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Assessment of genotoxic, cytotoxic, and protective effects of *Salacia crassifolia* (Mart. Ex. Schult.) G. Don. stem bark fractions in mice

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ABSTRACT. *Salacia crassifolia* (Mart. Ex. Schult.) G. Don., popularly known in Brazil as "bacupari", "cascudo", and "saputá", is a shrub of the Celastraceae family that is unique to the Brazilian Cerrado region. In folk medicine, this plant has been mainly used to treat skin cancer and gastric ulcers. In the present study, the genotoxic, cytotoxic, antigenotoxic, and anticytotoxic effects of *S. crassifolia* stem bark fractions (hexane, ethyl acetate, and hydroalcoholic extracts) were evaluated using the mouse bone marrow micronucleus test. Our results showed that none of the *S. crassifolia* fractions led to a significant increase in the frequency of micronucleated polychromatic erythrocytes (MNPCE) ($P > 0.05$), suggesting the absence of genotoxicity. In the antigenotoxicity assessment, a significant decrease in the MNPCE frequency was observed in all fractions of this plant ($P < 0.05$), demonstrating its protective action against genotoxicity induced by mitomycin C (MMC), which was used as the positive control. Only the

hexane fraction of *S. crassifolia* significantly decreased the poly- and normochromatic erythrocyte ratio (PCE/NCE) in all doses tested ($P < 0.05$), demonstrating its cytotoxic activity. In association with MMC, both ethyl acetate and hydroalcoholic fractions significantly increased the PCE/NCE ratio in almost all doses tested ($P < 0.05$), demonstrating the protective action of *S. crassifolia* against the cytotoxic effect of the positive control. In contrast, the hexane fraction presented a significant decrease in the PCE/NCE ratio in all treatments ($P < 0.05$), demonstrating an increase in this plant's cytotoxicity in mouse bone marrow cells.

Key words: *Salacia crassifolia*; Stem bark fractions; Micronucleus test; Cytotoxicity; Antigenotoxicity

INTRODUCTION

Brazil is known for its great biological and genetic diversity, with 40 to 55 thousand plant species distributed across several biomes, as well as for the great number of plants that are used by the population in folk medicine to treat a variety of diseases (de Oliveira et al., 2011).

Indeed, many plants contain good sources of compounds that are responsible for human health maintenance, reduction of oxidative damage, and protection against coronary heart diseases and cancer, including tocopherols, vitamin C, carotenoids, and phenolic compounds (Kilani et al., 2008). However, many of these compounds can also cause harm, including DNA damage, which may lead to genetic instability and related diseases, such as cancer (Kleter et al., 2009). Therefore, it is of vital importance to evaluate the asserted pharmacological and/or therapeutic properties of these plants, to isolate their active constituents, and to verify their possible toxicity (Marques et al., 2003).

Salacia crassifolia (Mart. Ex. Schult.) G. Don., popularly known in Brazil as "bacupari", "cascudo", and "saputá", is a shrub of the Celastraceae family that is unique to the Brazilian Cerrado region (Lombardi and Temponi, 2000). In folk medicine, the leaves, stems, seeds, and fruits of this species are used to treat pediculosis, kidney disease, gastric ulcers, skin cancer, malaria, chronic coughs, and headaches. The fruits are also used for fresh consumption and industrial purposes (Silva Júnior, 2005; Cavéchia and Proença, 2007). Previous studies have described the biological activities of this plant revealing cytotoxic, antitumoral, and antibacterial effects (Santana et al., 1971; Lima et al., 1969, 1972).

Several other studies have reported that various species of the family Celastraceae exhibit important pharmacological constituents, such as: quinone-methide triterpenes, which have antibiotic, cytotoxic, antitumor, antimalarial, and antioxidant properties (Santana et al., 1971; Lima et al., 1972; Figueiredo et al., 1998; Alvarenga et al., 1999; Jeller et al., 2004); sesquiterpenes, which show insecticide activity (Dagang et al., 1992); flavonoids, which have antioxidant properties (Corsino et al., 2003); and alkaloids, which show antitumor and insecticide activities (Corsino et al., 1998; Jinbo et al., 2002). Quinone-methide triterpenes are natural constituents whose occurrence is restricted to the family Celastraceae (Carvalho et al., 2005). Some compounds of this class have already been isolated from *S. crassifolia*, such as maytenin and pristimerin, which have been proven to present not only significant cytotoxic

and antimicrobial activities, but also potent antitumor activity (Santana et al., 1971; Lima et al., 1969, 1972). It is also believed that these molecules are potential antineoplastic agents (Buffa Filho et al., 2004; Lu et al., 2010; Yadav et al., 2010).

