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Genotoxicity Testing of *Plantago major* Extracts in Somatic Cells of *Drosophila melanogaster*

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Plantago major is used in many parts of the world for the treatment of diseases and to promote the healing of wounds. In the present study, the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* was used to evaluate the genotoxic activity of an aqueous extract of *P. major*. The following Drosophila crosses were made: standard (ST) cross, in which virgin *flare* females (*flr*^{3}/*TM3*, *Bd*^{s}) were mated with *mwh/mwh* males, and high-bioactivation (HB) cross, in which virgin *ORR* females (*ORR/ORR*; *flr*^{3}/*TM3*, *Bd*^{s}) were mated with *mwh/mwh* males. Each cross produced two types of descendents, marker-transheterozygous (MH) (*mwh +/+ flr*^{3}) and balancer-heterozygous (BH) (*mwh +/+ TM3*, *Bd*^{s}) flies. Three-day-old larvae of both types of descendents were treated with undiluted and diluted (1:1 and 1:2 in water) aqueous extracts of *P. major*. The extracts were genotoxic in both crosses, producing similar induced frequencies in ST and HB flies. Comparison of the frequencies of wing spots in the BH and MH descendents indicated that recombination was a major response. The results indicate that, under these experimental conditions, aqueous extracts of *P. major* are genotoxic (recombinagenic). Environ. Mol. Mutagen. 45:56–61, 2005. © 2004 Wiley-Liss, Inc.

Key words: ***Plantago major*; recombinagenic activity; somatic mutation and recombination test; *Drosophila melanogaster***

INTRODUCTION

Plant extracts are commonly used in Brazilian folk medicine. The active substances of the plants are used as remedies for the treatment of many diseases (Oka and Roperto, 1999; http//www.couanet.com.br/eco/herb/tanch.nun). Oleanolic acid and ursolic acid, for example, are triterpenoid compounds that exist widely in foods, medicinal herbs, and other plants. The most notable effect of these two triterpenoids is their antitumor activity [Liu, 1995]. Polyphenolic compounds also appear to have anticarcinogenic potential [Lohman et al., 2001].

Despite increasing research on flora, only a small percentage of the approximately 250,000 species of higher plants has been chemically and pharmacologically investigated [Ruffa et al., 2002], and data on the mutagenic properties of plants commonly used in folk medicine are limited [Ruiz et al., 1996]. Green plants in general contain mutagenic and carcinogenic substances [Sandermann, 1988; Velemínský and Gichner, 1988; Kanaya et al., 1992; Plewa and Wagner, 1993], but there is little information on the biological effects of herbal medicines [Basaran et al., 1996]. Many plants also have antimutagenic and/or anticlastogenic properties [Rasquinha et al., 1988; Mitscher et al., 1996]. Alternative approaches to reducing breast cancer, for example, involve the inclusion of high levels of green tea or soybean in the diet [Lohman et al., 2001]. Plants exist in numerous varieties [Edenharder et al., 1998], which greatly complicates the analysis of their biological properties.

The *Drosophila melanogaster* somatic mutation and recombination test (SMART) has been used for investigating the genotoxicity of single compounds [Spanó et al., 2001], but also to study the genotoxicity of complex mixtures of various origins [Guzmán-Rincón and Graf, 1995; Sousa et al., 2003]. For instance, the wing spot test has been used for evaluating the genotoxicity of several kinds of beverages used for human consumption, such as different types of coffees, various herbal teas, as well as wine

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and brandy [van Schaik et al., 1984; Graf and Würgler, 1986; Abraham, 1994].

The present study uses the SMART to investigate the genotoxic and antigenotoxic properties of an aqueous extract of *Plantago major*, which is used in the treatment of many diseases around the world [Samuelsen, 2000]. *P. major* extracts have produced contradictory results in toxicity tests [Samuelsen, 2000]. They are toxic in the brine shrimp test (*Artemia salina*) [Schmeda-Hirschmann et al., 1992] and produce DNA damage in the alkaline Comet assay using human lymphocytes [Basaran et al., 1996]. The extracts were inactive, however, in *Salmonella typhimurium* strains TA100 and TA98 [Basaran et al., 1996], in the plate incorporation assay with *Aspergillus nidulans* D-30 and in the somatic segregation assay [Ruiz et al., 1996], as well as in tests of cytotoxic activity in the human hepatocellular carcinoma cell line Hep G2 [Ruffa et al., 2002]. In the present study, we find that *P. major* extracts are genotoxic in the wing SMART assay of Drosophila.

