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Evaluation of the genotoxic potential of *Mangifera indica* L. extract (Vimang), a new natural product with antioxidant activity

I. Rodeiro [a,*], L. Cancino [b], J.E. González [c], J. Morffi [a], G. Garrido [a], R.M. González [a], A. Nuñez [a], R. Delgado [a]

[a] *Laboratory of Pharmacology, Department of Biomedical Investigations, Center of Pharmaceutical Chemistry, 200 and 21, Atabey, P.O. Box 16042, Havana, Cuba*
[b] *Laboratory of Genotoxicology, Center of Biomedical Investigations (CIBIOMED), 25 and 158, Cubanacan, P.O. Box 16042, Havana, Cuba*
[c] *Laboratory of Genotoxicology, Faculty of Pharmacy, University of Havana, 222 street, La Coronela, Havana, Cuba*

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Abstract

Mangifera indica L. extract (Vimang) consists of a defined mixture of components (polyphenols, terpenoids, steroids, fatty acids and microelements). It contains a variety of polyphenols, phenolic esters, flavan-3-ols and a xanthone (mangiferin), as main component. This extract has antioxidant action, antitumor and immunemodulatory effects proved in experimental models in both *in vitro* and *in vivo* assays. The present study was performed to investigate the genotoxicity potential activity of Vimang assessed through different tests: Ames, Comet and micronucleus assays. Positive and negative controls were included in each experimental series. Histidine requiring mutants of *Salmonella typhimurium* TA1535, TA1537, TA1538, TA98, TA100 and TA102 strains for point-mutation tests and *in vitro* micronucleus assay in primary human lymphocytes with and without metabolic activation were performed. In addition, genotoxic effects were evaluated on blood peripheral lymphocytes of NMRI mice of both sexes, which were treated during 2 days with intraperitoneal doses of *M. indica* L. extract (50–150 mg/kg). The observed results permitted to affirm that Vimang (200–5000 μg/plate) did not increase the frequency of reverse mutations in the Ames test in presence or not of metabolic activation. Results of Comet assay showed that the extract did not induce single strand breaks or alkali-labile sites on blood peripheral lymphocytes of treated animals compared with controls. On the other hand, the results of the micronucleus studies (*in vitro* and *in vivo*) showed Vimang induces cytotoxic activity, determined as cell viability or PCE/NCE ratio, but neither increased the frequency of micronucleated binucleate cells in culture of human lymphocytes nor in mice bone marrow cells under our experimental conditions. The positive control chemicals included in each experiment induced the expected changes. The present results indicate that *M. indica* L. extract showed evidences of light cytotoxic activity but did not induce a mutagenic or genotoxic effects in the battery of assays used.
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Keywords: *Mangifera indica* L.; Ames test; Comet assay; Micronucleus

1. Introduction

Mangifera indica L. (Anacardianceae) grows in tropical and sub-tropical regions of the world and it is commonly used in folk medicine for a wide variety of remedies (Coe and Anderson, 1996). The chemical composition of this plant has been well studied during the last years and the extract yield triterpenes, flavonoids, phytosterols and polyphenols, in general (Anjaneyulu et al., 1994; Khan et al., 1994).

In Cuba, the aqueous extract of *M. indica* L. (Vimang) is used to improve welfare on patients suffering from elevated stress (Guevara et al., 2002). Vimang consists of a defined mixture of components (polyphenols, terpenoids, steroids, fatty acids and microelements) (Capote et al., 1998; Nuñez et al., 2002). Vimang contains a variety of polyphenols, which include phenolic acids (gallic acid, 3–4-dihydroxy

* Corresponding author. Fax: +53 7 336471.
E-mail address: idania.rodeiro@infomed.sld.cu (I. Rodeiro).

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benzoic acid), phenolic esters (methyl gallate, propyl gallate, propyl benzoate), flavan-3-ols ((+)-catechin and (−)-epicatechin) and a xanthone (mangiferin).

The main polyphenol present in the extract, a *C*-glucosylxanthone (1,3,6,7-tetradroxyxanthone-C2-D-glucoside) mangiferin, was reported to have antioxidant action (Sato et al., 1992; Sanchez et al., 2000), inhibitory effects in bowel carcinogenesis of male F344 rats (Yoshimi et al., 1996), antitumor (Guha et al., 1996) and immunemodulatory effects (Leiro et al., 2003).

