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# Genotoxicity of the medicinal plant *Maytenus robusta* in mammalian cells *in vivo*

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**ABSTRACT.** Plants belonging to the Celastraceae family have been used in traditional medicine for their analgesic, anti-inflammatory and anti-ulcerogenic properties, among others. *Maytenus ilicifolia* is the principal species of this family, and is used in the treatment of gastric ulcers. However, owing to its inadequate management in Brazil, the species is becoming extinct and is being substituted with *Maytenus robusta*, which also displays gastroprotective activity. The aim of this study was to evaluate the genotoxic effects of *M. robusta* hydroalcoholic extract *in vivo*, using the comet assay and micronucleus test. Three doses (50, 250 and 500 mg/kg body weight) were administered to mice orally 2 times at 24-h intervals. Cytotoxicity was assessed by scoring 200 consecutive total polychromatic and normochromatic erythrocytes to calculate their ratio. Parametric (analysis of variance/Tukey) and non-parametric (Kruskal-Wallis/Dunn *post hoc*) tests were used to evaluate the results according to the nature of the data distribution. The results

 

showed a significant increase in the frequency of DNA damage on leukocytes at the 2 higher doses tested, but the extract did not enhance micronucleus frequency in bone marrow cells. Our findings showed that after 48 h of treatment, *M. robusta* hydroalcoholic extract had weak genotoxic effects but no clastogenic effects in mice cells.

**Key words:** *Maytenus robusta*; Micronucleus test; Comet assay; Celastraceae; Genotoxic activity

## INTRODUCTION

Medicinal plants are of special importance for their role in health protection as preventive or supportive therapy for numerous diseases and disorders (Farnsworth and Soejarto, 1991). Although herbal remedies are often perceived as being natural and therefore safe, they are not free from adverse effects, and evaluation of their safety, efficacy and quality is necessary (WHO, 1991; Ernst, 2000).

Plants of the Celastraceae family have been the subject of continued and growing interest owing to their pharmacological activities. The leaves of some Celastraceae are used in traditional medicine for their analgesic, anti-inflammatory and anti-ulcerogenic activities, among others (Corrêa, 1984). Some species of this family have been shown to have anti-ulcer activity, e.g.: *Maytenus ilicifolia* (Jorge et al., 2004), *M. aquifolium* (Gonzalez et al., 2001), *M. truncata* (Silva et al., 2005), and *Austroplenckia populnea* (Andrade et al., 2006). Phytochemical characterizations of plants belonging to the *Maytenus* genus have demonstrated the presence of many classes of constituents, including flavonoids (Leite et al., 2001; Tiberti et al., 2007), pentacyclic triterpenes principally from the friedelane, oleanane, lupane, and ursane series (Oliveira et al., 2006), sesquiterpenes, alkaloids (Brüning and Wagner, 1978), and condensed tannins (Gonzalez et al., 1982).

*M. ilicifolia* is used in the treatment of gastric ulcers; however, its populations in Brazil are presently in a clear decline owing to inadequate management (Niero et al., 2001). *M. robusta* is other species from this genus that is well adapted to the ecosystem of southern Brazil (Niero et al., 2001) and is used in Brazilian folkloric medicine to treat stomach ulcers (Balbach, 1980; de Andrade et al., 2007). Thus, its use is suggested in phytotherapeutic preparations, as a substitute for *M. ilicifolia*. To the best of our knowledge, no genotoxic studies of *M. robusta* extracts have been carried out. Therefore, we report herein the genotoxic evaluation of the aerial parts of this plant on mice cells using the comet assay and micronucleus test.

## MATERIAL AND METHODS

### Chemicals

N-nitroso-N-ethylurea (ENU, Sigma, Canada, CAS No. 759-73-9) was used as the DNA-damaging agent in the micronucleus test and comet assay with Swiss mice and was dissolved in phosphate buffer, pH 6. The other main chemicals were obtained from the following suppliers: Tween 80 (Dinâmica, Brazil, CAS No. 9005-65-6); dimethyl sulfoxide

(Synth, Brazil); ethidium bromide (Sigma, USA, CAS No. 1239-45-8); ethylenediaminetetraacetic acid (EDTA, Dinâmica, CAS No. 6381-92-6); low melting point agarose (Invitrogen, USA); normal melting point agarose (Invitrogen); sodium *N*-lauroylsarcosine (Sigma, UK); Tris(hydroxymethyl)aminomethane (Tris, Vetec, Brazil) and Triton X-100 (Merck, USA).