Although studies involving species of the family Celastraceae are prominent, only a few have focused on *S. crassifolia* specifically. Caramori et al. (2004) demonstrated that *S. crassifolia* seed extracts contain a considerable amount of polyphenols and tannins, as well as compounds with nutritional value, such as proteins, carbohydrates, and lipids.

Therefore, further studies with *S. crassifolia* are needed in order to prove its effectiveness in treating a variety of diseases and to know the risks associated with its intake, as well as the pharmacological effects of its active constituents.

Due to the large utilization of *S. crassifolia* and the relevant pharmacological constituents present in this plant, the present study aimed to evaluate the genotoxic, cytotoxic, antigenotoxic, and anticytotoxic effects of the stem bark fractions [hexane (HEF), ethyl acetate (EAF), and hydroalcoholic extracts (HAF)] of this species using the mouse bone marrow micronucleus test.

MATERIAL AND METHODS

Plant material

Samples of *S. crassifolia* stem bark were collected in Anápolis, GO, Brazil (16°12'49"S, 48°57'57"W). The plant was identified by Dr. Mirley Luciene dos Santos and a voucher specimen (No. 5910) was deposited in the Central Herbarium of Universidade Estadual de Goiás, in Anápolis.

The stem bark samples were dried in a forced-air oven at 45ºC and ground in a fraction mill to obtain a dry powder that was then subjected to extraction with methanol in an exhaustive manner. The organic solvent was evaporated at reduced pressure with a rotary evaporator to a residue we named crude methanolic extract. The crude methanolic extract was resuspended in 1:3 methanol:water and partitioned according to the polarity of the solvents hexane, dichloromethane, and ethyl acetate. The following fractions of *S. crassifolia* stem bark were obtained: HEF, dichloromethane, EAF, and HAF. Dichloromethane was excluded from this study, because its mass was insufficient to perform the experiments. HEF, EAF, and HAF were stocked at 5ºC until use, at which point they were dissolved in dimethylsulfoxide (DMSO).

Chemical agents

The following chemicals were used in the experiments: mitomycin C (MMC, $C_{15}H_{18}N_4O_5$; Bristol-Myers Squibb, No. 237AEL), dibasic sodium phosphate ($Na_2HPO_412H_2O$; Vetec, No. 982162), monobasic sodium phosphate ($NaH_2PO_4H_2O$; Vetec, No. 983831), methanol (CH_4O; Synth, No. 55026), Giemsa (Doles, No. 1081), and fetal calf serum (FCS; Laborclin, No. 30721063).

Animals

This study was approved by the Research Ethics Committee of Universidade Federal

de Goiás (CoEP-UFG, No. 225/11). Healthy, young male adult (8-12 weeks) outbred mice (*Mus musculus*, Swiss Webster), weighing 25-30 g, obtained from the Central Animal House of UFG, were used in this study. All animals were brought to the laboratory 5 days before the experiments and housed in plastic cages (40 x 30 x 16 cm) at 24° ± 2°C and 55 ± 10% relative humidity, with a light-dark natural cycle of 12 h. The animals were fed appropriate commercial rodent diet (Labina, Ecibra Ltda., Santo Amaro, SP, Brazil) and water *ad libitum*.

