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Evaluation of genotoxicity and DNA protective effects of mangiferin, a glucosylxanthone isolated from *Mangifera indica* L. stem bark extract

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ABSTRACT

Mangiferin is a glucosylxantone isolated from *Mangifera indica* L. stem bark. Several studies have shown its pharmacological properties which make it a promising candidate for putative therapeutic use. This study was focused to investigate the *in vitro* genotoxic effects of mangiferin in the Ames test, SOS Chromotest and Comet assay. The genotoxic effects in bone marrow erythrocytes from NMRI mice orally treated with mangiferin (2000 mg/kg) were also evaluated. Additionally, its potential antimutagenic activity against several mutagens in the Ames test and its effects on CYP1A1 activity were assessed. Mangiferin (50–5000 μg/plate) did not increased the frequency of reverse mutations in the Ames test, nor induced primary DNA damage (5–1000 μg/mL) to *Escherichia coli* PQ37 cells under the SOS Chromotest. It was observed neither single strand breaks nor alkali-labile sites in blood peripheral lymphocytes or hepatocytes after 1 h exposition to 10–500 μg/mL of mangiferin under the Comet assay. Furthermore, micronucleus studies showed mangiferin neither induced cytotoxic activity nor increased the frequency of micronucleated/binucleated cells in mice bone marrow. In short, mangiferin did not induce cytotoxic or genotoxic effects but it protect against DNA damage which would be associated with its antioxidant properties and its capacity to inhibit CYP enzymes.

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1. Introduction

Natural products have lately been in the focus of several studies for determining their beneficial effects on human health (Torres et al., 2002; Murthy et al., 2002; Chen and Ping, 2008; Link and Balaguer, 2010). Xanthones are the biologically active plant phenols and they are found in some tropical plants as *Mangifera indica*, *Swertia mussotii* and *Swertia franchetiana*. Natural xanthones and its derivatives provide health benefits to humans, like flavonoids do (Pinto et al., 2005). Mangiferin (1,3,6,7-tetrahydroxyxanthone C_2-β-D-glucoside), is a glucosylxanthone found in large quantities in leaves, fruits and the stem bark of *M. indica* L. It exhibits several beneficial pharmacological properties like anti-viral, anti-inflammatory, hypoglycemic, antidiabetic, analgesic, hepatoprotective, immunomodulative and antitumor activities (Guha et al., 1996; Zheng and Lu, 1990; Ojewole, 2005; Muruganandan et al., 2005; Rajendran et al., 2008; García et al., 2003, 2011; Garrido et al., 2007; Rodeiro et al., 2008a,b). The strong antioxidant activity of mangiferin is also well demonstrated at *in vitro* and *in vivo* experimental models (Sanchez et al., 2000; Pardo et al., 2008, 2005a,b; Nuñez et al., 2009). In Cuba, mangiferin has been extracted and purified from MSBE, a standardized stem bark extract from *M. indica* L. and a pharmaceutical formulation of it is under development as new phytomedicine with potential use in cancer and degenerative diseases treatment.

Meanwhile, experimental toxicology studies are essential for the determination of the risk to benefit ratio of any product under development, but few data are available in relation to the toxicological potential of mangiferin. Intraperitoneal (i.p.) DL_{50} of mangiferin in DBAxC57BL mice is 400 mg/kg (Jagetia and Baliga, 2005). Additionally, LD_{50} is higher than 5000 mg/kg in rats and mice under oral acute doses of mangiferin obtained from the MSBE, while only some transient toxicity signs like dyspnoea, abdominal position, piloerection and reduced locomotion activity were observed. The same toxicity signs are observed when the product is administered by i.p. route, but under these conditions mangiferin is considered a toxic product for mice according to the Acute Toxic method.

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On the other hand, rats tolerate it well when exposed to 2000 mg/kg i.p. doses (author's unpublished data).

Studies of Govindaraj et al. (2009) show that mangiferin is no mutagenic to *Salmonella typhimurium* strains by using the Ames assay. However, it is crucial to perform a thorough toxicological evaluation of the genotoxic effects of any chemical intended to be used in humans as part of the determination of potential hazards. Consequently, a battery of *in vitro* and *in vivo* assays was used in order to establish the genotoxic effects of mangiferin. In addition, DNA-protective properties of mangiferin and its effects on CYP1A1 were also evaluated.

2. Materials and Methods

2.1. Material

Mangiferin (2-b-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone C_2-β-D-glucoside) was supplied by the Laboratory of Chemistry for Natural Products at the Cuban Center of the Development and Research of Medicines (CIDEM, Cuba). It was purified from MSBE by extraction with methanol (Núñez et al., 2002) and its purity was assessed as 95%.