## Plant material and extraction

*M. robusta* was collected at the Morro do Baú Ecological Park, Ilhota, Santa Catarina, Brazil, and identified by Dr. Ademir Reis (Department of Botany, Universidade Federal de Santa Catarina, Florianópolis, Brazil). A voucher specimen was deposited at the Barbosa Rodrigues Herbarium (Itajaí, Santa Catarina, Brazil) under number V.C. Filho 016. Air-dried and powdered aerial parts of *M. robusta* (500 g) were extracted with methanol for 1 week. The macerated leaves were filtered and concentrated under reduced pressure, yielding 38.0 g (7.6%) crude methanol extract.

## Phytochemistry study

The concentrated hydroalcoholic extract (38.0 g) was diluted with 300 mL water and successively partitioned with hexane and ethyl acetate. Evaporation of the 2 organic extracts gave 2.8 and 8.3 g residue, respectively. Part of ethyl acetate fraction (6.0 g) was chromatographed on silica gel column (0.063-0.20 mm, 200 g) and eluted with a gradient of MeOH in $CHCl_3$ to give minor fractions (1-170). The similar fractions (51-75) eluted 98:2 were combined, giving 2.0 g of a brown residue, which was rechromatographed using the above system, furnishing steroid stigmasterol and the triterpenes friedelin, friedelanol, 3,15-dioxo friedelane, and 3,15-dioxo-21α-hydroxyfriedelane. The compounds were identified by Co-thin-layer chromatography and nuclear magnetic resonance spectral data comparisons with authentic samples.

## Animals and dosing

The experiments were carried out using 12-week-old male Swiss albine mice (*Mus musculus*), weighing 25-30 g. The animals were acquired from the Universidade Estadual Paulista (UNESP), Botucatu, São Paulo State, Brazil, and housed in polyethylene boxes in a climate-controlled environment (25° ± 4°C, 55 ± 5% humidity) with a 12-h light/dark cycle (7:00 am to 7:00 pm). Food (Nuvilab CR1, Nuvital) and water were available *ad libitum*. The mice were divided into 5 experimental groups of 6 animals each. *M. robusta* hydroalcoholic extract was dissolved in 1% Tween 80 aqueous solution. A fixed volume of 0.5 mL was administered twice by gavage at 24-h intervals (48-h treatment). The doses administered were 50, 250, and 500 mg/kg body weight, chosen on the basis of gastroprotective activity in rodents (de Andrade et al., 2007). The negative control group received 1% Tween 80 aqueous solution, and the positive control group received an intraperitoneal injection of ENU (50 mg/kg). The animals used in this study were sacrificed by cervical dislocation. The animal Bioethics Committee of the Faculdade de Medicina de Marília in Marilia, Brazil, approved the study on October 15, 2008 (protocol No. 468/08).

## Comet assay

A comet assay [single-cell gel electrophoresis (SCGE)] was carried out following a method described by Speit and Hartmann (1999), which is based on the original study of Singh et al. (1988) and includes modifications introduced by Klaude et al. (1996) as well as additional modifications. Peripheral blood samples were obtained from animals in each group 24 h after treatment. An aliquot was removed from the peripheral blood cell suspension to determine cell viability. Cell counting was performed using a hemocytometer. Cell viability was determined using Trypan blue dye exclusion. The number of Trypan blue-negative cells was considered the number of viable cells and was greater than 90%. A 10-µL aliquot of cells from each animal was mixed with 120 µL 0.5% low melting point agarose at 37°C and immediately spread onto 2 microscope slides per animal pre-coated with 1.5% normal melting point agarose. The slides were coverslipped and allowed to gel at 4°C for 20 min. The coverslips were gently removed, and the slides were immersed in cold, freshly prepared lysing solution consisting of 89 mL stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 mL distilled water, and 1% sodium lauryl sarcosine), 1 mL Triton X-100 (Merck), and 10 mL dimethyl sulfoxide (Merck). The slides, which were protected from light, were allowed to stand at 4°C for 1 h and then placed in a gel box, positioned at the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH, 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) at 4°C for 20 min before electrophoresis to allow the DNA to unwind. Electrophoresis was carried out in an ice bath (4°C) for 20 min at 300 mA and 25 V (0.722 V/cm). The slides were then submerged in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 15 min, dried at room temperature and fixed in 100% ethanol for 10 min. The slides were dried and stored overnight or longer before staining. For staining process, the slides were briefly rinsed in distilled water, covered with 30 µL 1X ethidium bromide staining solution prepared from a 200 µg/mL 10X stock solution, and coverslipped. The material was evaluated immediately at 400X magnification using a fluorescence microscope (Olympus BX 50) with a 515-560-nm excitation filter and a 590-nm barrier filter.