Experimental procedure

The experiments were performed according to methods described in von Ledebur and Schmid (1973). For each treatment, groups of 5 animals were treated orally with 50, 100, or 150 mg/kg HEF, EAF, or HAF, according to body weight, for the evaluation of *S. crassifolia* genotoxicity. The same doses of the three fractions were co-treated with MMC for the evaluation of this species' antigenotoxicity. A positive control group (MMC, 4 mg/kg intraperitoneally), a negative control group (sterile distilled water) and a solvent control group (DMSO, 0.1 mL/10 g body weight) were also included. The animals were euthanized 24 or 48 h after the administration of HEF, EAF, or HAF by cervical dislocation, and their bone marrow cells were flushed from both femurs in fetal calf serum. After centrifugation (300 *g*, 5 min), the bone marrow cells were smeared on glass slides, coded for blind analysis, air-dried, and fixed with absolute methanol for 5 min at room temperature. The smears were stained with Giemsa, dibasic sodium phosphate, and monobasic sodium phosphate. For each animal, we prepared three slides and counted 2000 polychromatic erythrocytes (PCE) to determine the frequency of micronucleated polychromatic erythrocytes (MNPCE). Genotoxicity and antigenotoxicity were assessed by the frequency of MNPCE, whereas cytotoxicity and anticytotoxicity were evaluated by the ratio of PCE to normochromatic erythrocytes (NCE). The slides were analyzed using a microscope (Olympus BH-2 10x100, Tokyo, Japan).

Statistical analysis

To analyze *S. crassifolia* genotoxic activity, we compared the frequency of MNPCE detected in the treated groups with that of the solvent control group using one-way analysis of variance (ANOVA). To assess the antigenotoxic activity of *S. crassifolia*, the frequency of MNPCE in the treated groups was compared with the positive control group (ANOVA). The Tukey test was applied for a multiple comparison after ANOVA, and P values lower than 0.05 ($P < 0.05$) were considered to be indicative of significance. To evaluate the cytotoxicity of *S. crassifolia*, the PCE/NCE ratio of all treated groups was compared with the solvent control group using the chi-squared test (χ^2). To evaluate *S. crassifolia* anticytotoxicity, the PCE/NCE ratio of all treated groups was compared with the positive control group using the χ^2 test. P values lower than 0.05 ($P < 0.05$) were considered to be indicative of significance.

RESULTS

The results of HEF, EAF, and HAF genotoxicity, cytotoxicity, antigenotoxicity, and anticytotoxicity are shown in Tables 1, 2, and 3, respectively.

In this study, the negative control and the solvent control groups demonstrated low

MNPCE values, as expected, and the positive control group's MNPCE frequency was significantly higher ($P < 0.05$), confirming the sensitivity of the test.

The HEF genotoxicity analysis, for all doses tested (50, 100, and 150 mg/kg), indicated no significant increase in the MNPCE frequency at 24 and 48 h when compared with the solvent control group ($P > 0.05$), leading to the conclusion that the HEF of *S. crassifolia* stem bark does not have genotoxic effects (Table 1).

Table 1. Frequency of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio in mice bone marrow cells treated with different doses of the hexane fraction (HEF) of *Salacia crassifolia* stem bark and co-treated with mitomycin C (MMC) and their respective controls.

Sampling time/treatment	Individual data (MNPCE/2000 PCE)	Total MNPCE	Means ± SD (MNPCE)	Means ± SD (PCE/NCE)
24 h				
Water[1]	3, 5, 3, 4, 4	19	3.8 ± 0.74[c]	1.06 ± 0.06[c]
DMSO[2]	4, 4, 4, 4, 5	21	4.2 ± 0.4[c]	1.1 ± 0.05[c]
MMC[3]	34, 32, 33, 30, 34	163	32.6 ± 1.67[a]	0.7 ± 0.01[a]
HEF alone				
50 mg/kg	3, 4, 3, 3, 4	17	3.4 ± 0.49[b]	0.94 ± 0.02[a]
100 mg/kg	2, 2, 2, 2, 3	11	2.2 ± 0.4[b]	0.81 ± 0.05[a]
150 mg/kg	3, 2, 2, 3, 1	11	2.2 ± 0.75[b]	0.87 ± 0.05[a]
HEF + MMC				
50 mg/kg	9, 9, 11, 13, 9	51	10.2 ± 1.78[c]	0.58 ± 0.05[c]
100 mg/kg	8, 9, 5, 9, 4	35	7.0 ± 2.09[c]	0.59 ± 0.05[c]
150 mg/kg	4, 4, 5, 4, 4	21	4.2 ± 0.4[c]	0.62 ± 0.06[c]
48 h				
DMSO	4, 3, 3, 3, 4	17	3.4 ± 0.49[c]	1.09 ± 0.09[c]
MMC	10, 12, 11, 12, 14	59	11.8 ± 1.48[a]	0.55 ± 0.01[a]
HEF alone				
50 mg/kg	3, 2, 3, 3, 3	14	2.8 ± 0.4[b]	0.98 ± 0.16[a]
100 mg/kg	2, 1, 2, 2, 3	10	2.0 ± 0.63[b]	0.86 ± 0.08[a]
150 mg/kg	3, 2, 1, 2, 3	11	2.2 ± 0.75[b]	0.82 ± 0.05[a]
HEF + MMC				
50 mg/kg	6, 6, 5, 5, 4	26	5.2 ± 0.83[c]	0.5 ± 0.06[c]
100 mg/kg	3, 3, 4, 3, 3	16	3.2 ± 0.4[c]	0.32 ± 0.02[c]
150 mg/kg	2, 2, 2, 3, 4	13	2.6 ± 0.8[c]	0.36 ± 0.05[c]

