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Assessment of toxic, genotoxic, antigenotoxic, and recombinogenic activities of *Hymenaea courbaril* (Fabaceae) in *Drosophila melanogaster* and mice

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ABSTRACT. *Hymenaea courbaril* L., popularly known as jatobá, is a plant species that grows in the forests of South America. The species has been used for culinary purposes and in folk medicine to treat arthritis and inflammations. Due to the increasing use of this plant globally, the present study aimed to evaluate the toxic, genotoxic, recombinogenic, and antigenotoxic effects of *H. courbaril* sap (Hycs) using the mouse bone marrow micronucleus test and the somatic mutation and recombination test (SMART) in *Drosophila melanogaster*. To evaluate the aneugenic and clastogenic activities revealed by the micronucleus test, the animals were treated with 3 doses of Hycs (5, 10, and 15 mL/kg body weight). To evaluate the antianeugenic and anticlastogenic activities, the animals were simultaneously treated with Hycs and mitomycin C (4 mg/kg body weight). To assess the mutagenic and recombinogenic activities using SMART, 3-day-old larvae derived from standard and high bioactivation crosses were treated with 3 doses of Hycs (3.0, 1.5, and 0.3 mL) for approximately 48 h. To evaluate antimutagenic and antirecombinogenic

activities, larvae derived from both crosses were co-treated with 3 doses of Hycs (3.0, 1.5, and 0.3 mL) and doxorubicin (0.125 mg/mL). The mouse bone marrow micronucleus test revealed that Hycs exhibited no cytotoxic, clastogenic and/or aneugenic effects, but did show anticytotoxic, anticlastogenic and/or antianeugenic activities. The SMART revealed no mutagenic or recombinogenic effects, but antimutagenic and antirecombinogenic activities were observed in somatic cells of *D. melanogaster* from both crosses.

Key words: Jatobá; Antianeugenic; Anticlastogenic; Antimutagenic; Antirecombinogenic

INTRODUCTION

For centuries, plants have been widely used as food and for medicinal purposes in several cultures. In the last few years, interest in medicinal plants has increased worldwide. Because of the immense diversity of flora around the world and cultural factors, the use of medicinal plants in the form of crude extracts, infusions, or plasters has experienced a revival as a common approach for the treatment of several diseases (Samuelsson, 2004; Marques and Farah, 2009). Despite the increased use of plants as complementary or alternative medicines (Meijerman et al., 2006), in many cases, scientific evidence of their therapeutic efficacy or their potential health risks is still limited (Pereira et al., 2008; Vilar, 2011).

Consequently, the indiscriminate use of plants can pose risks to human health, since many plants possess toxic compounds that can induce mutation in somatic or germ cells. In contrast, plant consumption could decrease the rate of DNA damage accumulation and may be an effective strategy for either the modulation or the inhibition of mutagenic and carcinogenic processes (Ferguson et al., 2004; De Flora and Ferguson, 2005). Therefore, it is very important to assess the mutagenic potential or modulating activity of plant extracts (Siddique et al., 2008).

Hymenaea courbaril L. (Fabaceae), popularly known in Brazil as jatobá, is a tropical species that grows in semi-deciduous forests in South America. For centuries, the species has been used in Brazil for culinary purposes and in folk medicine to treat arthritis, gastric dysfunction, inflammations, and respiratory diseases. Plants of the genus *Hymenaea* are commonly used in traditional Brazilian medicine to treat inflammatory processes, bacterial infections, rheumatism, and anemia (Gazzaneo et al., 2005; Gonçalves et al., 2005; Agra et al., 2007).

The trunk of *H. courbaril* exudes a sap that is used in folk medicine for the treatment of wounds, bronchitis, and stomach disorders. Analgesic and anti-inflammatory activities have also been observed in the hydroalcoholic extract obtained from the bark of this species. The jatobá fruit produces a high-fiber flour containing 66 g/kg protein, which offers potential for the production of high-fiber snacks (Nogueira et al., 2001).

A phytochemical analysis of *H. courbaril* revealed the presence of phenolic compounds, such as tannins, flavonoids and procyanidins, essential oils, and terpenes, in the resin exuded by the trunk, as well as in the extracts of bark, leaves, fruits, and sap. Flavonoids have antioxidant activities, remove free radicals, and exhibit antigenotoxic activity. Procyanidins show a variety of activities, such as antioxidative, anti-inflammatory, and tyrosinase inhibition (Miyake et al., 2008); however, they may have genotoxic effects in high concentrations

(Nogueira et al., 2001; Park et al., 2004).

