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Lack of *in vivo* embryotoxic and genotoxic activities of orally administered stem bark aqueous extract of *Mangifera indica* L. (Vimang®)

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Abstract

Mango (*Mangifera indica* L.) stem bark aqueous extract (MSBE) is a new natural product with antioxidant, anti-inflammatory and immunomodulatory effects known by the brand name of its formulations as Vimang®. Previously, the oral toxicity studies of the extract showed a low toxicity potential up to 2000 mg/kg. This work reports the results about teratogenic and genotoxicologic studies of MSBE. For embryotoxicity study, MSBE (20, 200, or 2000 mg/kg/day) was given to Sprague–Dawley rats by gavage on days 6–15 of gestation. For genotoxicity, MSBE was administered three times during 48 h to NMRI mice. Cyclophosphamide (50 mg/kg) was used as a positive control. No maternal or developmental toxicities were observed when the rats were killed on day 20th. The maternal body-weight gain was not affected. No dose-related effects were observed in implantations, fetal viability or external fetal development. Skeletal and visceral development was similar among fetuses from all groups. No genotoxicity was observed in bone marrow erythrocytes and liver cells after administration. MSBE appears to be neither embryotoxic nor genotoxic as measured by bone marrow cytogenetics in rodents.

Keywords: Stem bark; *Mangifera indica* L.; Vimang®; Teratogenesis; Genotoxicity

1. Introduction

Mango (*Mangifera indica* L., Anacardiaceae) stem bark aqueous extract (MSBE) has been developed in Cuba as a new natural product under de brand name of Vimang® with a defined composition. Phytochemical studies of MSBE by spectrochemical techniques (IR, UV, MS and H, ^{13}C NMR) revealed that it is a mixture of polyphenols, terpenoids, steroids, fatty acids and microelements (Nuñez-Sellés et al., 2002). The polyphenols identified in MSBE are phenolic acids (gallic acid, 3-4-dihydroxy benzoic acid), phenolic esters (methyl gallate, propyl gallate, propyl benzoate), flavan-3-ols [(+)-catechin and (−)-epicatechin] and a xanthone (mangiferin) as a major component. Mangiferin, (1,3,6,7-tetradroxyxanthone-C2-D-glucoside) is present in MSBE in about 10–20%. Also the elemental composition of MSBE by induced coupled plasma (ICP) spectrometry has been reported recently. Ca, Mg, K and Fe as main components, and Cu, Zn and Se as microelements were correlated to varietal difference and plant age. Toxic elements (As, Cd and Hg) were not detected and Pb was rather below its toxic dose for human consumption (0.07 mg/100 mL in plasma) (Nuñez-Sellés et al., 2007).

MSBE has been shown to be a potent antioxidant effect both, *in vitro* and *in vivo* (Martinez et al., 2001a,b). It has scavenger activity on hydroxyl radicals and hypochlorous acid, presented a significant inhibitory effect on the peroxidation of rat brain phospholipid, and inhibited DNA damage by bleomycin or copper-phenanthroline assays. Also, it

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has analgesic, anti-inflammatory and immunomodulatory effects in several experimental models manifested as the protection against the activation-induced cell death in human T-cell (Hernandez et al., 2006, 2007), the inhibition of eosinophil generation and migration (Sa-Nunes et al., 2006), the inhibition of IgG production (Garcia et al., 2003a), antiallergic effect (Rivera et al., 2006; Garcia et al., 2003b), the inhibitions of the inducible cyclooxygenase isoforms (cyclooxygenase-2) and nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF), interleukin-1beta, granulocytes/macrophage colony-stimulating factor, nitric oxide, phospholipase A2, prostaglandin E2, leukotriene B4, and the activation by TNF of the nuclear transcription factor NF-kappaB (Garrido et al., 2006, 2005, 2004a,b, 2001; Beltran et al., 2004; Leiro et al., 2004; Garcia et al., 2002).

In acute and subchronic toxicity studies performed with MSBE (up to 2000 mg/kg of body weight) no signs of drug-related toxicity were observed (data not showed). In addition, the *in vitro* genotoxic evaluation of MSBE showed neither mutagenic (Ames test) nor clastogenic (micronucleus assay) activities (Rodeiro et al., 2006).

