhya and Devraj, 1999). The presence of many molecules in plants may be advantageous, as some of them may counteract the toxicity of others so that the net effect may be therapeutically beneficial. For example, the effect of various concentrations (200, 250, 300, 350 and 400 mg/kg body weight) of *Aegle marmelos* on DXR-induced mutagenicity in mouse bone marrow was studied (Venkatesh *et al.*, 2007). Mice treated with different concentrations of DXR (5, 10 or 15 $mg.kg^{-1}$ body weight) showed a dose-dependent elevation in the frequency of PCE and NCE in their bone marrow, and this was accompanied by a DXR-mediated dose-dependent decline in the PCE/NCE ratio. In contrast, the treatment of mice with *A. marmelos* orally once a day for five consecutive days before treatment with DXR significantly reduced the frequency of DXR-induced micronuclei and significantly increased the PCE/NCE ratio at all time intervals. This chemoprotective effect may reflect the sum of interactions between different components of this complex mixture. The degree of protection may depend on the individual or collective interaction of components with the genotoxic agent. The plausible mechanisms of action of *A. marmelos* in protecting against DXR-induced damage included the scavenging of $O_2^{\bullet-}$, $^{\bullet}OH$ and other free radicals, an increase in antioxidant status, restoration of topoisomerase II activity and inhibition of the formation of the DXR-iron complex (Venkatesh *et al.*, 2007). More recently, Alves *et al.* (2012) evaluated the genotoxic potential of a hydroalcoholic extract of *Copaifera lansdorffii* Desf. leaves and its influence on the genotoxicity of DXR (MN test) in peripheral blood from Swiss mice. Their finidngs demonstrated that *C. lansdorffii* Desf. was not genotoxic but that the extract significantly reduced the number of micronuclei in DXR-treated mice. The putative antioxidant activity of one or more of the active compounds of *C. lansdorffii* Desf., including two major flavonoid heterosides (quercitrin and afzelin), may explain the effect of this plant on DXR genotoxicity.

Conclusions

This study used the MN assay to evaluate the mutagenic (clastogeny and/or aneugeny) and antimutagenic activity of an extract of *Z. joazeiro* bark in mouse bone marrow. The *Z. joazeiro* bark extract was not mutagenic at the doses and time intervals tested, although sex-related variation was observed. The antimutagenic effect (anticlastogeny and/or antianeugeny) of *Z. joazeiro* bark extract against DXR-induced genotoxicity was observed at a high dose of extract (2 $g.kg^{-1}$), but was independent of the duration of treatment and animal sex. Low concentrations of GEZJ (0.5-1 $g.kg^{-1}$) were not toxic, regardless of mouse gender and duration of treatment, whereas moderate toxic-

ity was observed at doses of 1.5-2 $g.kg^{-1}$. Together, these findings indicate that phytochemical compounds in *Z. joazeiro* bark can attenuate DRX-induced mutagenicity and that a high dose of extract (2 $g.kg^{-1}$) showed no toxicity in the conditions tested here.

Other studies on the genotoxicity and mutagenicity of *Z. joazeiro* extracts are needed to characterize the (anti)genotoxic effects and mechanisms, and to determine the potential health risks of this extract in humans. Such investigations will be useful for implementing strategies related to the use of *Z. joazeiro* bark in chemoprevention.

Acknowledgments

This research was supported by Rede Mineira de Ensaios Toxicológicos e Farmacológicos de Produtos Terapêuticos (REDE MINEIRA TOXIFAR - 2012) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

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Associate Editor: Daisy Maria Fávero Salvadori