Due to the broad utilization of jatobá herein described, the aim of the present study was to evaluate the toxic, genotoxic, recombinogenic, and antigenotoxic activities of *H. courbaril* sap (Hycs) using the micronucleus test in mice (Heddle, 1973) and the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* (Graf et al., 1984).

The micronucleus test in mice is used to detect the genotoxicity of chemicals through their ability to induce the formation of small membrane-bound DNA fragments. The evaluation of micronucleus induction is very important in *in vivo* genotoxicity tests and it is recommended by regulatory agencies around the world as part of product safety assessments. The assay detects both clastogenic and aneugenic effects (Ouanes et al., 2003).

SMART, using wings of *D. melanogaster*, is a flexible and sensitive short-term test for the detection of genotoxicity of inductor agents, and has been proven to be the most suitable technique for the detection of recombinogenic activity of genotoxic agents (Graf et al., 1984, 1989; Graf and van Schaik, 1992; Spanó et al., 2001). Furthermore, this assay allows the simultaneous detection and quantification of mitotic recombination and mutations (Andrade et al., 2004).

MATERIAL AND METHODS

Plant material

The Hycs was collected in Pirenópolis, in the State of Goiás, Brazil. A voucher specimen was deposited at the Herbarium of Universidade Federal de Goiás (No. 22.972/UFG). The sap was extracted through incisions in the trunk of *H. courbaril*, transferred to glass flasks, and kept at 5°C until use in experiments.

Mouse bone marrow micronucleus test

Animals

This protocol was approved by the Animal Research Ethics Committee of Universidade Federal de Goiás (CEP/UFG No. 183/2011).

Healthy, young male adult outbred mice (*Mus musculus*, Swiss-Webster strain), obtained from the Central Animal House of Universidade Federal de Goiás, were randomly allocated to treatment groups. All animals were brought to the laboratory 7 days before the experiments and housed in plastic cages (40 x 30 x 16), in groups of 5 animals, at 24 ± 2°C and 55 ± 10% humidity, with a 12-h light-dark natural cycle. All animals received food (appropriate commercial rodent diet, Labina, Ecibra Ltda., Inhumas, GO, Brazil) and water *ad libitum*. On the day of dosing, the animals were approximately 7-9 weeks old and weighed 25-35 g.

Experimental procedure

The experiments were performed according to protocols described in Heddle (1973) and Schmid (1975). To assess genotoxicity, 3 doses of Hycs (5, 10, and 15 mL/kg body weight) were orally administered to groups of 5 animals for each treatment. To evaluate antigenotox-

icity, groups of 5 animals were orally treated with 3 doses of Hycs (5, 10, and 15 mL/kg) simultaneously with mitomycin C (MMC, 4 mg/kg, lot No. 237AEL, Bristol, Mayers Squibb, São Paulo, SP, Brazil) administered intraperitoneally. For both experiments, a positive control group (PC, 4 mg/kg MMC) and a negative control group (NC, sterile distilled water) were included. Mice were euthanized by cervical dislocation 24 and 48 h after Hycs administration.

After the treatment period (24 and 48 h), mice femurs were dissected, opened, and the bone marrow cells were gently flushed out with fetal calf serum (lot No. 30721063, Laborclin, Campinas, SP, Brazil), and centrifuged (300 *g*, 5 min). The bone marrow cells were smeared on glass slides, coded for blind analysis, air-dried, and fixed with absolute methanol (CH_4O, lot No. 55026, Synth, Diadema, SP, Brazil) for 5 min at room temperature. The smears were stained with Giemsa (lot No. 1081, Doles, Goiânia, GO, Brazil), dibasic sodium phosphate ($Na_2HPO_412H_2O$, lot No. 982162, Vetec, Duque de Caxias, RJ, Brazil), and monobasic sodium phosphate ($NaH_2PO_4H_2O$, lot No. 983831, Vetec) for the detection of micronucleated polychromatic erythrocytes (MNPCE) (Heddle, 1973).

In order to evaluate the genotoxic and/or antigenotoxic effects, we prepared 4 slides for each mouse, and scored 2000 polychromatic erythrocytes (PCE) to determine the frequency of MNPCE. Cytotoxicity was evaluated by the ratio of PCEs to normochromatic erythrocytes (NCE) (PCE/NCE). We counted the frequency of both PCE and NCE; when the PCE count reached 2000 cells, the frequency for NCE was scored. The slides were analyzed by microscopy (10 x 100, BH-2 microscope, Olympus, Center Valley, PA, USA).