2.2. Chemicals

Nicotinamide adenine dinucleotide phosphate disodium salt, glucose 6-phosphate, L-histidine monohydrate and D-Biotin were purchased from Sigma (St. Louis M.O. USA) and minimal essential and LB media supplied from Gibco. Mutagens were purchased from the following suppliers: benzo(a)pyrene, cyclophosphamide (CP), cisplatin and bleomycin from Sigma (St. Louis M.O. USA), sodium azide (NaAz) and 2-aminofluorene (2 AAF) from BDH (Chemicals Ltd., England). Colchicine was purchased from Sigma (St. Louis M.O. USA) and sodium phenobarbital and 5,6-β-naphtoflavone from BDH (Chemicals Ltd., England) were used for inducing the S_9 fraction.

Collagenase was obtained from Roche (Barcelona, Spain), Ham's F-12 and Lebovitz L-15 medium and calf serum were acquired from Gibco (Madrid, Spain), bovine serum albumin, fraction V, dimethylsulfoxide (DMSO), were purchased from Sigma Chemical Co. (Madrid, Spain). All other reagents used were of the purest available grade.

2.3. Cells

2.3.1. Isolation and culture of hepatocytes

Hepatocytes were obtained from 200–300 g Sprague Dawley male rats by perfusion of the liver with collagenase as described elsewhere (Gómez-Lechón and Castell, 1998). Cell viability of suspension was determined by the trypan blue exclusion test and it was higher than 85%. Cells were seeded at a density of 8×10^4 viable cells/cm^2 in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with sodium selenite (170 μg/mL), 2% calf serum, 0.2% bovine serum albumin, 50 mU/mL of penicillin, 50 μg/mL of streptomycin and 10 nM insulin and were later incubated at 37 °C in a 5% CO_2 humidified atmosphere. Unattached cells were removed by changing the medium 1 h after plating and cells were exposed to treatment. Cells were incubated with different concentrations of mangiferin (10, 100 and 500 μg/mL) during 1 h.

2.3.2. Isolation and culture of lymphocytes

Human peripheral blood was obtained by venous puncture from two healthy voluntaries, no smokers, between 25 and 35 years old. Blood peripheral lymphocytes were purified by using the Lymphoprep kit (Nycomed) and cell concentration was adjusted to 1×10^6 cells/mL. Cells were incubated with different concentrations of mangiferin (10, 100 and 500 μg/mL) in presence and in absence of metabolic activation at 37 °C during 1 h.

2.4. In vitro assays

2.4.1. Ames test

The *S. typhimurium* strains used in the experiments were supplied by Dr. Javier Espinosa Aguirre, UNAM, Mexico, they were: TA1535, TA1537, TA1538, TA98, TA100 and TA102. Liver cytosolic fractions were prepared from young adult male Sprague Dawley rats. According to INVITTOX Protocol (INVITTOX, 1990), animals were sacrificed after 5 days of receiving daily i.p. injections of sodium phenobarbital at 30 mg/kg (day 1) and 60 mg/kg (days 2–5). On the third day, 80 mg/kg of 5,6 β-naphtoflavone were also administrated. The 9000*g* liver supernatant (S9) was split into 1 mL aliquots, frozen and stored at −80 °C.

The standard plate-incorporation method in the presence and absence of S9 was performed according Maron and Ames (1983). For this study, mangiferin was prepared in DMSO at stock concentration of 50 mg/mL and it was added to the cultures at 50, 500, 1000, 2000 and 5000 μg/plate (Maron and Ames, 1983). Negative (vehicle) and positive controls were included. Dimethylnitrosamine (DMNA) (5 μL/plate), sodium azide (NaAz) (2 μg/plate) and 2-aminofluorene (2 AAF) (5 μg/plate) were used as positive control.

The toxicity effects of the product were investigated first. For each test, 2 mL of top agar containing 0.6% agar, 0.5% NaCL, 0.5 mM biotin and 0.05 mM L-histidine were mixed with 0.1 mL of the compound (or the controls), 0.1 mL of the overnight culture (about 10^8 cells) and 0.5 mL S9 mix (or 0.5 mL of phosphate buffer). Afterward, all the plates were incubated at 37 °C for 48 h. After incubation the number of revertants colonies was determined.

Protective effects of mangiferin (50, 250, and 500 μg/plate) against different mutagenic agents were also assessed by using the standard plate incorporation assay. The mutagenicity of AFB_1 (20 μg/plate), benzo(a)pyrene (10 μg/plate), 2 AAF (5 μg/plate) were monitored against TA98 in presence of the S9 mixture, meanwhile, bleomycin (0.5 μg/plate) and DMNA (5 μg/plate) were monitored against TA102 and cyclophosphamide (500 μg/mL) using TA100 also in the presence of the S9 mixture. The direct acting mutagens H_2O_2 (100 μg/plate) and mitomycin C (20 μg/plate) were tested by using TA102 strain, while sodium azide (2 μg/plate) and cisplatin (100 μg/plate) were tested by using TA100. Control plates containing only DMSO (vehicle) were also included to obtain the background or spontaneous revertant counts. The % inhibition of the mutagenicity was calculated as: [1 − (number colonies/plates with mutagen plus mangiferin)/(number colonies/plate with mutagen along)] × 100.