MATERIALS AND METHODS

Preparation of *Plantago major* Leaf Extract

Leaves of *P. major* were collected in the month of September from the experimental garden of the Federal University of Uberlândia in the State of Minas Gerais, Brazil. These plant materials were identified by a botanist at the Federal University of Uberlândia, and their voucher specimens (HUFU 3457-8) have been deposited at the herbarium of the institution. They were used in assays the same day. Freshly collected leaves were weighed, washed, dried at room temperature, and used for the preparation of juices. Water extracts were prepared as described by Ito et al. [1986]. Leaves were homogenized using a home mixer (Black and Decker SB30T). Juice thus obtained was filtered through gauze and termed ''undiluted extract.'' The volume of undiluted extract obtained from 500 g of vegetable motley was 250–300 mL. This extract was diluted 1:1 (one part water to one part undiluted extract) and 1:2 (one part undiluted extract to two parts water). The aqueous extracts were then used immediately in experiments.

Urethane

Ethylcarbamate or urethane (URE) ($NH_2COOCH_2CH_3$; CAS 51-79-6; MW 89.1) was obtained from Fluka (Buchs, Switzerland). It was used as a positive control since it is clearly genotoxic to *D. melanogaster* in the SMART assay using the standard (ST) cross and displays increased genotoxicity in the high-bioactivation (HB) cross. URE, a recombinagenic compound, is metabolically activated by the cytochrome P-450 enzyme system [Frölich and Würgler, 1990]. A 10 mM concentration of URE was prepared in sterile water (Mille-Q) immediately before the treatment.

Somatic Mutation and Recombination Test

Stocks of three mutant lines of *D. melanogaster* were maintained: multiple wing hairs (*mwh/mwh*), *flare*^{3} (*flr*^{3}/*TM3*, *Bd*^{s}), and *ORR* (*ORR*/*ORR*; *flr*^{3}/*TM3*, *Bd*^{s}) [Graf et al., 1984]. The *ORR* (Oregon R) strain is useful for its high constitutive expression of cytochrome P450 [Frölich and Würgler, 1989].

TABLE I. Genotoxicity of *Plantago major* Extract in the Drosophila SMART Assay: Larvae From Standard Cross

		Spots per fly (total number of spots)[a]					Clone induction frequency (per 10^5 cells per cell division)[b]	
Treatment	Number of flies	Small single spots (1–2 cells), m = 2	Large single spots (> 2 cells), m = 5	Twin spots, m = 5	Total spots, m = 2	*mwh* clones	Observed	Control corrected
MH								
Control (water)	30	0.57 (17)	0.07 (02)	0.07 (02)	0.70 (21)	20	1.37	
Extract diluted 1:2	30	1.03 (31) +	0.20 (06) i	0.17 (05) i	1.37 (41) +	40	2.73	1.36
Extract diluted 1:1	30	1.10 (33) +	0.37 (11) +	0.33 (10) +	1.80 (54) +	53	3.62	2.25
Undiluted extract	30	1.10 (33) +	0.13 (03) i	0.23 (07) i	1.43 (43) +	42	2.87	1.50
URE	30	1.73 (52) +	0.03 (01) i	0.10 (03) i	1.87 (56) +	56	3.82	2.45
BH								
Control (water)	20	0.45 (09)	0.10 (02)		0.55 (11)	11	1.13	
Extract diluted 1:2	20	0.35 (07) −	0.10 (02) i		0.45 (09) −	9	0.92	−0.21
Extract diluted 1:1	20	0.35 (07) −	0.10 (02) i		0.45 (09) −	9	0.92	−0.21
Undiluted extract	20	0.30 (06) −	0.15 (03) i		0.45 (09) −	9	0.92	−0.21
URE	20	0.90 (18) i	0.20 (04) i		1.10 (22) +	22	2.25	1.12

[a]Statistical diagnosis according to Frei and Würgler [1988]. m, multiplication factor; +, positive; −, negative; i, inconclusive. Kastenbaum-Bowman test, one-sided, probability: $\alpha = \beta = 0.05$.
[b]Frequency of clone formation: clones/flies/48,800 cells (without size correction).