Statistical analysis

To analyze the genotoxic activity of Hycs, we compared the frequency of MNPCE detected in the treated groups to the results obtained from the NC. To analyze the antigenotoxic activity of Hycs, the frequency of MNPCE in the treated groups was compared with the results of the PC. One-way analysis of variance (ANOVA) was applied, followed by all pairwise comparisons via a multiple comparison procedure (Tukey test) and a value of $P < 0.05$ was used as the criterion of statistical significance.

To evaluate the cytotoxicity of Hycs, the PCE/NCE ratio of all treated groups was compared with the results obtained from the NC. To evaluate the anticytotoxicity of Hycs, the PCE/NCE ratio of all treated groups was compared with the results obtained from the PC. A nonparametric χ^2 test was applied to determine the statistical significance of the results and a value of $P < 0.05$ was considered to be significant.

SMART

Drosophila strains and crosses

Three strains of *D. melanogaster* were used for the SMART wing assay: 1) multiple wing hairs (mwh): *y;mwh j* (*mwh*, 3-0.3); 2) flare^3 (*flr^3*, 3-38.8) [*flr^3/In*(*3LR*)*TM3*, *ri p^p sep I*(*3*)*89Aabx^{34e}* and *Bd^S*]; 3) ORR/ORR; *flr^3/In*(*3LR*)*TM3*, *ri p^p sep I*(*3*)*89Aabx^{34e}* and *Bd^S*. To produce the standard (ST) cross, stocks of flare^3 virgin females were crossed with stocks of mwh males (Graf et al., 1989). The high bioactivation (HB) cross was obtained by crossing *ORR;*flare^3 virgin females with mwh males (Graf and van Schaik, 1992). The HB cross is

characterized by a high sensitivity to promutagens and procarcinogens because the ORR;flr^3/TM3,Bd^S strain carries chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line, which is characterized by an increased level of cytochrome P-450 (Saner et al., 1996).

Both crosses produce two types of progeny phenotypically distinguished by the *Bd*^{S} marker, i.e., marker-heterozygous (MH) flies (*mwh +/+ flr*^{3}) with phenotypically wild-type wings or balancer-heterozygous (BH) flies (*mwh/TM3*, *Bd*^{S}) with phenotypically serrate wings. A detailed analysis of genetic markers is provided in Lindsley and Zimm (1992).

Experimental procedure

The experiments were performed according to methods described in Graf et al. (1984). Eggs from both crosses were collected over an 8-h period and maintained in culture bottles containing a solid agar base (3%, w/v) covered with a layer of live yeast (*Saccharomyces cerevisiae*) supplemented with sucrose. Third instar (72 ± 4 h) larvae were removed from the culture bottles, washed in tap water, and collected in a stainless steel strainer.

Batches of equal numbers of larvae (100 larvae/dose) were collected from the two crosses and transferred to glass vials containing 0.9 g mashed potato flakes hydrated with Hycs or PC and NC. To assess toxic potential, we employed different doses of Hycs (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2, 0.25, 0.28, 0.3, 1.5, and 3.0 mL) and generate larval survival curves. To assess genotoxicity, 3 doses of Hycs (0.3, 1.5, and 3.0 mL) were administered to the larvae. To evaluate antigenotoxicity, 3 doses of Hycs (0.3, 1.5, and 3.0 mL) were administered to the larvae simultaneously with doxorubicin (DXR, 0.125 mg/mL, Doxolen lyophilized, Eurofarma Laboratórios Ltda., São Paulo, SP, Brazil, CAS No. 23214-92-8). NC (distilled water) and PC (DXR, 0.125 mg/mL) treatments were included in both experiments. Larvae were allowed to feed on the medium for the remainder of their larval life (~48 h). The experiments were performed at 25°C and 60% relative humidity.

After hatching, flies were killed and stored in 70% ethanol, in which they remained until used for analysis. Wings of the MH flies were removed, mounted on glass slides with Faure's solution (30 g gum arabic, 20 mL glycerol, 50 g chloral hydrate, and 50 mL water), and examined for spots using a compound microscope at 400X magnification. The wings of BH flies were mounted and analyzed whenever a positive response was obtained in the MH progeny. During the analysis, the positions of the spots were recorded according to wing section (Graf et al., 1984).

Single spots resulted from point mutations, chromosome aberrations, or recombination events, while twin spots (flare and mwh) were produced by mitotic recombination between the proximal flare marker and the centromere of chromosome 3. A comparison of the results obtained from MH and BH flies was used to quantify the recombinogenic potential (Frei et al., 1992; Frei and Würgler, 1995; Spanó et al., 2001).