Accordingly, as part of the safety evaluations of MSBE, embryotoxic and genotoxic studies (Comet assay and a mouse bone marrow micronucleus test) were conducted. These tests included the evaluation of MSBE administered to rodents by oral route for developmental toxicity during organogenesis, primary DNA damage and clastogenic activity and/or disruption of the mitotic apparatus by detecting strand breaks, alkali labile sites in liver cells and micronuclei in polychromatic bone marrow erythrocytes.

2. Materials and methods

2.1. Chemicals

Agarose, fetal calf serum, tungstosilicic acid, HBSS, silver nitrate, sodium carbonate, formaldehyde, and cyclophosphamide (6055-19-2) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of reagent grade.

2.2. Plant material

Mango stem bark (*Mangifera indica* L.) was collected from a cultivated field located in the region of Pinar del Rio, Cuba (cultivar M11). Archived specimens of the plant (code: 41722) were deposited at the Herbarium of the Academy of Sciences, guarded by the Institute of Ecology and Systematic, Ministry of Science, Technology and Environmental, La Habana, Cuba. MSBE was prepared by decoction for 1 h. The extract was concentrated by evaporation and then spray-dried to obtain a fine brown powder (MSBE), which is used as the standardized active ingredient of Vimang® formulations. It melts at 210–215 °C with decomposition. The chemical composition of MSBE has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry, NMR, and UV–Vis spectrophotometry (Nuñez-Sellés et al., 2002). The elemental inorganic composition has been determined by ICP spectrometry (Nuñez-Sellés et al., 2007).

2.3. Animals

2.3.1. Embryotoxicity

Seven week old male and female Sprague–Dawley rats were obtained from the National Centre for the Production of Laboratory Animals (CENPALAB, La Habana, Cuba). Animals were housed in a room with a constant day–night cycle, at 22 ± 5 °C temperature and 55 ± 15% relative humidity. Animals were acclimated to the laboratory for at least two weeks prior to breeding. At the start of the study, female rats were approximately 11 weeks old and weighed approximately 220 g. Food (CENPALAB; La Habana, Cuba) and tap water were available *ad libitum*. On mating two females were randomly mated with one male. The following morning, the presence of sperm in the vaginal smear was considered to be evidence of copulation and the sperm-positive females were considered to be at day 0 of gestation. Random assignment of animals to test groups, grouped according to their day 0 of gestation was performed.

2.3.2. Genotoxicity

Adult female and male mice of NMRI strain (20–22 g body weight) were obtained from CENPALAB. The animals were adapted to experimental conditions and acclimated (25 ± 2 °C, 50–70% relative humidity) under a 12 h light/dark cycle for two weeks before use. They were housed individually in polycarbonates cages. Clinical signs (daily) and body weights (at the beginning and at the end of the study) were recorded. At the end of the experiment were recorded the organ weights and liver samples of each animal was collected and analyzed by histopathological studies.

2.4. Dose preparation

MSBE was suspended in a solution of Arabic gum at 1% in distilled water. Dosing suspension was prepared fresh daily and administered by gavage (10 mL/kg b.w.) at doses of 20, 200 and 2000 mg/kg/day, a vehicle group was also included. Cyclophosphamide used as a positive control for genotoxicity, embryotoxicity was dissolved in saline solution and administered by intraperitoneal injection at a dose of 50 mg/kg/day.

2.5. Study design

2.5.1. Embryotoxicity

Groups of 26 bred rats were administered by gavages with 20, 200 and 2000 mg/kg/day of MSBE from days six through 15 of gestation. Control group was dosed with vehicle under the same conditions. Another group of 13 animals was given 50 mg/kg/day cyclophosphamide intraperitoneally as positive control on gestation day 13.