All results were compared with the respective control group at the respective time. [1]Negative control = distilled water. [2]DMSO = solvent control. [3]Positive control = 4 mg/kg MMC. [a]Significant difference compared to the solvent control group ($P < 0.05$). [b]No significant difference compared to the solvent control group ($P > 0.05$). [c]Significant difference compared to the positive control group ($P < 0.05$). [d]No significant difference compared to the positive control group ($P > 0.05$).

Regarding HEF cytotoxicity, all doses tested (50, 100, and 150 mg/kg) at both treatment times (24 and 48 h) caused a significant reduction in the PCE/NCE ratio relative to the solvent control group ($P < 0.05$), although this reduction was relatively small at the 50 mg/kg dose level (Table 1).

In the HEF antigenotoxic evaluation, for all doses tested (50, 100, and 150 mg/kg HEF + MMC), the mean MNPCE values (per 2000 PCE) were 10.2, 7.0, and 4.2 at 24 h, and 5.2, 3.2, and 2.6 at 48 h, respectively, whereas for the positive control group, they were 32.6 at 24 h and 11.8 at 48 h (Table 1). These results show that HEF strongly modulated the genotoxic activity of MMC at both treatment times ($P < 0.05$), demonstrating its antigenotoxic effect.

With respect to HEF anticytotoxicity, the observed that PCE/NCE ratios were 0.58, 0.59, and 0.62 at 24 h and 0.5, 0.32, and 0.36 at 48 h in treatments with doses of 50, 100, and 150 mg/kg HEF + MMC, respectively, whereas for the positive control group, they were 0.7

at 24 h and 0.55 at 48 h (Table 1). The statistical analysis showed that all doses of HEF at both times of treatment caused significant reductions in the PCE/NCE ratio compared with those of the positive control group ($P < 0.05$). Therefore, we observed that the HEF of *S. crassifolia* stem bark significantly increased the cytotoxicity induced by MMC.

The EAF genotoxic activity assessment showed that none of the doses tested (50, 100, and 150 mg/kg) caused significant increases in the MNPCE frequency at 24 or 48 h when compared with that of the solvent control group ($P > 0.05$), indicating no genotoxic effect under these experimental conditions (Table 2).

Table 2. Frequency of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio in mice bone marrow cells treated with different doses of the ethyl acetate fraction (EAF) of *Salacia crassifolia* stem bark and co-treated with mitomycin C (MMC) and their respective controls.