Statistical analysis

The frequencies of spots per fly were compared with the concurrent control series according to Frei and Würgler (1988, 1995). Statistical comparisons were made using the conditional binomial test, according to Kastenbaum and Bowman (1970) and followed a multiple-decision procedure (Frei and Würgler, 1988), in which four potential diagnoses could

be obtained: positive, weakly positive, negative, or inconclusive. A value of $P < 0.05$ was considered to be significant.

Because of the weak expression of the flr^3 marker in small clones and its lethality in large clones of mutant cells (Graf, 1995), only the mwh clones (mwh single and mwh twin spots) were used to calculate the clone formation frequencies per 10^5 cells. These values were then employed to estimate the contribution of recombination and mutation to the incidence of total mutant spots per fly in trans-heterozygous flies (Andrade et al., 2004). The inhibition percentages (IP) were calculated using the total number of spots per wing with the following formula (Abraham, 1994):

$$IP = \frac{[\text{genotoxin alone} - \text{genotoxin plus Hycs}] \times 100}{\text{genotoxin alone}}$$

RESULTS

Mouse bone marrow micronucleus test

The results obtained from mouse bone marrow cells 24 and 48 h after administration of Hycs and Hycs co-treated with MMC are shown in Table 1.

Table 1. Frequency of micronucleated polychromatic erythrocytes and polychromatic and normochromatic erythrocyte ratio observed in bone marrow of mice treated with *Hymenaea courbaril* sap and co-treated with mitomycin C and their respective controls.

Treatment	MNPCE/2000 PCE		PCE/NCE
	Individual data	Means ± SD	
24 h NC	3, 2, 3, 4, 4	3.2 ± 0.84	1.20 ± 0.07
Hycs			
5 mL/kg	1, 2, 2, 3, 2	2.0 ± 0.7[a]	1.25 ± 0.08[a]
10 mL/kg	3, 3, 4, 1, 2	2.6 ± 0.89[a]	1.36 ± 0.07[a]
15 mL/kg	4, 4, 3, 3, 3	3.4 ± 0.55[a]	1.21 ± 0.11[a]
24 h PC	34, 32, 33, 30, 34	32.6 ± 1.67	0.55 ± 0.03
Hycs + 4 mg/kg MMC			
5 mL/kg	28, 26, 25, 27, 28	26.8 ± 1.3[d]	0.54 ± 0.02[c]
10 mL/kg	18, 21, 13, 17, 11	16.0 ± 4.0[d]	0.67 ± 0.17[d]
15 mL/kg	10, 12, 14, 14, 14	12.8 ± 1.79[d]	0.83 ± 0.09[d]
48 h NC	3, 2, 3, 4, 4	3.2 ± 0.84	1.20 ± 0.07
Hycs			
5 mL/kg	1, 3, 4, 3, 3	2.8 ± 1.09a[b]	1.23 ± 0.09[a]
10 mL/kg	2, 3, 2, 3, 1	2.2 ± 0.84[a]	1.20 ± 0.17[a]
15 mL/kg	3, 3, 4, 4, 5	3.8 ± 0.84[a]	1.27 ± 0.06[a]
48 h PC	10, 12, 11, 12, 14	11.8 ± 1.48	0.70 ± 0.06
Hycs + 4 mg/kg MMC			
5 mL/kg	6, 7, 6, 7, 7	6.6 ± 0.55[d]	0.92 ± 0.07[d]
10 mL/kg	5, 3, 8, 8, 7	6.2 ± 2.17[d]	0.87 ± 0.12[d]
15 mL/kg	6, 5, 3, 6, 7	5.4 ± 1.51[d]	0.95 ± 0.04[d]

MNPCE = micronucleated polychromatic erythrocyte; PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte; SD = standard deviation; NC = negative control, distilled water; Hycs = *Hymenaea courbaril* sap; PC = positive control, mitomycin C (MMC, 4 mg/kg). All results were compared with their respective control group at the respective time. [a]No significant differences compared with the NC ($P > 0.05$); [b]Significant differences compared with the NC group ($P < 0.05$); [c]No significant differences compared with the PC group ($P > 0.05$); [d]Significant differences compared with the PC group ($P < 0.05$).

The NC treatment resulted in a low MNPCE value (3.2), and PC caused a significant increase in MNPCE compared with the NC (32.6 and 11.8 at 24 and 48 h, respectively; $P < 0.05$). This result is in accordance with the expected value and confirms the sensitivity of the test.