The general appearance and well-being of the rats were monitored daily. The rats were weighed daily during the treatment and food consumption was measured on days 0, 6, 15 and 20 of gestation. On day 20 of gestation the dams of all experimental groups were sacrificed by diethyl ether inhalation. The uteri were extracted and examined. The position and number of implantations live and dead fetuses and resorption sites were recorded. Live fetuses were weighed individually, sexed and examined for external malformations and thereafter were euthanized with an overdose of diethyl ether. One-half of the fetuses were selected for visceral examination using the method of Stuckhart and Poppe (1984). The remaining fetuses were fixed in ethanol, cleared and stained with alizarin red S and examined for skeletal malformations (Dawson, 1926). The uteri of the dams were immersed in 10% ammonium sulfide solution to identify early entire litter resorption.

2.5.2. Genotoxicity

The study was conducted using five groups of five mice of both sexes. Three groups of both sexes were administered MSBE (20, 200 and 2000 mg/kg/day, p.o. during 4 days) and the fourth and fifth groups were administered with vehicle (1% Arabic gum in distilled water, p.o. during 4 days) and cyclophosphamide (50 mg/kg/day, i.p. 24 h before the end of the experiment) as positive control, respectively.

2.5.2.1. Comet assay. The Comet assay was essentially performed as described by Singh et al. (1988). Blood peripheral lymphocytes were purified using the Lymphoprep kit (Nycomed) and liver samples were collected, disaggregated and homogenized for analyze. Concentration cell

was adjusted to 1×10^6 cells/mL. Then, 10 μL of cell suspension was embedded in 75 μL of 0.5% low melting point agarose (LMPA) and spread on a slide pre-coated with 150 μL of 0.5% normal melting point agarose. Two slides were prepared for each sample. A cover slip was added to each slide and allowed to solidify at 4 °C. Finally, another layer of 75 μL of 0.5% LMPA was added to each slide. Slides were placed on ice for 10 min to accelerate gelling of the agarose layer and then transferred to lysis solution. Slices were incubated overnight at 4 °C and placed in an electrophoresis chamber, exposed to alkali for 20 min. Then, electrophoresis was performed for 20 min at 1.25 V/cm and 300 mA. Assay variability was reduced by ensuring that replicate slides, which were randomly distributed among multiple electrophoresis runs in a fully balanced design. After electrophoresis, slides were neutralized, dried and stained with ethidium bromide (20 μg/mL) prior to analysis.

The nucleoids were silver stained according to silver stain protocol García et al. (2004). Two hundred silver stained nucleoids per animal were evaluated with an Olympus A_2 fluorescence microscope (Fig. 1).

The stained nucleoids from each animal were classified in five levels of DNA damage and a score was calculated following the formula: DNA damage score (AU) = Nucleoids in level $1 + 2 \times$ Nucleoids in level $2 + 3 \times$ (Nucleoids in level 3) $+ 4 \times$ (Nucleoids in level 4). Then if all nucleoids were in level 0 (undamaged) the score was 0 and if all the nucleoids were in level 4 (maximally damage) the score is 400 expressed in Arbitrary Units (AU) (Collins et al., 1993).

2.5.2.2. Micronucleus test. One femur was removed from each animal at sacrifice and marrow samples were obtained by flushing a small drop of fetal bovine serum through the marrow cavity to obtain a cell suspension. The cell suspension from each mouse was centrifuged at 1000 rpm for 5 min, suspended and spread on coded slides. Then smears were prepared, fixed and stained with Giemsa (1:6 distilled water) for 10 min; air dried, cleared and analyzed for the frequency of micronuclei determined as the number of micronucleated PCEs (MnPCEs) in 2000 PCEs per mouse. The percentage of PCE from total erythrocytes was considered as a cytotoxicity index (OECD, 1997).

2.6. Statistical analysis

2.6.1. Embryotoxicity

The litter was taken as experimental unit and data were expressed as averages per litter and per group (Wier and Ventre, 1990). Maternal initial body weight, maternal weight gain and fetal body weights were analyzed using a parametric analysis of variance followed by Tukey's multiple comparison test. Data on numbers of *corpora lutea*, implantations, resorptions, dead and alive fetuses and sex ratio were analyzed by the Kruskal–Wallis (non-parametric) test, followed by Mann–Whitney *U*-test, to determine which treatment groups differed from the control. The incidence of fetuses and litters with malformations was compared to the control group by using Chi square and Fischer's exact probability tests, respectively. Linear regression from ANOVA evaluated linear trends on continuous data and the Cochran–Armitage test evaluated linear trends on proportional data. Tests of statistical significance were made at the $p < 0.05$ level.