The following crosses were made: ST cross, in which *flr*^{3}/*In(3LR)TM3*, *ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{s} females mated with *mwh*/*mwh* males [Graf et al., 1989]; and HB cross, in which *ORR*; *flr*^{3}/*In(3LR)TM3*, *ri p*^{p} *sep I(3)89Aa bx*^{34e} *e Bd*^{s} females mated with *mwh*/*mwh* males [Graf and van Schaik, 1992]. These crosses produce two types of progeny: marker-transheterozygous (MH) flies and balancer-heterozygous (BH) flies. The descendents are phenotypically distinct based on the *TM3*, *Bd*^{s} marker. The MH (*mwh* +/+ *flr*^{3}) flies have structurally normal chromosomes, while the BH (*mwh* +/+ *TM3*, *Bd*^{s}) flies possess a balancer chromosome with multiple inversions (*TM3*, *Bd*^{3}). MH descendents develop normal wings with smooth borders, while BH descendents have poorly formed wings with the appearance of torn or sawed borders, referred to as serrate [Guzmán-Rincón and Graf, 1995].

Mutation and recombination are detected in adult MH descendents as single or twin spots on their wings. Single spots (*mwh* or *flr*^{3}) are produced by point mutation, deletion, crossing-over at mitosis, or other causes, while twin spots (*mwh* adjacent to *flr*^{3}) are produced exclusively by mitotic recombination [Graf et al., 1984; Frei and Würgler, 1996]. The BH descendents do not detect recombination due to the multiple inversions of the balancer chromosome; BH flies detect only mutational events [Graf and Singer, 1992]. Therefore, a comparative quantitative analysis of the frequencies of wing spots in the two types of descendents permits a determination of the recombinagenic effects of genotoxins [Graf et al., 1996].

Larval Feeding

ST and HB crosses were made simultaneously and under the same conditions. Over a period of 8 hr, eggs were collected in culture bottles containing a solid agar base (4% w/v agar-agar in water) covered completely with an approximate 5 mm layer of live baker's yeast supplemented with sucrose. Three-day-old larvae in the third stage of embryonic development were collected, washed in running water, and fed for a period of 48 hr with 1.5 g of instant mashed potatoes (Yoki Alimentos, São Bernardo do Campo, Brazil) and 5.0 ml of the different concentrations of the aqueous *P. major* extract (undiluted extract, 1:1 dilution and 1:2 dilution). At these concentrations, *P. major* was not toxic to larvae. Concurrent positive controls were fed 10 mM URE. Frölich and Würgler [1990] indicate that this is the maximum concentration of URE without toxic effects in these Drosophila crosses. All experiments were conducted at 25°C and 65% relative humidity. The surviving adults were collected from the treatment vials and stored in 70% ethanol.

Slide Analysis

The fly wings were prepared for analysis in Faure's solution (30 g gum arabic, 20 ml glycerol, 50 g chloral hydrate, 50 ml water). The wings were mounted on slides with the help of entomological tweezers and a stereoscopic microscope (40×). The wings (both dorsal and ventral surfaces) were analyzed for wing spots using a compound microscope (400×). During the analysis, the positions of spots were noted according to wing sections [Graf et al., 1984, 1989].

Statistical Analysis

The frequencies of spots per fly in treated flies were compared with those of the negative control using a two-tailed chi-square test (with $\alpha \leq 0.05$) [Frei and Würgler, 1988].

RESULTS

Treatment with *P. major* extract resulted in significant increases in the total mutant spot frequency in MH des-

TABLE II. Genotoxicity of *Plantago major* Extract in the Drosophila SMART Assay: Larvae From High-Bioactivation Cross

Treatment	Number of flies	Spots per fly (total number of spots)[a] Small single spots (1–2 cells), m = 2	Large single spots (> 2 cells), m = 5	Twin spots, m = 5	Total spots, m = 2	*mwh* clones	Clone induction frequency (per 10^5 cells per cell division)[b] Observed	Control corrected
MH								
Control (water)	64	1.08 (69)	0.19 (12)	0.08 (05)	1.34 (86)	84	2.69	
Extract diluted 1:2	48	1.46 (70) w+	0.27 (13) i	0.29 (14) +	2.02 (97) w+	91	3.88	1.19
Extract diluted 1:1	60	1.40 (84) −	0.27 (16) i	0.28 (17) +	1.92 (115) w+	113	3.86	1.17
Undiluted extract	69	1.71 (118) +	0.26 (18) i	0.20 (14) +	2.17 (150) +	144	3.38	0.69
URE	60	7.28 (437) +	1.35 (81) +	0.98 (59) +	9.62 (577) +	560	19.12	16.43
BH								
Control (water)	20	0.60 (12)	0.10 (02)		0.70 (14)	14	1.43	
Extract diluted 1:2	20	0.60 (12) i	0.05 (01) i		0.65 (13) −	13	1.33	−0.1
Extract diluted 1:1	20	0.85 (17) i	0.10 (02) i		0.95 (19) i	19	1.95	0.52
Undiluted extract	20	0.70 (14) i	020 (04) i		0.90 (18) i	18	1.84	0.41
URE	20	6.60 (132) +	0.45 (09) +		7.05 (141) +	141	14.45	13.02