The groups that received 5, 10, and 15 mL/kg Hycs showed means of 2.0, 2.6, and 3.4 (24 h), and 2.8, 2.2, and 3.8 (48 h) of MNPCE (per 2000 PCEs), respectively. Consequently, none of the doses tested indicated significant increases in MNPCE frequencies (either at 24 or 48 h) when compared with the NC ($P > 0.05$), leading to the conclusion that Hycs does not harbor a genotoxic effect.

With respect to cytotoxicity, the PCE/NCE ratio was 1.25, 1.36, and 1.21 (24 h), and 1.23, 1.20, and 1.27 (48 h) in groups treated with 5, 10, and 15 mL/kg Hycs, respectively, whereas it was 1.20 in the NC group. Thus, Hycs exhibited no cytotoxicity effect 24 or 48 h after treatment ($P > 0.05$).

In the antigenotoxicity evaluation, the MNPCE means (per 2000 PCEs) were 26.8, 16.0, and 12.8 (24 h), and 6.6, 6.2, and 5.4 (48 h) for the groups exposed to 5, 10, and 15 mL/kg Hycs added with MMC, respectively, whereas in the PC group they were 32.6 (24 h) and 11.8 (48 h). These results demonstrate that the simultaneous treatment with Hycs and MMC led to a significant reduction in the frequency of MNPCE at all doses, compared with treatments of MMC alone ($P < 0.05$). The results indicate decreases in the MNPCE frequency of 17, 50, and 55% (24 h), and 33, 35, and 43% (48 h). Thus, our results showed that Hycs strongly modulates the genotoxic activity of MMC, demonstrating its antigenotoxic effect.

In the evaluation of the anticytotoxic activity of Hycs, we observed increases in the PCE/NCE ratio at all doses of Hycs co-treated with MMC compared with the PC group ($P < 0.05$). Therefore, using the simultaneous treatment (Hycs + MMC), Hycs attenuated the cytotoxic action of MMC.

SMART

The larval survival curves according to Hycs dosage are shown in Figure 1. Based on the survival curves generated, none of the Hycs doses used (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2, 0.25, 0.28, 0.3, 1.5, and 3.0 mL) had significant effects on the number of survivors and, consequently, the doses tested were not toxic to *D. melanogaster* larvae.

In this study, DXR alone produced a positive response in both MH and BH descendants of ST and HB crosses, indicating that this compound was genotoxic in this assay (Tables 2 and 3). This finding is in accordance with the expected value and confirms the sensitivity of the test.

The frequencies of mutant spots observed in MH and BH descendants from ST cross individuals treated with Hycs alone or co-treated with DXR are shown in Table 2. In the genotoxicity evaluation, the 3 doses of Hycs employed (0.3, 1.5, and 3.0 mL) did not result in significant increases ($P > 0.05$) in the frequency of any category of spots or total spots, leading to the conclusion that, at these doses, Hycs is not genotoxic.

In the antigenotoxicity evaluation in the ST cross (Table 2), Hycs reduced the genotoxicity induced by DXR in all mutant spot categories in MH and BH descendants. Inhibition in the MH descendants from the ST cross ranged from 67.00 to 75.46%.

The frequencies of mutant spots observed in MH and BH descendants from HB cross individuals treated with Hycs alone or co-treated with DXR are shown in Table 3. In the genotoxicity evaluation, none of the 3 doses of Hycs (0.3, 1.5, and 3.0 mL) resulted in significant

increases (P > 0.05) in the frequency of any category of spots or total spots, leading to the conclusion that, at the doses tested, Hycs is not genotoxic to the HB cross.

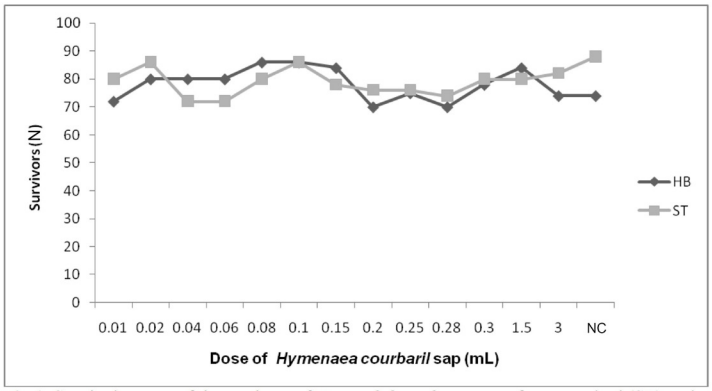

Figure 1. Survival curves of descendants of *Drosophila melanogaster* from standard (ST) and high bioactivation (HB) crosses fed different doses (0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.15, 0.2, 0.25, 0.28, 0.3, 1.5, and 3 mL) of *Hymenaea courbaril* sap; NC = negative control.