Different authors have reported varied therapeutic and chemoprotective activities for polyphenols, generally ascribed to their antioxidant properties (Fujiki et al., 2000; Yang et al., 2001; Duthie and Crozier, 2000). Vimang, which is rich in this kind of molecules, has potent antioxidant effect in both *in vitro* and *in vivo* systems (Martinez et al., 2000a,b, 2001). It showed a powerful scavenger activity on hydroxyl radicals and hypochlorous acid, presented a significant inhibitory effect on the peroxidation of rat brain phospholipids and inhibited DNA damage by bleomycin or copper–phenanthroline assays. It has also analgesic, anti-inflammatory and immunemodulatory effects in different experimental models (Garrido et al., 2001; Garcia et al., 2002; Leiro et al., 2004; Garrido et al., 2004a,b).

Experimental toxicology is essential for the assessment of the putative risk to benefit ratio of any new research product. Previous acute oral toxicity studies of *M. indica* L. aqueous extract in rats and mice showed that LD_{50} of the extract is superior to 5000 mg/kg of weight, but some transient toxicity signs such as dyspnoea, abdominal position, piloerection and reduced locomotor activity were observed after single oral administration of Vimang at higher dose tested (unpublished data). This toxicity effects were also observed when Vimang was administered by intraperitoneal (ip) route, but in these experimental conditions the LD_{50} in mice is 273.14 mg/kg in females and 219.67 mg/kg in males, so Vimang was considered a toxic product (unpublished data). Subchronic oral studies conducted in Sprague Dawley rats showed that doses up to 2000 mg/kg did not induce drug-related toxicity. On the other hand, teratogenic studies conducted by oral route in rats until 2000 mg/kg show no toxicity related with the exposure to the extract (unpublished data). Nevertheless, on the toxicological evaluation of mutagenic and genotoxic effects of any new chemical intended to be used in humans, is a crucial part of its assessment for potential hazards. Consequently, in the present study the *in vitro* and *in vivo* potential genotoxicity of the *M. indica* L. (Vimang) were investigated.

2. Materials and methods

2.1. Plant material

M. indica L. was collected from a determined cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code: 41722) were deposited at the Herbarium of Academic of Sciences, guarded by the Institute of Ecology and Systematic from Ministry of Science, Technology and Environmental, La Habana, Cuba. Stem bark extract of *M. indica* was prepared by decoction for 1 h. The extract was concentrated by evaporation and dried to obtain a fine brown powder, which was used as the standardized active ingredient of Vimang formulations. It melts at 210–215 °C with decomposition. The chemical composition of this extract has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV–Vis spectrophotometry (Nuñez et al., 2002). The solid extract was dissolved in distilled water for the studies.

2.2. Chemicals

The mutagenic chemicals used in this study were benzo(a)pirene, cyclophosphamide (CP) and bleomicin from Sigma (St. Louis, MO, USA), sodium azide (NaAz) and 2-aminofluorene (AF) were supplied by BDH (Chemicals Ltd., England). Colchicine was purchased from Sigma (St. Louis, MO, USA). Additionally, sodium phenobarbital and 5,6-β-naphtoflavone (BDH) were used for the induction of the S_9 fraction.

2.3. In vitro assay

Ames test. The *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, TA100 and TA102 were provided by the Center for Research Medical Technology and Devices, Havana City, Cuba, which were a kindly gift to them by Dr. B.N. Ames, Berkeley, CA, USA.

Liver cytosolic fractions were prepared from young adult male Sprague Dawley rats. According to INVITTOX Protocol (1990), animals were sacrificed 5 days after receiving daily intraperitoneal (ip) injections of sodium phenobarbital at 30 mg/kg (day 1) and 60 mg/kg (days 2–5). Also, a single administration of 5,6,β-naphtoflavone (80 mg/kg) was given on third day. The $9000 \times g$ liver supernatant (S_9) was split into 1 mL aliquots, and frozen and stored at −80 °C.