Sampling time/treatment	Individual data (MNPCE/2000 PCE)	Total MNPCE	Means ± SD (MNPCE)	Means ± SD (PCE/NCE)
24 h				
Water[1]	3, 5, 3, 4, 4	19	3.8 ± 0.74[c]	1.06 ± 0.06[c]
DMSO[2]	4, 4, 4, 4, 5	21	4.2 ± 0.4[c]	1.1 ± 0.05[c]
MMC[3]	34, 32, 33, 30, 34	163	32.6 ± 1.67[a]	0.7 ± 0.01[a]
EAF alone				
50 mg/kg	3, 4, 2, 3, 3	15	3.0 ± 0.63[b]	1.09 ± 0.04[b]
100 mg/kg	3, 3, 3, 4, 3	16	3.2 ± 0.4[b]	1.14 ± 0.07[b]
150 mg.kg	3, 2, 2, 2, 3	12	2.4 ± 0.49[b]	1.05 ± 0.05[b]
EAF + MMC				
50 mg/kg	16, 20, 19, 21, 22	98	19.6 ± 2.3[c]	0.82 ± 0.04[c]
100 mg/kg	15, 14, 12, 09, 13	63	12.6 ± 2.3[c]	0.76 ± 0.04[c]
150 mg/kg	11, 08, 13, 13, 11	56	11.2 ± 2.04[c]	0.78 ± 0.01[c]
48 h				
DMSO	4, 3, 3, 3, 4	17	3.4 ± 0.49[c]	1.09 ± 0.09[c]
MMC	10, 12, 11, 12, 14	59	11.8 ± 1.48[a]	0.55 ± 0.01[a]
EAF alone				
50 mg/kg	5, 3, 4, 4, 4	20	4.0 ± 0.63[b]	1.08 ± 0.03[b]
100 mg/kg	2, 2, 3, 2, 2	11	2.2 ± 0.4[b]	1.12 ± 0.05[b]
150 mg/kg	3, 3, 3, 2, 3	14	2.8 ± 0.4[b]	1.08 ± 0.02[b]
EAF + MMC				
50 mg/kg	9, 9, 12, 9, 8	47	9.4 ± 1.35[c]	0.67 ± 0.007[c]
100 mg/kg	9, 7, 10, 9, 8	43	8.6 ± 1.02[c]	0.65 ± 0.01[c]
150 mg/kg	7, 8, 9, 8, 5	37	7.4 ± 1.35[c]	0.68 ± 0.05[c]

All results were compared with the respective control group at the respective time. [1]Negative control = distilled water. [2]DMSO = solvent control. [3]Positive control = 4 mg/kg MMC. [a]Significant difference compared to the solvent control group ($P < 0.05$). [b]No significant difference compared to the solvent control group ($P > 0.05$). [c]Significant difference compared to the positive control group ($P < 0.05$). [d]No significant difference compared to the positive control group ($P > 0.05$).

With respect to EAF cytotoxicity, no significant differences were observed between each of the PCE/NCE ratios for all doses tested (50, 100, and 150 mg/kg) and the respective doses of the solvent control groups at either time of treatment ($P > 0.05$) (Table 2). Thus, the EAF of *S. crassifolia* stem bark did not exhibit genotoxic or cytotoxic activities under our experimental conditions.

According to our results, the mean MNPCE values (per 2000 EPC) of the groups treated with 50, 100, and 150 mg/kg EAF + MMC were 19.6, 12.6, and 11.2 at 24 h, and 9.4, 8.6, and 7.4 at 48 h, respectively, whereas in the positive control group, they were 32.6 at 24 h and 11.8 at 48 h (Table 2). These results show that the EAF of *S. crassifolia* stem bark protected the mouse cells against MMC genotoxicity ($P < 0.05$) in all doses tested at both treatment times, demonstrating its strong antigenotoxic effect.

In the anticytotoxic evaluation, the PCE/NCE ratios of the groups treated with 50, 100, and 150 mg/kg EAF + MMC were 0.82, 0.76, and 0.78 at 24 h, and 0.67, 0.65, and 0.68 at 48 h, respectively, and in the positive control group they were 0.7 at 24 h and 0.55 at 48 h, demonstrating a moderate anticytotoxic effect of EAF at all doses tested ($P < 0.05$) (Table 2).

No significant differences in the MNPCE frequency were observed during the genotoxic evaluation of HAF when compared with that of the solvent control group at 24 and 48 h at any of the doses tested ($P > 0.05$), suggesting a lack of genotoxicity of this fraction (Table 3).

No significant differences were observed in the PCE/NCE ratios of HAF compared with those of the solvent control group at any dose tested (50, 100, and 150 mg/kg), or at either treatment time ($P > 0.05$), indicating that this fraction presented no cytotoxic effects.