Table 2. Frequency of mutant spots observed in marker-heterozygous (MH) and balancer-heterozygous (BH) descendants of *Drosophila melanogaster* from standard (ST) cross treated with *Hymenaea courbaril* sap and co-treated with doxorubicin.

Treatment and genotype		Flies (N)	Frequency of mutant spots per individual (No. of spots)/statistical diagnosis[a]				Spots with mwh clone (n)[c]	Frequency of clone formation/10^5 cells[d] (n/NC)[e]	Recombination (%)[f]	Inhibition (%)
DXR (mg/mL)	Hycs (mL)		Small single spots (1-2 cells)[b] (m = 2)	Large single spots (>2 cells) (m = 5)	Twin spots (m = 5)	Total spots (m = 2)				
mwh/flr^3										
0[g]	0	40	0.58 (23)	0.20 (8)	0.05 (2)	0.83 (33)	33			
0	0.3	40	0.15 (6)/-	0.05 (2)/-	0.00 (0)/i	0.20 (8)/-	8			
0	1.5	40	0.08 (3)/-	0.25 (10)/-	0.00 (0)/i	0.33 (13)/-	13			
0	3.0	40	0.13 (5)/-	0.05 (2)/-	0.00 (0)/i	0.18 (7)/-	7			
0.125	0	40	2.80 (112)/+	3.65 (146)/+	3.1 (124)/+	9.55 (382)/+	330	16.91	84.86	
0.125	0.3	40	1.88 (75)/*	0.85 (34)/*	0.45 (18)/*	3.18 (127)/*	109	5.58	82.62	67.00
0.125	1.5	40	1.63 (65)/*	0.90 (36)/*	0.53 (21)/*	3.05 (122)/*	101	5.17	86.07	69.43
0.125	3.0	40	1.48 (59)/*	0.55 (22)/*	0.30 (12)/*	2.33 (93)/*	81	4.15	86.51	75.46
mwh/TM3										
0	0	40	0.18 (7)/-	0.10 (4)/-	[h]	0.28 (11)/-	11	0.56		
0.125	0	40	0.93 (37)/+	0.33 (13)/+		1.25 (50)/+	50	2.56		
0.125	0.3	40	0.30 (12)/*	0.18 (7)/*		0.48 (19)/*	19	0.97		
0.125	1.5	40	0.25 (10)/*	0.10 (4)/*		0.35 (14)/*	14	0.72		
0.125	3.0	40	0.20 (8)/*	0.08 (3)/*		0.28 (11)/*	11	0.56		

Marker-heterozygous flies (*mwh/flr^3*) and balancer-heterozygous flies (*mwh/TM3*) were evaluated. DXR = doxorubicin (0.125 mg/mL). Hycs = *Hymenaea courbaril* sap. [a]Statistical diagnosis according to Frei and Würgler, 1995: - = negative; + = positive; i = inconclusive; m = multiplication factor. *Significant difference compared with the negative control group (NC) (P < 0.05). [b]Including rare *flr^3* single spots. [c]Considering *mwh* clones from *mwh* single and twin spots. [d]Frequency of clone formation: clones/wings/24,400 cells (Abraham and Graf, 1996). [e]n = number of spots mwh; NC = number of cells; [f]Percentage of recombination (R) calculated according to Frei and Würgle, 1995: R = 1 - [(n/NC in mwh/TM3 flies) / (n/NC in mwh/flr^3 flies)] x 100. Control corrected frequencies were used for these calculations. [g]Negative control: distilled water. [h]Only mwh single spots can be observed in balancer-heterozygous individuals.

Table 3. Frequency of mutant spots observed in marker-heterozygous (MH) and balancer-heterozygous (BH) descendants of *Drosophila melanogaster* from high bioactivation (HB) cross treated with *Hymenaea courbaril* sap and co-treated with doxorubicin.