2.4.2. SOS Chromotest assay

At first, the following toxicity assay was conducted: The exponential phase cultures of strain PQ37 grown in LB medium at 37 °C, were diluted 1:10 in a medium LB(2X). Aliquots of 500 μL were distributed into sterile tubes. Final concentrations of the product were: 5, 250, 500 and 1000 μg/ml. Positive controls received 150 Gy of gamma irradiation or bleomycin (2 mg/ml) with S9 mix. Negative controls (vehicle) were made. To estimate cell survival per cent the following formula was used: $S = N/N_0 \times 100\%$, where *S*: Cell survival, *N*: Number of colonies in the treated plates, N_0: Number of colonies in the negative control (Iwanami and Oda, 1985). The alkaline phosphatase assay in treated *Escherichia coli* PQ37 cells (protein synthesis inhibition indices) was also used as toxicity criterion (Quillardet and Hofnung, 1993).

Afterward, the Chromotest assay was conducted. The original procedure described by Quillardet et al. (1982) was performed with some modifications for avoiding interferences caused by colors present in the plant products with the data measurement. Modifications were: the use of fluorescent substrates and a modified substrate buffer for the alkaline phosphatase assay according to Salvo et al., 1994. Mangiferin was considered mutagenic if it produced a dose–response effect and a 2-fold increase in the induction factor (IF) respect to control. IF was calculated as: [β-galactosidase (induced)/phosphatase (induced)]/[β-galactosidase (control)/phosphatase (control)] (Quillardet et al., 1989).

2.4.3. Comet assay

The Comet assay was performed as described elsewhere (Singh et al., 1988; Collins et al., 1997) in order to detect DNA damage in lymphocytes and liver cells. Briefly, after incubation with mangiferin, samples were taken and cell concentration 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, 0.5 % LMPA was added to each slide to produce another layer of 75 μL and slides were placed on ice for 10 min to accelerate agarose jellification. Afterward they were incubated overnight in lysis solution at 4 °C. Slides were placed inside of an electrophoresis chamber filled with alkaline buffer and 20 min later it was applied 1.25 V/cm and 300 mA. Assay variability was reduced as follows: replicate slides were randomly distributed among multiple electrophoresis and they run under a fully balanced design. After electrophoresis, slides were neutralized, dried and stained with ethidium bromide (20 μg/mL) prior to analysis. Stained nucleoids were evaluated with an Olympus A_2 fluorescence microscope. Fifty cells from each slide were analyzed and classified in five levels according to the DNA damage; data from both slides per treatment were combined. The DNA damage score was calculated as: (Nucleoids in level 1) + (2 × Nucleoids in level 2) + (3 × Nucleoids in level 3) + (4 × Nucleoids in level 4). The score was normalized as follows: it was 0 AU if all nucleoids were at level 0 (undamaged) and it was 400 AU if all nucleoids were at level 4 (maximally damage), where AU is Arbitrary units (García et al., 2004).

2.4.4. Effects on CYP1A1 enzyme in rat microsomes

Microsomes were obtained from the phenobarbital and 5,6 β-naphtoflavone induced S9 fraction. The latter it was centrifuged at 105,000*g* for 1 h at 4 °C. The supernatant was then resuspended in PBS at pH 7.4 and centrifuged again under the same conditions; the resuspension was made in PBS plus EDTA (1 mM), dithiothreitol (0.1 mM) and glycerol (20%). Protein concentrations were determined with the Bradford (1976) assay, and the microsomal fraction was kept at −80 °C until use.

The CYP1A1 associated ethoxyresorufin-O-dealkylation (EROD) activity was measured according to the method described by Burke et al. (1994) with some modifications. The production of resorufin was spectrofluorometrically assessed using the microsomes previously obtained. For measuring EROD activity we used 2 ml of the reaction mixture (at 4 °C) constituted by 7-ethoxyresorufin (25 μM), 1 mg/ml of microsomal protein, and buffer (50 mM tris and 25 mM $MgCl_2$, pH 7.4). The mixture was pre-incubated at 37 °C for 3 min and the reaction started with the addition of 0.2 ml of 50 mM NADPH. The spectrofluorometric readings were recorded every 15 s for 3 min using excitation and emission filters of 530 and 585 nm, respectively. A resorufin solution was used for the calibration curve (5–250 pmol). Microsomes were incubated with concentrations of mangiferin (4, 40 and 400 μg/mL) or controls (only medium-treated microsomes). All measurements were made in triplicate.