2.6.2. Genotoxicity

Comet data was analyzed with Jonckheere test for trend and Mann–Whitney test for individual group comparisons. The frequency of micronucleated PCE was compared with Cochran–Armitage trend test and Dunnett test. The percentage of PCE was compared using ANOVA, linear trend test and Dunnett test. In the case of non-sex effect, the data was pooled to obtain more statistical significance. All tests were conducted at α level of 5%.

3. Results

3.1. Embryotoxicity

During the course of the experiment, the dams in the control- and MSBE-administered groups were in good physical condition; no maternal death occurred. However, maternal toxicity was evident in the cyclophosphamide-exposed group, where body weights were significantly lower than controls for intervals of gestation on days 15–20 and 0–20 (Table 1).

Feed consumption on days 0, 6, 15 and 20 of gestation was comparable among control- and MSBE-administered groups (Table 2). There were no significant effects on litter size, resorption rate, or fetal body weight in MSBE-administered groups of rats. A summary of external, skeletal and visceral anomalies of fetuses is presented in Table 3. The incidence of major malformations, when considering fetuses and litters with malformation, was not significantly increased ($p < 0.05$) for any of the MSBE administered

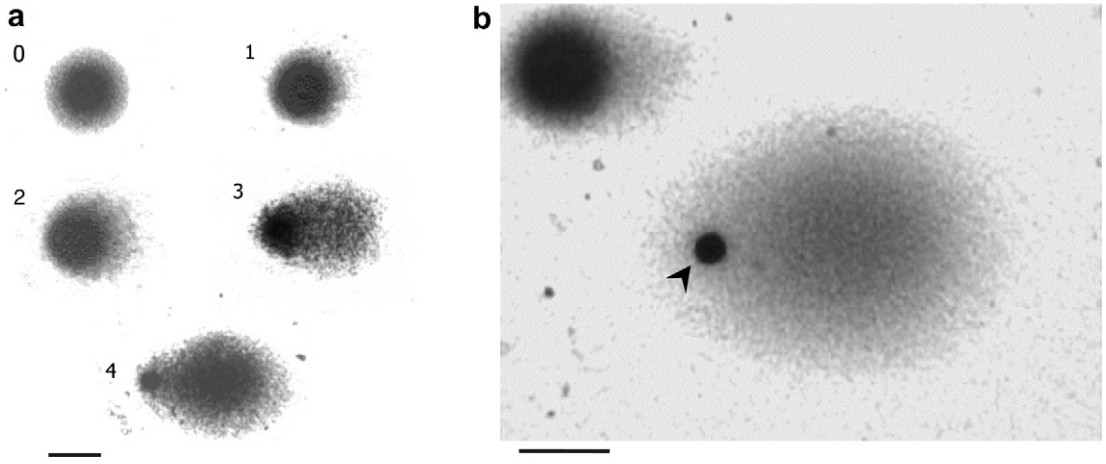

Fig. 1. (a) Silver stained nucleoids with different level of damage between category 0 (no damage) and category 4 (maximally damage). (b) Detail in a category 4 comet with very small head (arrow). The increase in the frequency of type 4 comets was considered as alert of possible apoptosis induction, which requires confirmation. Lines represent 20 μm.

Table 1
Average body weight gain during pregnancy in rat developmental toxicity study: effect of oral MSBE administration

Treatment	Dose (mg/kg)	No. of females pregnant	Body weight day = 0	Body weight gain (g)[a]			
				0–6	6–15	15–20	0–20
MSBE	0	24	236 ± 17	26 ± 9	35 ± 16	61 ± 17	122 ± 24
	20	20	235 ± 17	26 ± 6	33 ± 10	59 ± 10	118 ± 17
	200	25	240 ± 21	24 ± 12	34 ± 12	64 ± 19	122 ± 27
	2000	19	231 ± 19	25 ± 14	37 ± 13	53 ± 25	116 ± 36
CP	50	9	233 ± 23	16 ± 19	29 ± 17	10 ± 5*	55 ± 10*

CP, cyclophosphamide (positive control group); MSBE, mango stem bark extract.
* $p < 0.05$ (Mann–Whitney *U*-test).
[a] Mean ± SD.