Table 3. Frequency of micronucleated polychromatic erythrocytes (MNPCE) and polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio in mice bone marrow cells treated with different doses of the hydroalcoholic fraction (HAF) of *Salacia crassifolia* stem bark and co-treated with mitomycin C (MMC) and their respective controls.

Sampling time/treatment	Individual data (MNPCE/2000 PCE)	Total MNPCE	Means ± SD (MNPCE)	Means ± SD (PCE/NCE)
24 h				
Water[1]	3, 5, 3, 4, 4	19	3.8 ± 0.74[c]	1.06 ± 0.06[c]
DMSO[2]	4, 4, 4, 4, 5	21	4.2 ± 0.4[c]	1.1 ± 0.05[c]
MMC[3]	34, 32, 33, 30, 34	163	32.6 ± 1.67[a]	0.7 ± 0.01[a]
HAF alone				
50 mg/kg	3, 3, 4, 4, 4	18	3.6 ± 0.49[b]	1.08 ± 0.04[b]
100 mg/kg	2, 3, 4, 3, 4	16	3.2 ± 0.75[b]	1.01 ± 0.02[b]
150 mg/kg	2, 2, 2, 3, 2	11	2.2 ± 0.4[b]	1.05 ± 0.03[b]
HAF + MMC				
50 mg/kg	20, 22, 18, 17, 23	100	20.0 ± 2.28[c]	0.72 ± 0.05[d]
100 mg/kg	16, 15, 13, 15, 16	75	15.0 ± 1.09[c]	0.78 ± 0.02[c]
150 mg/kg	11, 10, 10, 10, 13	54	10.8 ± 1.16[c]	0.82 ± 0.04[c]
48 h				
DMSO	4, 3, 3, 3, 4	17	3.4 ± 0.49[c]	1.09 ± 0.09[c]
MMC	10, 12, 11, 12, 14	59	11.8 ± 1.48[a]	0.55 ± 0.01[a]
HAF alone				
50 mg/kg	2, 4, 4, 3, 3	16	3.2 ± 0.74[b]	1.05 ± 0.04[b]
100 mg/kg	4, 4, 3, 4, 3	18	3.6 ± 0.49[b]	1.07 ± 0.04[b]
150 mg/kg	3, 2, 3, 3, 4	15	3.0 ± 0.63[b]	1.05 ± 0.04[b]
HAF + MMC				
50 mg/kg	10, 9, 8, 8, 9	44	8.8 ± 0.75[c]	0.58 ± 0.05[d]
100 mg/kg	11, 7, 7, 8, 8	41	8.2 ± 1.47[c]	0.6 ± 0.02[c]
150 mg/kg	10, 6, 7, 8, 7	38	7.6 ± 1.35[c]	0.65 ± 0.03[c]

All results were compared with the respective control group at the respective time. [1]Negative control = distilled water. [2]DMSO = solvent control. [3]Positive control = 4 mg/kg MMC. [a]Significant difference compared to the solvent control group ($P < 0.05$). [b]No significant difference compared to the solvent control group ($P > 0.05$). [c]Significant difference compared to the positive control group ($P < 0.05$). [d]No significant difference compared to the positive control group ($P > 0.05$).

Regarding the HAF antigenotoxicity, our results showed that the mean MNPCE values (per 2000 EPC) of the groups treated with 50, 100, and 150 mg/kg HAF + MMC were 20.0, 15.0, and 10.8 at 24 h, and 8.8, 8.2, and 7.6 at 48 h, respectively, whereas for the positive control group, they were 32.6 at 24 h and 11.8 at 48 h (Table 3). According to these results, the HAF modulated the genotoxic activity of MMC at both treatment times, demonstrating its antigenotoxic effect ($P < 0.05$).