2.5. In vivo assays

2.5.1. Animals

NMRI mice (20–25 g) of both sexes were purchased from the National Center for Laboratory Animals Production (CENPALAB, Havana, Cuba) and adapted during 5 days to laboratory conditions. A standard rodent chow manufactured at CENPALAB was supplied *ad libitum*. Animals were fasted 12 h before sacrifice. Access to tap water was freely allowed. All manipulations were performed according to the ethical principles for animal care and management recommended by Cuban Guidelines and Standard Operational Procedures established for such aim.

2.5.2. Comet assay

For studying the effects of mangiferin on the bleomycin induced DNA damage, BalbC male mice were orally administered during 14 days with 100 mg/kg of mangiferin, which is a pharmacological effective dose for this specie (Garrido et al., 2007). The negative control group received 10 mg/kg of acacia gum, the vehicle used for diluting mangiferin. At the end of this period, animals were administered by i.p. route with a dose of bleomycin (50 mg/mL) and 24 h later they were sacrificed by cervical dislocation. Another group administered only with bleomycin was also included. Each group included 6 animals. Blood samples were taken from the aorta and the Comet assay was conducted as described in Section 2.4.3.

2.5.3. Micronucleus test

Animals received a 2000 mg/kg single oral dose of mangiferin, which is well tolerated under the same experimental conditions and it is considered limit for this kind of studies (OECD, 1997). Micronuclei induction in mangiferin treated animal was compared with the one induced in animals which received only acacia gum and they were used as negative control. We used another group of animals as positive control group, they received 100 mg/kg of CP by a single i.p. injection and they provide information about the validity of the experimental design. Each group included 6 animals. Bone marrow from the femora was down 24 h after administration for mangiferin treated and vehicle groups and 48 h after the single injection for CP in the positive controls.

Both femora bones were removed from sacrificed animals and bones were freed from muscle and by gentle traction. The distal epiphyseal portion was torn off together with the rest of the tibia and the surrounding muscle. The proximal end of the femur was carefully shortened until a small opening to the marrow canal became visible. Then, 2 mL of serum were introduced into the bone canal and the femur was submerged in a centrifuge tube filled with fetal calf serum. The marrow was aspirated and flushed for several times. Cells were centrifuged at 1000 rev/min for 5 min. Two drops of cell suspension were placed onto clean, dry slides and smeared, fixed in methanol and stained with Giemsa 5%(v/v) for 12 min. The presence of micronuclei and the percentage of cells containing micronucleus were determined in a sample of 2000 polychromatic erythrocytes (PCE). Normochromatic erythrocytes (NCE) were also scored in 200 erythrocytes samples to determine the PCE/NCE ratio (Hayashi et al., 1994).

2.6. Statistical analysis

Ames test: statistical analysis was performed after to determine the mean values of revertants/plate using a non-parametric method (Kolmogorov–Smirnov test) (Maron and Ames, 1983) and the SALANAL statistical package software. Comparisons between groups were made using Mann Whitney U test. SOS Chromotest: the alkaline phosphatase activity, the percentage of cell survival and the SOS induction factor data were tested for normality by using the Kolmogorov–Smirnov test. Means were compared by using the Mann Whitney *U* test. Comet, micronucleus and CYP1A1 activity data were analyzed by using the Kruskal Wallis and Dunnett non-parametric test. Data was expressed as mean ± SD and the statistical significance *p* level used was 0.05.

3. Results and discussion

A balance between therapeutic and toxicological effects of a new product is important when verifying its applicability as a pharmacological drug. Thus, the genotoxicological evaluation of the product is a necessary step as part of the preclinical evaluation of the candidate.

This study was performed using histidine requiring mutants of *S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100 and TA102 strains, with and without metabolic activation as a measure of point-mutation DNA. No toxicity was observed after exposition to 5000 μg/mL of mangiferin in any tested *Salmonella* strain (Table 1). The frequency of spontaneous reversion did not differ from the historically observed in our laboratory for the same strains, whereas the mutagens used as positive controls significantly increased the revertant frequency. On the other side, the addition of mangiferin at doses from 50 up to 5000 μg/plate did not induce significant changes in the reversion frequency compared with controls, similar results were obtained with or without metabolic activation. These results showed that mangiferin did not induce mutagenesis in the different strains of *S. typhimurium* used. Our results are in agreement with other studies (Govindaraj et al., 2009), which reported no mutagenic activity for mangiferin when tested in the Ames assay. They are also in concordance with the SOS Chromotest assay results (Fig. 1).

The bacterial SOS Chromotest with *E. coli* has been widely used as part of the genotoxicity screens. It is based on the SOS response induction, which is activated in the presence of DNA damage and leads to replication blockage (Janion, 2001). The cytotoxicity study showed mangiferin did not inhibit (Student *t* test, $p < 0.05$) neither the cell survival percent nor the protein synthesis at concentrations up to 2000 μg/ml, in the presence or absence of metabolic activation when compared to control cells. These results indicated

Table 1
Mutagenicity of mangiferin in the *Salmonella typhimurium* assay (mean ± SD) in presence or absence of metabolic activation.