Table 2
Overall mean daily diet consumption (g/animal) of female rats in the MSBE-orally administered peri-postnatal study

Diet consumption (g)[a]	Control	MSBE (mg/kg)			CP
		20	200	2000	50
Day 0	17 ± 4	17 ± 4	18 ± 3	18 ± 2	20 ± 2
Day 6	19 ± 3	18 ± 2	20 ± 4	19 ± 5	22 ± 2
Day 9	20 ± 2	19 ± 2	21 ± 2	20 ± 3	19 ± 6
Day 12	21 ± 3	21 ± 2	20 ± 4	21 ± 3	21 ± 5
Day 15	21 ± 3	22 ± 3	22 ± 3	22 ± 5	21 ± 3
Day 20	21 ± 3	22 ± 3	24 ± 3	21 ± 5	21 ± 3

CP, cyclophosphamide (positive control group); MSBE, mango stem bark extract.
[a] Mean ± SD.

groups compared to the control group (Table 4). Abnormal fetuses were observed in one 200 mg/kg and one 2000 mg/kg litters. Malformation in the mid-dose pup consisted in a single fetus with craneorachischisis. The high-dose fetuses had tail agenesis. No visceral or skeletal major malformations were observed in any of the fetuses examined. Skeletal examination revealed similar incidence of delayed ossification of sternebrae in the fetuses of each group. In the control group, one of the fetuses presented also a pair of extra ribs. Treatment-related developmental effects were not observed at all evaluated endpoints.

An increase in the number of resorptions and dead fetuses, and a decrease in the average weight of the pups, were observed in the group treated with cyclophosphamide. Micrognathia, exophthalmos, adactilia, ectrodactilia, gastroschisis, and umbilical hernia showed an incidence that was statistically significant ($p < 0.05$) as related to the control group.

Table 3
Maternal and fetal observations in rat during MSBE-orally administered developmental toxicity study

	Control	MSBE (mg/kg)			CP
		20	200	2000	50
Pregnant/timed-mated females	26	26	26	26	13
Pregnant females	24	20	25	19	9
Maternal mortality	0	0	0	0	0
Females with viable fetuses	24	20	25	19	9
Females with total reabsortions	0	0	0	0	0
Females with malformed fetuses	0	0	1	1	9*
Corpora lutea[a]	15.1 ± 3.9	15.6 ± 2.0	14.6 ± 4.1	14.8 ± 1.8	15.3 ± 2.5
Implantations[a]	13.5 ± 4.1	14.1 ± 2.9	14.1 ± 3.9	13.4 ± 3.7	14.1 ± 2.9
Resorptions					
Early[a]	0.4 ± 1.6	0	0	0	0.8 ± 0.8**
Late[a]	0.04 ± 0.2	0.05 ± 0.2	0	0	5.1 ± 3.5**
Dead fetuses[a]	0	0	0	0	1.4 ± 1.3**
Live fetuses[a]	13.1 ± 4.6	14.1 ± 2.9	14.1 ± 3.9	13.4 ± 3.7	6.8 ± 6.0**
Pre-implantation loss (%)[b]	10	10	3	10	8
Post-implantation loss (%)[c]	3	0.3	0	0	55**
Sex ratio (male/total)	0.54	0.50	0.56	0.54	–
Body weight live fetuses (g)					
Male[a]	3.6 ± 0.3	3.6 ± 0.2	3.6 ± 0.3	3.7 ± 0.4	–
Female[a]	3.4 ± 0.3	3.4 ± 0.3	3.4 ± 0.3	3.5 ± 0.5	–
Total[a]	3.5 ± 0.3	3.5 ± 0.2	3.5 ± 0.3	3.6 ± 0.5	1.0 ± 0.2**

CP, cyclophosphamide (positive control group); MSBE, mango stem bark extract.
* $p < 0.05$ (Fisher exact probability test).
** $p < 0.05$ (Mann–Whitney *U*-test).
[a] Mean ± SD.
[b] [(No. of *corpora lutea* − No. of implants)/No. of *corpora lutea*] × 100.
[c] [(No. of implants − No. of live fetuses)/No. of implants] × 100.