The standard plate-incorporation method in the presence and absence of S_9 was performed according Maron and Ames (1983). For these, Vimang was prepared in *N*,*N*-dimethylformamide (Sigma) at stock concentration of 50 mg/mL, concentration limit which Vimang is soluble and it was added to the cultures at concentrations of 200, 500, 1000, 2000 and 5000 μg/plate, the maximum limit of concentration recommended for this assay (INVITTOX Protocol, 1990; Maron and Ames, 1983). In addition, negative (vehicle) and positive controls were included. Dimethylnitrosoamine (DMNA) (5 μL/plate), sodium azide (NaAz) (2 μg/plate) and 2-aminofluorene (AF) (5 μg/plate) were used as positive control. 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 successively with 0.1 mL of the compound solution (or controls), 0.1 mL overnight culture (about 10^8 cells) and 0.5 mL S_9 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. For data analysis, the validity of a test was assessed by comparing the control values with internal historical data.

First, an exploratory experiment for studding the toxicity effects of the extract was performed. In this case, it was only used the higher concentration (5000 μg/mL) on each strain used and after 48 h exposure no toxicity effects were observed (data no shown).

Micronucleus assay in human primary culture lymphocytes. Blood was collected from two health volunteers (donor one was a 27 year old female and donor two a 32 year old male) with EDTA as anticoagulant and it was cultured for 72 h in a humidified incubator with 5% CO_2 at 37 °C. Culture medium consisted of RPMI 1640 supplement with 10% fetal bovine serum (Sigma), 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin (Sigma) and 1% phytohemagglutinin (PHA, Sigma).

Cells were incubated for 24 or 48 h in the culture medium. Afterward, except for negative controls, the medium was replaced by the treatment medium, containing either different test compound concentrations (150, 500 and 1500 μg/mL) or positive control chemicals dissolved in medium. Cells were treated with the extract for 3 h (+S_9) or for 20 h (−S_9). Cytochalasin B (4 μg/mL) was added to the cultures at 44 h post initiation as

described Fenech (2000). Cytochalasin B prevents the cells from completing cytokinesis, resulting in the formation of multinucleated cells. Prior to harvesting, cell viability was determined by trypan blue exclusion, it was done approximately 72 h after the start of treatment. Colcemid (0.05 μM) a known aneuploidogen compound and bleomycin (5 μg/mL) a clastogen were used as positive controls under similar experimental conditions. Benzo(a)pirene (5 μg/mL), a undirect mutagen, was used when metabolic activation was introduced in the cultures. Cytokinesis-block proliferation index (CBPI) was determined as measure of cytotoxicity and calculated by the expression

$$\mathrm{CBPI} = (M1 + 2(M2) + 3(M3) + 4(M4))/N,$$

were $M1$–$M4$ means the number of cells with one to four micronuclei and N is the total number of viable cells.

2.4. In vivo assays

Animals. NMRI mice (20–25 g) of both sexes were purchased from the National Center for Laboratory Animals Production (CENPALAB, Havana, Cuba) and adapted for 5 days to laboratory conditions. A standard rodent chow manufactured in CENPALAB was supplied *ad libitum*, except before the sacrifice, wherein animals were fasted for 12 h. Access to top water was freely allowed during the experiment. 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.

Experimental design. The assays were performed using 6 animals/group. The doses of Vimang evaluated were 50, 100 and 150 mg/kg by ip route. The high dose tested represent about the 70% of LD_{50} reported for mice when this experimental route is used (LD_{50} females: 273.14 mg/kg and LD_{50} males: 219.67 mg/kg). Another experimental group was administered with 175 mg/kg, dose that represent about 80% of LD_{50} for analysing the data at 6, 24 and 48 h (3 animals/exposure time), but only could be analyzed the data of 6 and 24 h because the other animals dead before 48 h exposure to the extract and different toxicity signs were observed in all the animals administered (data no shown). For this reason, the high dose evaluated on this study was 150 mg/kg and the animals were administered during 2 days with the product and vehicle. Positive control group was administered with 100 mg/kg of CP receiving once ip injection (0.1 mL/animal). Samples (blood and femora) were down 24 h after the last administration for Vimang treated and vehicle groups and 48 h after the single injection for CP in the positive controls.