Strains	S9	Control	Mangiferin (μg/plate)					DMNA	AzNa	2 AAF
			5	500	1000	2000	5000	(5 μg/plate)	(2 μg/plate)	(5 μg/plate)
TA1535	–	9.67 ± 1.5	8.02 ± 1.2	9.67 ± 1.5	7.50 ± 3.9	8.00 ± 3.5	8.20 ± 1.4		42.5 ± 3.9*	
	+	10.7 ± 2.0	8.62 ± 1.3	9.23 ± 1.4	9.40 ± 3.6	10.6 ± 1.6	9.8 ± 1.8			
TA1537	–	9.03 ± 3.2	10.67 ± 1.5	7.00 ± 2.6	7.56 ± 2.0	6.40 ± 1.7	6.56 ± 2.6			
	+	6.00 ± 2.0	7.30 ± 4.5	7.33 ± 4.0	6.33 ± 1.9	6.90 ± 1.9	5.50 ± 1.0	54.1 ± 4.8*		
TA98	–	25.3 ± 2.9	25.7 ± 4.4	29.3 ± 3.6	28.7 ± 2.5	21.7 ± 4.5	23.20 ± 3.7			
	+	31 ± 2.5	32.3 ± 4.9	33.7 ± 1.1	34.0 ± 3.6	31.7 ± 3.1	30.9 ± 2.9			207.7 ± 10*
TA100	–	99.7 ± 17	102.7 ± 12	100.9 ± 11	98.8 ± 15	96.3 ± 9.0	101.3 ± 9.9		402.3 ± 20*	
	+	108.3 ± 12	122.0 ± 8	122.0 ± 15	121.3 ± 17	109.7 ± 19	87.00 ± 13	535.7 ± 27.8*		
TA 102	–	165.6 ± 38	164.3 ± 17	152.5 ± 32	149.3 ± 26	158.5 ± 17	150.2 ± 29		501.0 ± 16*	
	+	155.5 ± 19	159.5 ± 35	159.6 ± 23	160.5 ± 15	155.2 ± 32	149.7 ± 12	808.4 ± 35*		

Data represent the mean of the number of histidine revertant colonies by plate in each treatment; two independent experimental series carried out in triplicate (6 plates) were performed. Incubations were made in presence (+S9) and absence (−S9) of rat liver microsomal mix. *$p < 0.05$, comparison between groups by Mann Whitney *U* test. DMNA: dimethylnitrosamine, AzNa: sodium azide, 2 AAF: 2-aminofluorene.

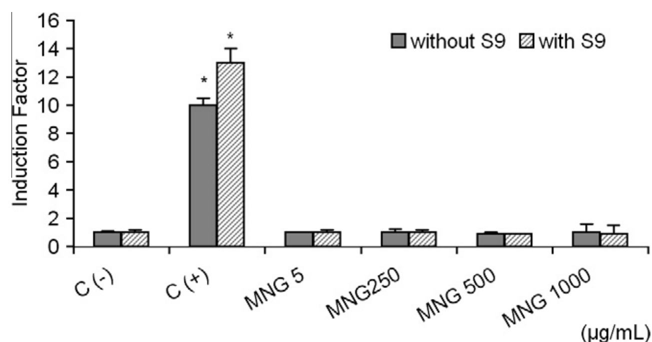

Fig. 1. Genotoxicity study of mangiferin by using SOS Chromotest. Data represent the mean ± S.D. of three independent experiments with three replicates. Positive controls received 150 Gy in absence of S9 and Bleomycin (2 mg/ml) in presence of S9. *$p < 0.05$ in regard to negative controls (Mann Whitney test).

Table 2
Effects of mangiferin on micronucleus assay in NMRI mice of both sexes. (mean ± SD).

Treatment	MN/PCE (%)	PCE/NCE
Male		
Vehicle control	0.22 ± 0.04	1.02 ± 0.05
Mangiferin (mg/kg)		
2000	0.26 ± 0.06	0.92 ± 0.06
CP 100 mg/kg	4.08 ± 0.45*	0.66 ± 0.04*
Female		
Vehicle control	0.30 ± 0.03	0.89 ± 0.05
Mangiferin (mg/kg)		
2000	0.28 ± 0.04	0.90 ± 0.04
CP (100 mg/kg)	5.88 ± 1.46*	0.54 ± 0.07*

PCE: polychromatic erythrocytes, %: represents number of micronucleus in PCE NCE: normochromatic erythrocytes, PCE/NCE: ratio PCE/NCE 2000 cells/animal were examined, CP cyclophosphamide *$p < 0.05$, comparison between treated groups and control by Kruskal Wallis and Dunnett non-parametric test.

that mangiferin was not toxic for *E. coli* PQ37 cells under these experimental conditions (data not shown). Mangiferin at the tested concentrations did not induce significant genotoxic activity ($p < 0.05$, Student t test) under our experimental conditions (Fig. 1). Mangiferin was unable to induce SOS function in PQ37 bacterial strain since the induction factor (IF) did not increase twice respective to the negative controls, thus indicating no genotoxicity according to the established criteria for this assay (Kevekordes et al., 1999).