[a]Statistical diagnosis according to Frei and Würgler [1988]. m, multiplication factor, +, positive; w+, weakly positive; −, negative; i, inconclusive. Kastenbaum-Bowman test, one-sided, probability: $\alpha = \beta = 0.05$.

[b]Frequency of clone formation: clones/flies/48,800 cells (without size correction).

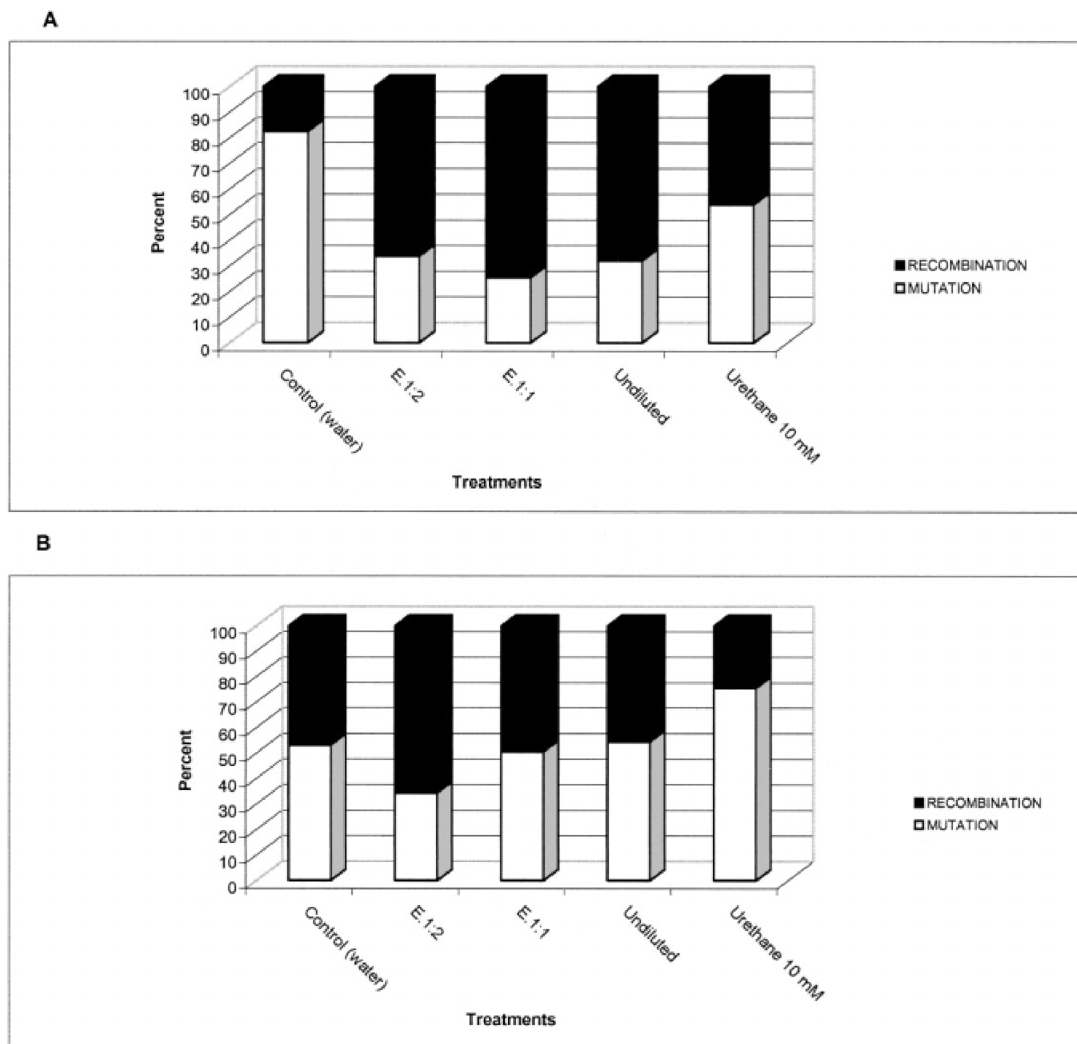

Fig. 1. Mutation and recombination in the Drosophila wing spot test after treatments with *Plantago major* and urethane. **A:** Treatment of larvae from ST cross. **B:** Treatment of larvae from HB cross.