Comet assay. The Comet assay was essentially performed as described by Singh et al. (1998). Blood peripheral lymphocytes were purified using the Lymphoprep kit (Nycomed). 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 were 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 were randomly distributed among multiple electrophoresis runs in a fully balanced design. After electrophoresis, slides were neutralized in TE buffer three times, dried and stained with ethidium bromide (20 μg/mL) prior to analysis. Stained nucleoids were evaluated with an Olympus A_2 fluorescence microscope and were analyzed 100 cells per animal (derived from two slides coded prior to analysis). 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 = nucleoids in level 1 + 2 × nucleoids in level 2 + 3 × nucleoids in level 3 + 4 × nucleoids in level 4, then if all nucleoids are in level 0 (undamaged) the score is 0 and if all the nucleoids are in level 4 (maximally damage) the score is 400 expressed in Arbitrary units (AU) (Hartmann et al., 2003).

Micronucleus test. Before the animal sacrifice, a 5 mL centrifuge tube was filled with fetal calf serum for each individual. From the freshly killed animal both femora were removed. The 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 was introduced into the canal of the bone with syringe. Immediately, femur was submerged in the tube and the marrow was aspirated, several aspirations and flushings were performed. Cells were centrifuged at 1000 rev/min for 5 min. Two drops of cell suspension from each animal were placed onto clean, dry slides and smeared, fixed in methanol and staining with Giemsa at 5% for 12 min. For each animal, 2000 polychromatic erythrocytes (PCE) were analyzed for determining the presence of micronuclei and the percentage of cells containing micronucleus on the total number of cells were evaluated. Also, the normochromatic erythrocytes (NCE) were scored in 200 erythrocytes from each animal to determine the ratio PCE/NCE (Hayashi et al., 1994).

Statistical analysis. Statistical analysis for Ames test was performed after to determine the mean ± DS values of revertants/plate using a nonparametric method (Kolmogorov–Smirnov test) (Maron and Ames, 1983) and the SALANAL statistical package software. *In vitro and vivo* series (micronucleus and Comet assays) the values of descriptive statistics are shown as mean ± SD. The data of *in vitro* micronucleus test was evaluated using a Cochran–Armitage test (Trend test) and for *in vivo* studies data was analyzed using non parametric Mann Whitney test. The *a priori p* level for statistical significance was $\alpha = 0.05$ was used in all cases.

3. Results and discussion

In the development of unknown products and chemicals is early necessary to determine the potential mutagenic effects. A balance between therapeutic versus toxicological effects of the compound is important when verifying its applicability as a pharmacological drug.

Histidine requiring mutants of *S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100 and TA102 strains, with and without metabolic activation were used for point-mutation tests (Table 1). No toxicity was observed after Vimang exposure (5000 μg/mL) in any strain of *Salmonella*. The frequencies of spontaneous reversions agree with those reported for such strains by other authors (Mitchell, 1982) and with our own historical values, included results observed using TA102 strain, which is an oxidative strain. The treatment with mutagenic substances such as AzNa, AAF and benzo(a)pirene significantly increased the frequency of revertants. On the contrary, the addition of Vimang at doses from 200 up to 5000 μg/plate, did not induce significantly changes in the reversion frequency compared with controls. Similar results were obtained with or without metabolic activation. These results showed that *M. indica* L. extract was not able to induce mutagenesis in the different strains of *S. typhimurium* used.

Comet assay was used to determine DNA damage measured as strand breaks and alkali-labile sites on blood peripheral lymphocytes of NMRI mice. Table 2 shows the percentage of damage cells and the distribution of damage in levels in the different treated groups. No single strand breaks or alkali-labile sites were induced in cells after Vimang treatments compared with controls. Bearing in mind that the increases of reactive oxygen species (ROS) concentrations have been associated with the induction of

Table 1
Results observed of *Mangifera indica* L. extract in Ames test (mean ± SD)[a]