Although the SOS Chromotest is less sensitive for identifying carcinogens than the Ames test, it provides more evidence about the lack of mutagenic activity of mangiferin. However, in order to go deep in the assessment of the genotoxic potential of mangiferin, Comet and micronucleus assays were also conducted. Comet assay allows determining DNA damage by detecting strand breaks and alkali-labile sites in rat hepatocytes cultures and human blood peripheral lymphocytes. As it can be observed in Fig. 2, no single strand breaks or alkali-labile sites were induced in hepatocytes or lymphocytes after mangiferin treatments when compared to controls. In contrast, damage in cells exposed to gamma radiation (positive control) was significantly different when compared to the rest of the groups.

The micronucleus is a simple cytogenetic assay based on scoring micronuclei in actively dividing cell populations. It is also a quantitative measure of chromosome damage and acts like an index of genomic damage (Hayashi et al., 1994). The frequency of PCE in mice after 24 h of oral administration with 2000 mg/kg of mangiferin was similar to the control groups (Table 2, data were presented as percent of micronucleus in PCE). This result shows that oral administration of up to 2000 mg/kg mangiferin did not induce genotoxic effects in mice. Furthermore, no clinical signs of toxicity were observed during treatment or in the macroscopic analysis after animal sacrifice. Therefore, mangiferin did not exhibit cytotoxic activity in this assay, determined as PCE/NCE ratio. As it was expected, the administration of CP (positive control) significantly increased PCE/NCE ratio and PCE in animals (Table 2), thus indicating the validity of the experimental design and the species selection for detecting *in vivo* cytotoxic and clastogenic effects.

Mangiferin is the main compound present in the *M. indica* L. stem bark extract (MSBE), a Cuban product polyphenol-rich with antioxidant, anti-inflammatory and immune-modulatory properties. MSBE is used in the treatment of cancer and degenerative diseases (Garrido et al., 2007; Nuñez et al., 2007, 2009). In previous papers, we showed that this extract is a product with a broad margin of security and neither mutagenic nor genotoxic effects were found in the performed assays (Rodeiro et al., 2006; Gonzalez et al., 2007). Meanwhile, recent experimental results revealed differences between MSBE and mangiferin safety. MSBE induced DNA strand breaks and alkali-labile sites in human lymphocyte cultures exposed to concentrations higher than 100 µg/mL after 1 h exposition (unpublished results). Therefore, our present results suggested that mangiferin should not be responsible for the DNA damage induced by MSBE in these experimental conditions.

There are increasing evidences that mutations in somatic cells are not only involved in the carcinogenesis process but also do play a role in the pathogenesis of other chronic degenerative diseases, which are the leading causes of death in the human population. In spite of the multiplicity and clinical diversity of the diseases, certain conditions are associated with common risk factors as well as with common protective factors. Additionally, they share common pathogenic determinants, such as genotoxic events or oxidative stress (De Flora et al., 1996). Prevention of cancer and other

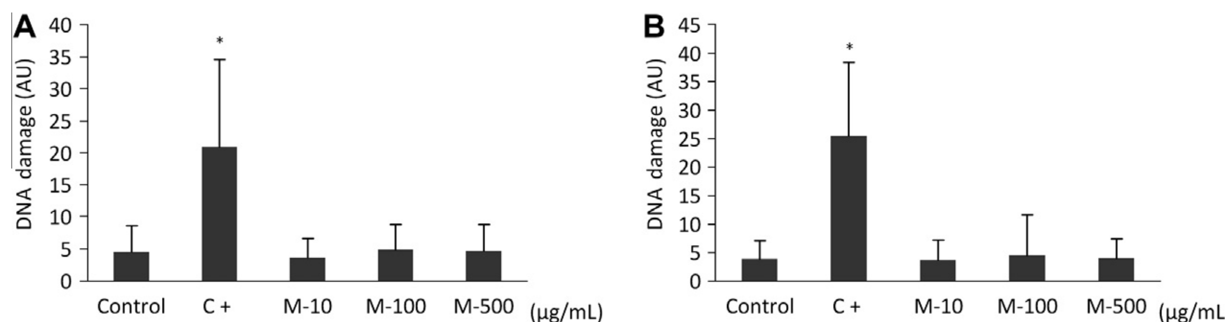

Fig. 2. Genotoxicity study of mangiferin by using Comet assay. rat hepatocytes in culture, B) lymphocytes of human peripheral blood in culture. Data represent the mean ± S.D. of at least two independent experiments with three replicates. Positive controls received 3 Gy. *$p < 0.05$ in regard to negative controls (Kruskal Wallis and Dunnett non-parametric tests).