Treatment and genotype		Flies (No.)	Frequency of mutant spots per individual (No. of spots)/statistical diagnosis[a]				Spots with mwh clone (n)[c]	Frequency of clone formation/10^5 cells[d] (n/NC)[e]	Recombination (%)[f]	Inhibition (%)
DXR (mg/mL)	Hycs (mL)		Small single spots (1-2 cells)[b] (m = 2)	Large single spots (> 2 cells) (m = 5)	Twin spots (m = 5)	Total spots (m = 2)				
mwh/flr[3]										
0[g]	0	40	0.80 (32)	0.15 (6)	0.08 (3)	1.03 (41)	41			
0	0.3	40	0.20 (8)/-	0.10 (4)/-	0.00 (0)/i	0.30 (12)/-	11			
0	1.5	40	0.13 (5)/-	0.05 (2)/-	0.00 (0)/i	0.18 (7)/-	5			
0	3.0	40	0.08 (3)/-	0.03 (1)/-	0.00 (0)/i	0.10 (4)/-	4			
0.125	0	40	4.60 (184)/+	2.08 (83)/+	1.70 (68)/+	8.38 (335)/+	267	13.68	86.92	
0.125	0.3	40	1.25 (50)/*	0.45 (18)/*	0.43 (17)/*	2.13 (85)/*	68	3.48	72.13	74.56
0.125	1.5	40	0.85 (34)/*	0.45 (18)/*	0.48 (19)/*	1.78 (71)/*	52	2.66	77.07	80.55
0.125	3.0	40	1.10 (44)/*	0.53 (21)/*	0.30 (12)/*	1.93 (77)/*	65	3.33	84.68	75.66
mwh/TM3					[h]					
0	0	40	0.18 (7)/-	0.10 (4)/-		0.20 (8)/-	8	0.41		
0.125	0	40	0.93 (37)/+	0.33 (13)/+		0.88 (35)/+	35	1.79		
0.125	0.3	40	0.30 (12)/*	0.18 (7)/*		0.48 (19)/*	19	0.97		
0.125	1.5	40	0.25 (10)/*	0.10 (4)/*		0.30 (12)/*	12	0.61		
0.125	3.0	40	0.20 (8)/*	0.08 (3)/*		0.25 (10)/*	10	0.51		

For letters a-h and abbreviations, see legend to Table 2.

In the antigenotoxicity evaluation in the HB cross (Table 3), Hycs reduced the genotoxicity induced by DXR in all mutant spot categories in MH and BH descendants. The inhibition in MH descendants from the HB cross ranged from 74.56 to 80.55%. Therefore, our results showed that Hycs strongly modulates the genotoxic activity of DXR, demonstrating its antigenotoxic effect.

Based on the clone induction frequency per 10^5 cells, we compared the number of observed spots in MH and BH flies and quantified the relative contribution (%) of mutation and recombination to the total number of observed spots (Frei et al., 1992; Graf et al., 1992; Abraham, 1994). Comparing the clone induction frequency obtained with DXR alone between genotypes, we observed that 15.14% of the mutant clones produced in ST flies were a result of mutation and 84.86% resulted from recombination. Similarly, 13.08% of the spots induced in HB flies occurred due to mutation and 86.92% due to recombination. In ST cross descendants, the recombination rates resulting from DXR in combination with 0.3, 1.5, and 3.0 mL Hycs were 82.62, 86.07, and 86.51%, respectively. Additionally, in HB cross descendants, the frequency of recombination produced by DXR in combination with 0.3, 1.5, and 3.0 mL Hycs were 72.13, 77.07, and 84.68%, respectively. These findings indicate that the induced spots were mainly due to recombination (Tables 2 and 3).

DISCUSSION

The aim of the present study was to evaluate the genotoxic, recombinogenic, antigenotoxic, toxic, and/or cytotoxic activities of Hycs using the micronucleus test in mice and SMART in *D. melanogaster*.

The mouse bone marrow micronucleus test consists of a short-term assay widely used to detect the genotoxic (clastogenic and/or aneugenic), antigenotoxic, and cytotoxic effects of

substances *in vivo*. Micronuclei in young erythrocytes arise primarily from acentric fragments or chromosomes that are unable to migrate and follow the mitotic spindle during cell division. An increase in the MNPCE frequency is an indication of induced chromosome damage (Ouanes et al., 2003).

SMART was developed using *D. melanogaster* to detect the loss in heterozygosity of suitable genetic markers that have detectable phenotypes expressed on the wings. This assay is an efficient and quick method for quantifying the recombinogenic and mutagenic potential of chemical and physical agents (Graf et al., 1996; Vogel et al., 1999; Spanó et al., 2001).

The genotoxic assessment of Hycs using the mouse bone marrow micronucleus test did not show any significant increase in MNPCE frequency in groups treated with the 3 different Hycs doses compared with the NC group ($P > 0.05$). This indicates that Hycs did not exhibit genotoxic (aneugenic and/or clastogenic) effects in PCE in mouse bone marrow.