Strains	S_9	Control	Vimang (μg/plate)					DMNA (5 μg/plate)	AzNa (2 μg/plate)	AF (5 μg/plate)
			200	500	1000	2000	5000			
TA35	–	9.67 ± 1.5	8.00 ± 1.0	8.67 ± 1.5	6.50 ± 3.5	7.00 ± 3.5	8.00 ± 1.4		30.5 ± 6.3*	
	+	8.67 ± 1.5	7.00 ± 3.6	7.00 ± 1.0	10.00 ± 3.6	10.33 ± 1.1	8.00 ± 1.4			
TA37	–	9.33 ± 3.0	10.67 ± 1.5	7.00 ± 2.6	5.00 ± 2.0	6.00 ± 1.7	5.00 ± 2.6			
	+	6.00 ± 1.0	8.33 ± 4.5	10.33 ± 4.0	3.33 ± 2.3	6.00 ± 1.4	6.40 ± 1.9	42.1 ± 5.8*		
TA38	–	9.67 ± 4.5	7.33 ± 2.5	8.33 ± 2.1	17.00 ± 4.2	8.33 ± 1.5	7.67 ± 1.5			
	+	9.00 ± 2.6	9.67 ± 2.5	11.00 ± 3.6	8.67 ± 2.5	9.67 ± 2.5	11.00 ± 2.0			131.0 ± 44.0*
TA98	–	22.3 ± 2.9	23.7 ± 6.3	20.3 ± 3.2	17.7 ± 2.5	18.7 ± 6.0	16.20 ± 2.7			
	+	25.7 ± 2.5	24.3 ± 6.6	17.7 ± 1.1	16.0 ± 6.5	22.7 ± 3.1	20.00 ± 3.7			140.7 ± 5.7*
TA100	–	104.7 ± 21	108.7 ± 8	120.7 ± 17	95.0 ± 5	88.3 ± 19	88.30 ± 15		378.3 ± 22*	
	+	108.3 ± 12	122.0 ± 8	122.0 ± 15	121.3 ± 17	109.7 ± 19	87.00 ± 13	535.7 ± 27.8*		
TA 102	–	135.6 ± 34	154.3 ± 14	132.5 ± 27	129.3 ± 21	155.5 ± 27	145.2 ± 19		499.0 ± 36*	
	+	140.5 ± 23	139.5 ± 35	146.6 ± 21	137.5 ± 17	150.2 ± 33	148.7 ± 21	775.4 ± 35*		

Mean of two experimental series, 6 plates. Incubations were made in presence ($+S_9$) and absence ($-S_9$) of rat liver microsomal mix.
AzNa: sodium azide, AF: 2-aminofluorene, DMNA: dimethylnitrosoamine.
* $p < 0.05$, comparison between groups by Mann Whitney U test.
[a] Number of revertant colonies by plate in each treatment.

Table 2
Effects of *Mangifera indica* L. extract on Comet assay in NMRI mice of both sexes (mean ± SD)

Treatment	Damage cells (%)	Level 0	Level 1	Level 2	Level 3	Level 4	DNA damage (UA)
Male							
Vehicle control	19.6 ± 4.0	80.4 ± 4	14.8 ± 6	4.0 ± 1	0.8 ± 1	0	23.8 ± 5.6
Vimang (mg/kg)							
50	19.9 ± 6.7	82.2 ± 5	12.5 ± 4	6.2 ± 4	0.1 ± 0.2	0.1 ± 0.2	28.0 ± 6.0
100	22.0 ± 5.0	81.2 ± 4	17.0 ± 7	2.8 ± 1	0	0	24.0 ± 3.7
150	20.0 ± 4.7	78.8 ± 9	16.4 ± 5	5.6 ± 3	0.2 ± 0.4	0.2 ± 0.4	26.0 ± 1.9
CP (100 mg/kg)	84.7 ± 8.0*	15.2 ± 8*	54.5 ± 6*	29.5 ± 7*	0.8 ± 0.2	0.8 ± 0.2	118.0 ± 1.3*
Female							
Vehicle control	18.8 ± 8.0	82.2 ± 5	12.8 ± 3	6.0 ± 3	0	0	24.8 ± 6.2
Vimang (mg/kg)							
50	20.0 ± 6.9	80.5 ± 4	14.1 ± 6	6.4 ± 5	0	0	24.2 ± 2.7
100	19.3 ± 7.0	82.5 ± 6	13.2 ± 6	5.3 ± 2	0.2 ± 0.2	0.2 ± 0.2	22.8 ± 6.4
150	19.9 ± 8.0	79.9 ± 6	14.0 ± 8	6.0 ± 3	0.1 ± 0.2	0.1 ± 0.2	29.0 ± 7.5
CP (100 mg/kg)	85.0 ± 8.1*	17.3 ± 5*	55.7 ± 5*	26.1 ± 4*	0.9 ± 0.4	0.9 ± 0.4	111.4 ± 6.5*

%: represents number of damage cells/ total cells, 100 cells were analyzed by slide, CP: cyclophosphamide (positive control).
Levels of damage: 0, undamaged cells and class 4, totally damaged cells. Damage degree between 0 and 4 were included by Collins et al. (1993).
* $p < 0.05$, comparison between treated groups and control by Mann Whitney U test.