Table 3
Effects of mangiferin on the DNA damage induced by different agents in *Salmonella typhimurium* strains.

Mutagenic agent	Control −S9 mix[a] (%)[b]	Control S9 mix[a] (%)[b]	Positive control[a] (%)[b]	Mangiferin (50 μg/plate)[a] (%)[b]	Mangiferin (250 μg/plate)[a] (%)[b]	Mangiferin (500 μg/plate)[a] (%)[b]
Bleomycin (0.5 μg/plate, TA102)		153 ± 19	889 ± 24	767 ± 25 (14)*	387 ± 21 (50)*	367 ± 27 (59)*
CP (500 μg/plate, TA100)		98 ± 7	403 ± 5	399 ± 6 (1)	304 ± 6 (24)*	266 ± 12 (13)*
AFB_1 (20 μg/plate, TA98)		82 ± 14	256 ± 12	245 ± 13 (4)	189 ± 8 (26)*	167 ± 18 (35)*
Benzo(a)pyrene (10 μg/plate, TA 98)		33 ± 4	830 ± 25	600 ± 12 (28)*	582 ± 9 (30)*	540 ± 17 (35)*
2 AAF (5 μg/plate, TA98)		90 ± 12	233 ± 14	189 ± 32 (19)	145 ± 13 (38)*	128 ± 5 (45)*
H_2O_2 (100 μg/plate, TA102)	121 ± 12		456 ± 14	408 ± 31(11)	313 ± 15 (23)*	207 ± 6 (54)*
Sodium Azide (2 μg/plate, TA100)	103 ± 17		423 ± 17	421 ± 21	389 ± 18 (8)	356 ± 20 (16)*
Cisplatin (100 μg/plate, TA100)	102 ± 20		801 ± 28	767 ± 14 (4)	434 ± 13 (43)*	232 ± 9 (60)*
Mitomycin C (20 μg/plate, TA102)	156 ± 17		756 ± 21	523 ± 29 (31)*	500 ± 8 (34)*	366 ± 9 (52)*
DMNA (5 μg/plate, TA102)		165 ± 15	768 ± 22	767 ± 23 (0)	567 ± 30 (26)*	499 ± 31 (35)*

[a] Values represent mean ± SD of histidine revertants of two independent experiments carried out in triplicate.
[b] Inside the parenthesis is the percentage inhibition of the revertants regarding to the positive control (plates containing the mutagenic agent alone). CP: cyclophosphamide, AFB_1: aflatoxin B_1, 2 AAF: 2-aminofluorene, DMNA: dimethylnitrosamine. *$p < 0.05$ compared to positive control, Mann Whitney test.

mutation-related diseases can be pursued by avoiding exposures to recognized mutagens/carcinogens, by favoring the intake of protective factors or by fortifying physiological defense mechanisms. Recent advances in the understanding of carcinogenesis at the cellular and molecular levels have led to the development of promising strategies for cancer prevention like chemoprevention. Over the last several years, there has been an increase in the number of studies reporting medicinal plants and dietary components as excellent source of chemopreventive agents (Ruhul et al., 2009; Ohno et al., 2009; Garcia et al., 2011; Rajendran et al., 2008).

In this study, the potential antimutagenic activity of mangiferin isolated from MSBE against DNA damage induced by different agents was assessed by using the Ames test and the Comet assay. As it is shown in Table 3, mangiferin protected against the mutagenic effects of various chemical mutagens and carcinogens when using the plate incorporation assay. Mangiferin was highly effective in reducing the mutagenicity caused by various agents including indirect and direct mutagens as: AFB_1, benzo(a)pyrene, 2-AAF, bleomycin, DMNA, cyclophosphamide, H_2O_2, mitomycin C, sodium azide and cisplatin (Table 3).

Protective effects of mangiferin against various agents which induced ROS-mediated DNA mutations were observed. As it have been described, H_2O_2 cause mutations by producing $OH^{\cdot}$ through the metal catalyzed Fenton reaction, while cisplatin induces $OH^{\cdot}$ and $O_2^{\cdot -}$ formation (Masuda et al., 1994; Weijl et al., 1997; Halliwell and Gutteridge, 1999). Mitomycin C is an alkylant agent which requires bioreductive transformation to form active species capable to produce crosslink DNA (Wang et al., 2007). Depending on the biotransformation pathway, metabolism of mitomycin C can generate ROS (Gustafson and Pritsos, 1992). On the other hand, bleomycin induces DNA degradation by the formation of a complex with ferric ions and which bind to DNA. In the presence of bleomycin, this complex is activated and produces ROS, such as $O_2^{\cdot -}$ and $OH^{\cdot}$, which cause single and double strand DNA breaks. In the presence of mangiferin, the DNA damage caused by these mutagens was effectively reduced, which reflected the capability of this product to interfere with their mutagenic mechanism. This capacity may be related to the antioxidant properties of the product: the scavenging activity of free radicals and the iron-chelating properties. These results are in accordance to previous reports of Pardo et al. (2005a,b) who showed the mangiferin capacity to inhibit the mitochondrial lipid peroxidation induced by Fe^{2+}– citrate *in vitro*.