Table 4
External, visceral and skeletal observations of fetuses in rat during MSBE-orally administered developmental toxicity study

	Control	MSBE (mg/kg)			CP
		20	200	2000	50
External observations					
No. of examined fetuses	315	218	353	255	61
Incidence of fetuses with malformations (%)	0	0	0.3	0.4	100
No. of fetuses with malformations	0	0	1	1	61*
Craneorachischisis	0	0	0	1	0
Tail agenesis	0	0	1	0	0
Other malformations	0	0	0	0	61
Visceral observations					
No. of examined fetuses	157	141	175	127	NE
No. of fetuses with malformations	0	0	0	0	NE
Skeletal observations					
No. of examined fetuses	158	140	178	128	NE
No. of fetuses with malformations	0	0	0	0	
Incidence of fetuses with poorly or no ossified sternebrae and extra-rudimentary ribs (%)	82	85	87	87	NE
No. of fetuses with rudimentary, asymmetric, dumbbell, poorly or no ossified sternebrae dumbbell	128	119	155	111	NE
No. of fetuses with supernumerary bilateral ribs	1	0	0	0	NE

CP, cyclophosphamide (positive control group); MSBE, mango stem bark extract.
NE, not evaluated.
%: (number of fetuses with malformations/total fetuses) × 100.
* $p < 0.05$ (Chi-square test).

3.2. Genotoxicity

3.2.1. Comet assay

DNA primary damage on liver cells was evaluated by use of the alkaline Comet assay at doses up to 2000 mg/kg (Table 5). No toxicity was detected after histopathology examination of all cell livers on any treatment groups (data no shown). MSBE did not induce a statistical significant ($p < 0.05$) increase of strand breaks and alkali labile sites in the DNA of livers cells as measured by the Comet assay.

Table 5
Comet assay data from liver cells of MSBE-orally administered mice

Test	Dose (mg/kg)	Sex	DNA damage (AU)[a]	HDC (%)[a]
Vehicle	0	F	69 (54–86)	7 (5–9)
MSBE	0	M	86 (80–112)	6 (4–7)
	20	F	76 (50–102)	5 (4–6)
	20	M	64 (50–80)	6 (4–9)
	200	F	76 (44–91)	4 (3–8)
	200	M	51 (32–92)	6 (3–8)
	2000	F	77 (46–107)	6 (3–9)
	2000	M	73 (40–96)	5 (4–10)
CP	50	F	191.5 (112–246)	16 (9–22)
	50	M	207 (187–228)	18 (16–20)

AU, arbitrary units; HDC, highly damage cells or comets in category four; F, female; M, male; CP, cyclophosphamide; MSBE, mango stem bark extract.
[a] Median (range) of five animals.

No evidence was found of a dose-related increase in the DNA damage, expressed as single-, double-strand breaks, and alkali labile sites (alkilated bases and abasic sites). The cyclophosphamide positive control induced statistically significant increases ($p < 0.05$) in DNA damage as compared to vehicle controls.

3.2.2. Micronucleus assay

The clastogenicity and genotoxicity of MSBE in bone marrow erythrocytes of mouse were evaluated at doses up to 2000 mg/kg. No clinical signs of toxicity were observed during the treatment or in the macroscopic analysis after the sacrifice (euthanasia) of animals. Toxicity on bone marrow was found since the decrease in the PCE percentage was around 10% but was dose-related and statistically significant at 2000 mg/kg ($p < 0.05$, Table 6). MSBE did not increase the incidence of MN PCEs at any examined dose. No dose-related increases were detected after performing trend analysis for PCE percentage and micronucleated PCEs.