DNA strand breaks (Labieniec et al., 2003), our results suggest that Vimang treatment does not induce increments of ROS or other compounds related with this endpoint at this level. In contrast, for animals treated with CP (positive control) damage was significantly different to the rest of the groups, which is similar to the results observed in NMRI mice treated with CP (Gámez et al., 2001).

On the other hand, as it is known, a micronucleus comprises a portion of chromatin surrounded by a separate nuclear membrane, which arises either by condensation of acentric chromosomes that remain separated at anaphase due to their inability to attach to the spindle at cell division, or by exclusion of intact centric chromosomes from anaphase segregation (Mitchell and Combes, 1997). Therefore, micronulcei represent the disruption of small amounts of decondensed chromatin. Thus, the existence of increased numbers of micronuclei is evidence of prior induction of structural chromosome damage or of changes in chromosome number.

In our study micronucleus analysis was performed in both human lymphocytes primary culture and on bone marrow in mice (Tables 3 and 4). Three concentrations of the extract (150, 500 and 1500 μg/mL) were used for testing the damage in primary human lymphocytes for 3 or 20 h in replicate cultures. As it can be seen, viability of treated cells decreased concentration dependent, when cultures were

Table 3
Effects of *Mangifera indica* L. extract on micronucleus assay in human lymphocytes primary cells

Treatment	Concentration (μg/mL)	Cytotoxicity[a]	MN[b] frequency (%)	Result[c]
Vimang 20 h, −S9, culture 1 (24 h)	0	0	0.8	−
	150	21	0.4	
	500	52	0.9	
	1500[b]	79	Toxic	
Vimang 20 h, −S9, culture 2 (24 h)	0	0	1.1	−
	150	10	0.8	
	500	51	1.5	
	1500	85	Toxic	
Vimang 20 h, −S9, culture 1 (48 h)	0	0	0.5	−
	150	0	0.5	
	500	0	0.9	
	1500	38	0.3	
Vimang 20 h, −S9, test 2 (48 h)	0	0	1.2	−
	150	0	1.5	
	500	23	0.8	
	1500	39	0.3	
Vimang 3 h, +S9, test 1 (24 h)	0	0	0.5	−
	150	12	1.2	
	500	40	1.4	
	1500	52	1.1	
Vimang 3 h, +S9, test 2 (24 h)	0	0	0.8	−
	150	3	1.4	
	500	50	1.3	
	1500	60	1.4	
Vimang 3 h, +S9, test 1 (48 h)	0	0	0.5	−
	150	0	0.7	
	500	0	0.9	
	1500	0	0.9	
Vimang 3 h, +S9, test 2 (48 h)	0	0	0.8	−
	150	0	0.9	
	500	0	0.9	
	1500	0	1.0	
Bleomicin, 20 h, test 1, (24 h)	5	43	3.5	+
Bleomicin, 20 h, test 2, (24 h)	5	40	5.6	+
Bleomicin, 20 h, test 1, (48 h)	5	47	4.3	+
Bleomicin, 20 h, test 2, (48 h)	5	47	5.0	+
Colchicine, 20 h, test 1, (24 h)	0.01	36	1.5[c]	+
Colchicine, 20 h, test 2, (24 h)	0.01	41	2.0[c]	+
Colchicine, 20 h, test 1, (48 h)	0.01	35	1.5[c]	+
Colchicine, 20 h, test 2, (48 h)	0.01	32	5.0[c]	+
B(a)P, 3 h, test 1, (24 h)	1	9	2.3	+
B(a)P, 3 h, test 2, (24 h)	1	10	5.1	+
B(a)P, 3 h, test 1, (48 h)	1	12	1.5	+
B(a)P, 3 h, test 2, (48 h)	1	13	1.9	+

[a] Cytotoxicity = 100 − 100 × (CBPIt − 1)/(CBPIc − 1); negative values assumed as no cytotoxicity.
[b] Micronucleus analyzed in mononucleated cells.
[c] Trend test (Cochran–Armitage test) or χ^2 for positive controls.