However, the present results showed mangiferin was effective to reduce the mutagenicity induced by 2-AAF, which causes frameshift mutation by forming adducts on the C8-position of guanine in the presence of metabolic bioactivation (Hoffmann and Fuchs, 1997). Similar evidence was observed for benzo(a)pyrene, AFB_1 and DMNA mutagens, that require bioactivation at hepatic level for inducing DNA damage. We have previous evidence that mangiferin inhibits the catalytic activity of some isoenzymes of the P450 system in hepatocytes culture and in human microsomes, like CYP1A2 and CYP3A4 isoforms, which are strongly involved in the bioactivation of many mutagens and carcinogens in the organism (Rodeiro et al., 2008b, 2009). In addition, now we show the inhibitory effect of the catalytic activity of the CYP1A1 in rat microsomes by the mangiferin (400 μg/mL) (Fig. 3). The results presented here constitute new evidence which remarks the modulation of P450 system by this product. As the activity of isoenzymes of the P450 system plays a significant role in the activation/detoxification balance of pro-carcinogens in the organism, the effects of mangiferin upon these isoenzymes could be relevant in explaining the antimutagenic properties of the product. On the other hand, alteration of the CYP basal activity may cause clinically important herb-drug interactions that must be broadly explored in the case of mangiferine (Hu et al., 2005). Further studies should be carried out in order to understand the mechanisms of action involved.

Thus, according to the present observations mangiferin can protect DNA by different mechanisms where the bioactivation process may be included. In addition, a decrease in the DNA damage induced by bleomycin in mice after treatment with mangiferin was observed in the Comet assay (Fig. 4), which is supported by the results observed by us in the *Salmonella* assay and by the observations of other authors, who reported mangiferin antigenotoxic properties under other experimental conditions (Rajendran et al., 2008; Satish et al., 2009; Govindaraj et al., 2009).

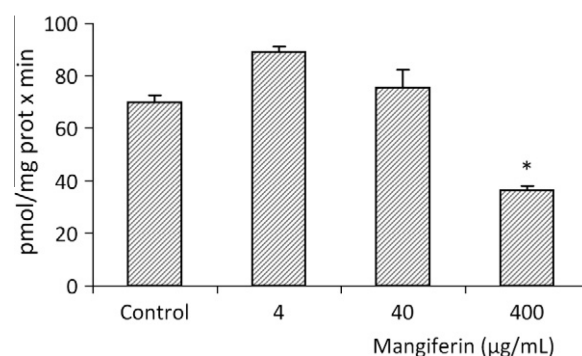

Fig. 3. Effects of mangiferin in rat liver CYP1A1 activity. CYP1A1 was assayed in rat microsomes incubated with 7-ethoxyresrorufin and mangiferin. The values represent mean ± SEM of three independent experiments. *$p < 0.05$, Kruskal Wallis and Dunnett non-parametric tests.

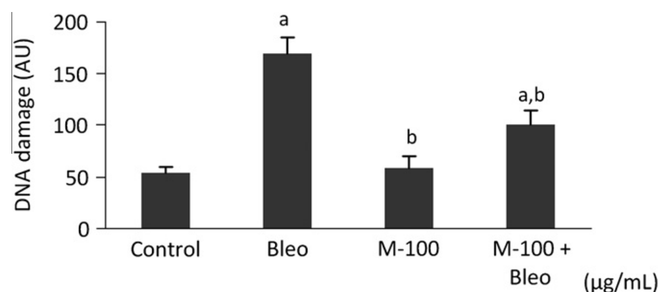

Fig. 4. Effects of mangiferin on bleomycin-induced DNA damage in mice by using Comet assay. Data represent the mean ± S.D. of damaged leucocytes of peripheral blood of male Balb C mice (2 slides per animal). (a) represents significant difference in regard to control group, (b) represents significant difference in regard to the bleomycin-treated group, $^{*}p < 0.05$, Kruskal Wallis and Dunnett non-parametric tests.

4. Conclusions

In summary, this study demonstrates that in the battery of assays used here, mangiferin extracted from *M. indica* L. stem bark did not induce cytotoxic, mutagenic or genotoxic effects. By contrast, it exhibited DNA protective properties, thus increasing the evidence about the chemopreventive properties attributed to this compound.

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