cendants from the ST cross (Table I). Flies treated with diluted (1:1) extract had significant increases in small single, large single, and twin spots. For the other treatments (1:2 and undiluted), significant increases occurred only for the small single spots. The extract did not significantly increase the total spot frequency in BH descendents.

Undiluted and diluted *P. major* extract also produced a statistically significant increase in the total number of spots among the MH descendents of the HB cross (Table II). These increases were only weakly positive for the two dilutions. All the concentrations of extract, however, produced statistically significant increases in the frequencies of twin spots. Descendents treated with undiluted extract and the 1:2 dilution also had increases in the frequencies of small single spots. Treatment-related effects for the BH descendents were either statistically inconclusive (total spot frequencies for the undiluted extract and the 1:1 dilution) or not significant (1:2 dilution).

The treatment-related increase in the frequencies of twin spots was considered important in that this endpoint is specifically induced by mitotic recombination. These spots result from crossing-over between the flr^3 locus and the centromere [Graf et al., 1984; Abraham and Graf, 1996]. To characterize the recombinagenic activity of the extract, comparisons between the MH and BH descendents were made for both the ST and the HB crosses. For the ST cross (Fig. 1A), the proportions of events indicate that 68% of spots produced with undiluted extract were due to recombination, 74.6% with the 1:1 dilution and 66.3% with the 1:2 dilution. For the HB cross (Fig. 1B), recombination accounted for 45.6% of the events in flies treated with the undiluted extract, 49.5% with the 1:1

dilution and 65.7% with the 1:2 dilution. In each case, the comparison indicates that the remaining events were due to mutation.

DISCUSSION

In this study, we evaluated the genotoxic activity of *P. major* leaf extracts in the somatic cells of *D. melanogaster*. Statistically significant increases in genotoxicity were found in MH descendent flies for all the concentrations of the extract that were tested. However, no clear dose response was observed. A quantitative comparative analysis of the frequencies of wing spots found in BH and MH flies, as described by Graf et al. [1996], permitted us to differentiate between the recombinagenic and mutagenic activities of the extract. This comparison demonstrated that the *P. major* extract induces a relatively high frequency of recombination. Recombinagenic activity was generally higher in ST than HB flies and was particularly high in ST flies exposed to the 1:1 dilution of the extract (74.6%). It can be concluded that the metabolic activities required for the activation of *P. major* extract are also present in sufficient amounts in the larvae of the ST cross. The results of these experiments suggest that the *P. major* extract, under these experimental conditions, is a genotoxic agent.

In light of the recombinagenic activity of *P. major* extract in Drosophila, it may be significant to note that previous studies demonstrated that saline extracts of *P. major* caused DNA strand breaks in human lymphocytes as determined by the alkaline Comet assay [Basaran et al., 1996], and a 70% ethanol extract was toxic in brine shrimp (*Artemia salina*) [Schmeda-Hirschmann et al., 1992]. In addition, *P. major* extract was negative for mutagenicity in *S. typhimurium* TA100 and TA98 [Basaran et al., 1996], which is consistent with the relatively weak mutagenic effects detected in the SMART assay. We suggest that the contradictory responses for *P. major* in previous studies [Samuelsen, 2000] may partially be due to the extract being a better recombinagen than mutagen.

The chemical composition of *P. major* is very complex [Guil et al., 1997; Samuelsen, 2000]. Some reports suggest that certain constituents of *P. major* contain toxic agents, such as oxalic acid, nitrates, and erucic acid [Guil et al., 1997]. We conclude that one of these components (or mixture of components) is genotoxic for *D. melanogaster*. The ability to detect genotoxic activity in aqueous extracts of *P. major* was in large part due to the sensitivity of the SMART assay to somatic recombination along with mutation. Since *P. major* is widely used in folk medicine, it is important that other studies be conducted in order to identify its principal genotoxic components and better characterize its toxicity in relevant test systems.

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