Table 4
Effects of *Mangifera indica* L. extract on micronucleus assay in NMRI mice of both sexes (mean ± SD)

Treatment	MN/PCE (%)	PCE/NCE
Male		
Vehicle control	0.23 ± 0.05	0.90 ± 0.05
Vimang (mg/kg)		
50	0.13 ± 0.04	0.73 ± 0.12*
100	0.15 ± 0.05	0.58 ± 0.05*
150	0.25 ± 0.08	0.56 ± 0.05*
CP 100 mg/kg	4.64 ± 0.43*	0.61 ± 0.05*
Female		
Vehicle control	0.17 ± 0.08	1.17 ± 0.05
Vimang (mg/kg)		
50	0.18 ± 0.05	0.62 ± 0.07*
100	0.16 ± 0.02	0.69 ± 0.05*
150	0.16 ± 0.04	0.66 ± 0.10*
CP (100 mg/kg)	6.48 ± 1.46*	0.74 ± 0.08*

PCE: polychromatic erythrocytes; %: represents number of micronucleus in PCE; NCE: normochromatic erythrocytes; PCE/NCE: ratio PCE/NCE 2000 cells per animal were examined; CP: cyclophosphamide.
* $p < 0.05$, comparison between treated groups and control by Mann Whitney U test.

treated during 20 h with the extract, which permit to affirm Vimang did induce cytotoxicity effects on human lymphocytes under these experimental conditions (Table 3). In all tested variants of treatments, there were no significant differences in total micronucleus frequency between treated and negative control experiments for either donor. These data suggest Vimang no induced genotoxic effects on primary human lymphocytes.

On the other hand, Vimang exposition to mice by ip route during 48 h did not significantly increase the frequency of PCE (data present as percent mPCE) compared to the control groups. This result reflects treatment with *M. indica* L. extract to mice did not induce genotoxic effects up to 150 mg/kg ip, doses that represent 70% of the LD_{50} of the extract in this specie. Meanwhile, Vimang did exhibit cytotoxic activity in this assay, determined as the PCE/

NCE ratio, which is in correspondence with the results observed on *in vitro* micronucleus assay and the results of the acute ip toxicity studies performed in rodents. It has been reported that single doses of Vimang is safe by oral route up to 5000 mg/kg body weight, but when it is administered by ip route, different toxicity signs are observed in mice and rats. These signs are dose dependent and included the dead of the animals into the firsts 72 h after the exposition at the higher doses tested. As it was expected, the administration of CP (positive control) significantly increased the ratios of PCE/NCE and PCE in both male and female animals, indicating the validity of the species selected and the study design to detect *in vivo* cytotoxic and clastogenic effects.

Neither mutagenic nor genotoxicity effects have been observed after exposition of *M. indica* L. extract in both *in vitro* and *in vivo* experimental models. Data about possible mutagenicity or genotoxicity effects from the main components of the extract have not been reported before. In addition, studies performed by Matsushima et al. (1985) showed that mangiferin, the main polyphenol present in the extract, is not mutagenic to *S. typhimurium* strains by using the Ames assay. However, many flavonoids have shown to be genotoxic in a variety of prokaryotic and eukaryotic cells and *in vivo* systems (Synder and Gillies, 2002; da Silva et al., 1997; Hodek et al., 2002; Walle et al., 2001; Labieniec et al., 2003). The mechanistic basis for this genotoxicity has not been fully elucidated, although structure–activity relationship studies have identified as a requisite the flavonoid structural features.

On the other hand, the probably cause of mutagenic effects observed in Ames and SOS assays after exposition to the crude extract of plants having polyphenols as components, is the formation of ROS associated with the presence of flavonoids inside cells (de Carvalho et al., 2003; Labieniec et al., 2003). Inside cell phenolic acids molecules (for example) bind with proteins and lipids, and they become quite easily oxidised by O_2, $O_2^{\cdot-}$ or H_2O_2. Free oxygen radicals can be generated during reactions between polyphenol–protein (lipid) complex and oxygen. These radicals may interact with DNA and induce a broad spectrum of DNA damage. It has been demonstrated that oxygen radicals can be generated by the reaction of a phenolic structure in the presence of oxygen (Bors and Michel, 1999). Up to date, there are no pro-oxidant effects reported for *M. indica* L. extract in cells. Additionally, our results showed Vimang did not induce any genotoxic effects in the three assays used.

In conclusion, this study shows that *M. indica* L. extract did not show any mutagenic or genotoxic effect under our experimental conditions.

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