In the evaluation of HAF anticytotoxicity, the PCE/NCE ratios obtained at doses of 50, 100, and 150 mg/kg HAF + MMC were 0.72, 0.78, and 0.82 at 24 h and 0.58, 0.6 and 0.65 at 48 h, respectively, whereas for the positive control group, they were 0.7 at 24 h and 0.55 at

48 h (Table 3). These results demonstrated a significant increase in the PCE/NCE ratio compared to that of the respective positive control group ($P < 0.05$) at doses of 100 and 150 mg/kg HAF co-administered with MMC, demonstrating a significant decrease in the cytotoxicity induced by MMC.

DISCUSSION

Although *S. crassifolia* has been broadly used in folk medicine to treat a variety of diseases, it remains unknown if this plant actually poses any risks to people and/or protect them against the genotoxic action of some compounds. Therefore, the aim of this study was to evaluate the genotoxic, cytotoxic, antigenotoxic, and anticytotoxic effects of the HEF, EAF, and HAF of *S. crassifolia* stem bark using the mouse bone marrow micronucleus test.

The mouse bone marrow micronucleus test is a short-term assay that is widely employed to detect genetic alterations arising from chromosomal damage and/or damage to the mitotic apparatus caused by clastogenic or aneugenic agents. As micronuclei are indicative of irreversible DNA loss, their frequency may be used as a mutation index. Moreover, positive correlations between increased micronucleus frequency and the occurrence of tumors in rodents and humans have been widely observed (Hayashi et al., 2000).

The results of the genotoxic assessment of *S. crassifolia* showed that for all doses tested (50, 100, and 150 mg/kg) and treatment times (24 and 48 h), neither the HEF, EAF, or HAF resulted in significant increases in the MNPCE frequency relative to the solvent control group ($P > 0.05$) and, therefore, did not exhibit genotoxic activity in PCE in mouse bone marrow cells.

To the best of our knowledge, so far, no studies have demonstrated genotoxic effects of *S. crassifolia.* However, studies with other species of the same genus have revealed such effects. Flammang et al. (2006, 2007) demonstrated that the root extract of *S. oblonga* did not present mutagenicity using the Ames test in strains of *Salmonella typhimurium* and a test of chromosomal aberrations in cultured rat peripheral blood lymphocytes (*in vivo* study). Previous studies have demonstrated that various extracts of *Salacia* species (*S. oblonga*, *S. chinensis*, and *S. prioidis*) show potent α-glucosidase-inhibitory activity, which may be used as adjunctive therapy in the treatment of individuals with diabetes mellitus (Matsuda et al., 2002; Muraoka et al., 2010).

Mangiferin is an active constituent of these species, and demonstrates antidiabetic properties based on observed decreases of insulin resistance in non-insulin-dependent mice (Miura et al., 2001). As a result, mangiferin isolated from *S. chinensis* was evaluated in a series of genotoxicity studies to confirm the safety of its usage, and showed no mutagenicity up to 5 mg/plate when tested in *S. typhimurium* strains TA 97a, TA 98, TA 100, TA 102, and TA 1535, both with and without metabolic activation (Govindaraj et al., 2009). Therefore, our results are in agreement with the findings of Flammang et al. (2006, 2007) and Govindaraj et al. (2009).

The micronucleus test used in our study also detects cytotoxic effects, based on the PCE/NCE ratio. When the proliferation of bone marrow cells is affected by a toxic agent, the number of immature erythrocytes (PCE) decreases in relation to that of mature erythrocytes (NCE), leading to a decrease in the PCE/NCE ratio (Hayashi et al., 2000). Our results showed that neither the EAF nor the HAF exhibited cytotoxic activity at any dose and time analyzed, whereas the HEF exhibited mild cytotoxic activity at the lowest dose (50 mg/kg) and moderate toxic effects at doses of 100 and 150 mg/kg at both time periods.

Previous studies conducted with species of the family Celastraceae have shown important cytotoxic activity related to some of their isolated constituents. Pristimerin, a very common quinone-methide triterpene in Celastraceae species, has been isolated from *Maytenus catigarum* root bark hexane fractions (Alvarenga et al., 1999), *S. beddomei* stem bark slightly polar fractions (Hisham et al., 1995), and *S. crassifolia* root bark hexane fractions (Lima et al., 1972). The cytotoxic activity of this compound has proven to be effective against certain bacteria, fungi, and tumor cells in several studies (González et al., 1998; Alvarenga et al., 1999; Gullo et al., 2012). Therefore, we believe that the HEF of *S. crassifolia* stem bark may contain chemical substances, such as pristimerin, that have a cytotoxic profile.