The cyclophosphamide positive control induced statistically significant increases ($p < 0.05$) in micronucleated PCEs as compared to vehicle controls.

4. Discussion

Administration of up to 2000 mg/kg of MSBE was not teratogenic in rats. The incidence of major malformations was not significantly increased on any MSBE-administered group as compared to the control group. Craneorachischisis and tail agenesis have been observed in large control groups of Sprague–Dawley rats in similar frequency (Manson and Kang, 1989); therefore the incidence of these fetal malformations was not significant ($p < 0.05$).

In this study, delayed ossification of the sternebrae was observed, which indicated a slight delay in fetal development disappearing during postnatal life in the rat (Khera, 1981; Wickramaratne, 1988; Chernoff et al., 1991).

MSBE was evaluated as negative in the Comet and micronucleus bone marrow assays under our experimental conditions. Only toxicity effects were observed on mice treated with 2000 mg/kg. It could be interpreted as evidence that MSBE reached the target organ and supported the validity of the test result. Cytotoxicity without genotoxicity has been found in plant extracts with polyphenols as meganatural brand grape seed extract (GSE), containing proanthocyanidin polyphenolic compounds. GSE was evaluated as negative in the mouse bone marrow micronucleus assay, but produced indication of cytotoxicity (decreased PCE:NCE ratio) at the 2000 mg/kg dose level for the 48 h harvest time point under these condition of assay (Erexson, 2003). One extract of a mixture of polymethoxylated flavones (PMFs) purified from citrus peel oil did not reveal any evidence of mutagenicity in five bacterial tester strains (*Salmonella typhimurium* TA98, TA100, TA102, TA1535 and TA1537) either in the absence or presence of S9 activation. Furthermore, in the presence of S9

Table 6
MSBE-orally administered genotoxicity study in mice

Treatment	Dose (mg/kg)	Micronucleated PCE per 1000 PCE from a total of 2000 PCE[a]	Linear trend[b]	PCE (%) from 200 ET[a]	Linear trend[c]
MSBE	0	2.5 ± 1.0	$b = -0.0002$ $p > 0.05$	54.0 ± 5.0	$R^2 = 0{,}13$ $p = 0.0266$
	20	2.8 ± 1.2		52.9 ± 3.5	
	200	2.6 ± 1.2		51.9 ± 3.9	
	2000	1.8 ± 1.2		$49.8 \pm 4.0^{*}$	
CP	50	$27.3 \pm 3.8^{*}$	–	$43.3 \pm 2.3^{*}$	–

Micronucleus data summary table (male plus female data).
PCE, polychromatic erythrocyte; ET, erythrocytes; CP, cyclophosphamide; MSBE, mango stem bark extract.
(–): Non applied.
* $p < 0.05$, Dunnett test.
[a] Mean ± SD, $N = 10$.
[b] Cochran–Armitage test.
[c] Linear trend (ANOVA, $df = 1$, $K' = \{-3, -1, 1, 3\}$).

there was no indication of genetic toxicity in L5178Y tk +/− cells. These results demonstrated that the PMF mixture was not genotoxic *in vitro*, but the cytotoxicity studies performed showed that the mixture was cytotoxic at high concentrations (Delaney et al., 2002).

The negative cytotoxicity found in liver of animals treated with MSBE (Comet data), as compared to a similar effect observed on bone marrow, could indicate that this effect was probably related to an antiproliferative activity of the extract. These results are consistent with the previously reported cytotoxicity data from the *in vitro* lymphocyte micronucleus assay, where a high reduction of lymphocyte proliferation was found together with a minor cytotoxicity, measured as membrane integrity (Rodeiro et al., 2006).

The embryotoxic assay evaluates established mechanisms of damage including DNA-damage (Peters, 1996). Testing of multiple genotoxic endpoints in the same animal is one of the best criteria to interpret a putative positive or negative result (Marzin, 1999). The performed genotoxicity assays evaluated the damage at different levels, primary and chromosomal-DNA damage. Negative results in these tests demonstrate that MSBE did not have any significant embryotoxic or genotoxic activity under these experimental conditions.

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