The bone marrow micronucleus test used in this study also detects cytotoxic effects through the PCE/NCE ratio. When the normal 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 indicated that Hycs did not present cytotoxicity at any dose applied since no significant reductions in the PCE/NCE ratio were observed compared with the NC group ($P > 0.05$).

In the antigenotoxicity evaluation, we observed that Hycs acted effectively against the genotoxic activity of the alkylating agent MMC at all doses tested, which suggests the possible presence of antigenotoxic compounds.

The results of the genotoxic assessment by SMART showed that Hycs did not induce a significant increase in the frequencies of any class of spots in either the ST or HB crosses. These results indicate that Hycs did not induce somatic mutation or recombination in *D. melanogaster*.

In the antigenotoxicity evaluation of Hycs by SMART, the chronic co-treatment of ST and HB larvae with DXR and different concentrations of Hycs led to significant reductions in all 3 categories of spots and in total spots in flies when compared with the PC group ($P < 0.05$). These results indicate that Hycs acts as an antigenotoxic agent on DXR-induced DNA lesions.

Previous studies have demonstrated that the wing spot test in *Drosophila* is suitable for the detection of recombinogenic activity of genotoxic chemicals (Spanó et al., 2001). Using MH and BH flies, it is possible to separate mutational events from recombinational events (Lehmann et al., 2003).

Comparing the number of observed spots in MH and BH flies in both crosses, we found that the induced spots were mainly due to recombination. We also observed that Hycs displays both antimutagenic and antirecombinogenic activity.

Some plants may possess substances that can modulate the genotoxicity of other compounds. Antigenotoxic activity of substances derived from plants may be due to a variety of mechanisms, such as inhibition of genotoxic effects, signal transduction, antioxidant activity, scavenging of free radicals, neutralization of premutagenic or mutagenic lesions by chemical compounds or by DNA repair mechanisms, or increase in metabolic inactivation of mutagens (Siddique et al., 2008).

In the present study, Hycs revealed antigenotoxic effect against chromosome damage induced by MMC and DXR in somatic cells. The antigenotoxic effect of Hycs observed in our study might be attributed to its action in reducing alkylation and also to its antioxidant activ-

ity, since the genotoxic action of MMC is related to its ability to alkylate DNA and/or produce reactive free radicals and the genotoxic action of DXR is also related to the production of reactive free radicals, which causes different types of cellular damage, including DNA breaks (Lehmann et al., 2003; Kang et al., 2006). Thus, the primary action of Hycs might be related to protection of the nucleophilic site in DNA.

Free radicals generated by exogenous chemicals, such as MMC and DXR, or endogenous metabolites may cause oxidative damage by oxidizing biomolecules, resulting in cell death and tissue damage. The genotoxic potential is directly proportional to the number of oxidative injuries to DNA that escape the repair mechanism (Valko et al., 2004). Data from numerous experiments suggest that oxidative damage to DNA contributes to aging as well as in the etiology of many human diseases, including atherosclerosis, neurodegenerative diseases, and even cancers (Olinski et al., 2002).

Antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases. Antioxidants that scavenge free radicals may retard the process of aging and decrease the risk of age-related degenerative diseases, such as cancer, cardiovascular diseases, immune system decline, and brain dysfunction (Baydar et al., 2007).

The phytochemical analysis of *H. courbaril* revealed the presence of phenolic compounds, such as tannins, flavonoids, and procyanidins, as well as essential oils and terpenes (Nogueira et al., 2001).

Several studies have demonstrated that phenolic compounds exhibit various physiological activities. Tannins have antigenotoxic effects and flavonoids have been shown to scavenge reactive oxygen species, inhibit tumor cell growth, induce apoptosis, and exert anticarcinogenic effects. The antioxidant activity of flavonoids may both inactivate lipid peroxidation and reactive oxygen species and also have an indirect effect on *in vivo* endogenous antioxidant systems. Terpenes are a major group of chemicals present in plants and have been shown to possess antioxidative properties, particularly against lipid peroxidation. Therefore, the antigenotoxic activity detected in our experiment might be mediated, at least partially, by the action of flavonoids, tannins, and/or terpenes (Manosroi et al., 2006).

In conclusion, the present results indicate that Hycs was not toxic, clastogenic, aneugenic, mutagenic and/or recombinogenic at the doses tested. Additionally, it protected cells against the clastogenic, aneugenic, mutagenic and/or recombinogenic effects of MMC and DXR in mouse bone marrow cells and in *D. melanogaster* somatic cells, respectively. The two assays used in the present study clearly indicated that Hycs is an efficient modulator of genotoxic action and could be used in primary health care.

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