The chemotherapeutic agent MMC was used in the assessment of *S. crassifolia* antigenotoxic and anticytotoxic effects in our study. The action of this compound can occur due to different mechanisms: alkylation of DNA, generation of free radicals, such as superoxides, and generation of hydroxyl radicals, which induce breaks and other lesions in DNA strands (Kumar et al., 1992). The lesions generated by MMC can also cause the blockage of DNA replication, resulting in the cytotoxic action of this compound.

In the antigenotoxic evaluation of *S. crassifolia* extract fractions, our results indicated that all doses tested (50, 100, and 150 mg/kg), at both 24 and 48 h, protected the mouse bone marrow cells against the genotoxic activity of MMC, suggesting the presence of antigenotoxic compounds in all three fractions of this plant.

Phytochemical studies with several species of the genus *Salacia* have demonstrated the presence of phenolic compounds, quinone-methide triterpenes, friedelane triterpenes, and flavonoids, which show antioxidant and free radical scavenging activities (Morikawa et al., 2003; Carvalho et al., 2005; Muraoka et al., 2010; Somwong et al., 2011). Caramori et al. (2004) demonstrated that the seed extract of *S. crassifolia* contains a considerable amount of polyphenols. Phenolic compounds derived from secondary metabolism of plants are important natural antioxidants (Atoui et al., 2005).

In general, terpenes with antioxidant properties are rare, but some examples are common in species of the family Celastraceae, such as rosmanol, celastrol, maytenin, and pristimerin (Corsino et al., 2000; Carvalho et al., 2005). Among these compounds, two quinone-methide triterpenes have already been isolated from *S. crassifolia*: pristimerin and maytenin (Lima et al., 1969; Santana et al., 1971; Carvalho et al., 2005). Therefore, *S. crassifolia* antigenotoxicity may be attributed, at least partially, to the action of phenolic compounds and quinone-methide triterpenes present in this species.

Regarding *S. crassifolia* anticytotoxicity, in our study, EAF and HAF protected the mouse bone marrow cells against the cytotoxic effects of MMC, while the HEF significantly increased the cytotoxicity induced by MMC at all doses and times analyzed. A compound that inhibits MMC genotoxicity can also inhibit its cytotoxicity and prevent irreparable damage to DNA that would lead to cell death. On the other hand, it has previously been demonstrated that the association of MMC with other antitumor agents may cause an increase in their cytotoxic effects due to an increase in apoptosis induction (Kraut and Drnovsek-Olup, 1996). This fact was also observed in our study using the HEF of *S. crassifolia*, suggesting a synergistic action of this fraction with the positive control.

Studies carried out with triterpenes isolated from *Maytenus diversifolia* (Celastraceae) showed significant antileukemic activity and high cytotoxicity against A-549 lung carcinoma cells (Nozaki et al., 1990). Other studies with species of the same family showed that sesquiterpene pyridine alkaloids from *M. aquifolium*, quinone-methide triterpenes from *S. liana*, and

phenolic triterpenes from *Cheiloclinium cognatum* are all promising secondary metabolites for the therapy and chemoprevention of cancer, particularly because they exhibit potent cytotoxic and antioxidant activities (Corsino et al., 1998; Jeller et al., 2004).

In summary, our results indicated that none of the fractions tested of *S. crassifolia* stem bark exhibited genotoxic effects in mice, but did demonstrate strong antigenotoxic effects, suggesting the presence of phenolic compounds and quinone-methide triterpenes. Furthermore, the cytotoxicity of *S. crassifolia* was only evident in HEF. In association with MMC, EAF and HAF protected the mouse bone marrow cells against the cytotoxic effect of this positive control, whereas the HEF increased the cytotoxicity induced by MMC, suggesting a synergistic action of this fraction with the positive control. Therefore, the antigenotoxicity demonstrated by *S. crassifolia*, as well as the increased cytotoxic action of HEF when co-administered with MMC, may provide important information for future developments of novel cancer therapies.

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