The extent and distribution of DNA damage indicated by the SCGE assay were evaluated by examining at least 100 randomly selected and non-overlapping cells on the slides (50 cells per slide) per animal. These cells were scored visually, according to tail size, into 4 classes according to tail size as follows: class 0: no tail; class 1: tail shorter than the diameter of the head (nucleus); class 2: tail length 1-2 times the diameter of the head, and class 3: tail longer than two times the diameter of the head. Comets with no heads and images with nearly all DNA in the tail or with a very wide tail were excluded from the evaluation because they likely represented dead cells (Hartmann and Speit, 1997). The total score for 100 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 300 (all maximally damaged).

## Bone marrow micronucleus test

The micronucleus test was performed following the method described by Krishna and Hayashi (2000), with some modifications. The rodent groups used for the comet assay test were also used in this test. The animals were killed 24 h after the second extract treat-

ment. The femur were immediately removed, and the bones were freed from the muscles. The epiphyses were cut and the bone marrow was flushed out using 2 mL saline solution (NaCl 0.9%). After centrifugation for 7 min, the supernatant was discarded. Formaldehyde (4%) was added to preserve the cytoplasm. A small drop of the resuspended cell pellet was spread onto a clean glass slide, which was air-dried and fixed in absolute methanol for 10 min. The smears were then stained with 5% Giemsa solution (Schmid, 1975). For the analysis of the micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored to determine the clastogenic properties of the *M. Robusta* extract. To detect possible cytotoxic effects, we calculated the PCE:normochromatic erythrocyte (NCE) ratio in 200 erythrocytes/animal (Gollapudi and McFadden, 1995). The cells were blindly scored using a light microscope at 1000X magnification. The mean number of micronucleated polychromatic erythrocytes (MNPCE) in the individual mice was used as the experimental unit, with variability (standard deviation) based on differences between animals within the same group.

## Statistical analysis

To compare the results, parametric (analysis of variance/Tukey) and non-parametric (Kruskal-Wallis/Dunn *post hoc*) tests were used according to the nature of the data distribution. The GraphPad Instat® version 3.01 software was used for this analysis. The positive control was not included in the analysis of variance procedure and was compared with the negative control separately. Difference with a P value $< 0.05$ was considered to be statistically significant.

## RESULTS AND DISCUSSION

The implications of the genotoxic effects of mixture complexes and chemicals include the initiation of carcinogenicity, the generation of hereditary defects via germ cell mutations, and teratogenicity (Mitchelmore and Chipman, 1998). An increase in genotoxicity is associated with an increased overall risk of cancer (Hagmar et al., 1998).

SCGE or comet assay and micronucleus test are the most useful means for detecting the potential genotoxicity of chemicals and mixture complexes (Krishna and Hayashi, 2000; Hartmann et al., 2003). They are considered markers of the early biological effects of carcinogen exposure (Lippman et al., 1990; Schabach et al., 2003).

The results of the comet assay in the evaluation of *M. robusta* extract, namely data on the total number of cells with damage and scores for mice treated with 50, 250 and 500 mg/kg - in negative (Tween) and positive controls (50 mg/kg ENU) are presented in Table 1. As expected, the positive control induced a significant increase in DNA migration in leukocytes ($P < 0.001$). A statistically significant increase in DNA damaged cells was observed for *M. robusta* extract at the 2 higher doses tested compared with that in undamaged cells. When cells were exposed to 3 concentrations of the extract, most of the cells examined on the slides were undamaged, a few cells showed minor damage (class 1) and very few showed a large amount of damage (classes 2 and 3). The cell viability for leukocytes was greater than 90% using Trypan blue staining, confirming the absence of cytotoxicity observed by the PCE:NCE ratio in the micronucleus test (Table 2).

**Table 1.** DNA migration in the comet assay for the assessment of genotoxicity of *Maytenus robusta* hydroalcoholic extract in peripheral blood cells (collected 24 h after treatment) from male Swiss mice (M) *in vivo*.

| Treatments | Animals | Total[c] | Comet classes | | | | Scores |
|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | |
| Vehicle control (Tween) | $M_1$ | 0 | 100 | 0 | 0 | 0 | 0 |
| | $M_2$ | 1 | 99 | 1 | 0 | 0 | 1 |
| | $M_3$ | 2 | 98 | 2 | 0 | 0 | 2 |
| | $M_4$ | 4 | 96 | 4 | 0 | 0 | 4 |
| | $M_5$ | 1 | 99 | 1 | 0 | 0 | 1 |
| | $M_6$ | 0 | 100 | 0 | 0 | 0 | 0 |
| | Mean ± SD | 1.33 ± 1.50 | | | | | 1.33 ± 1.50 |
| *M. robusta extract* (50 mg/kg) | $M_1$ | 1 | 99 | 1 | 0 | 0 | 1 |
| | $M_2$ | 0 | 100 | 0 | 0 | 0 | 0 |
| | $M_3$ | 0 | 100 | 0 | 0 | 0 | 0 |
| | $M_4$ | 7 | 93 | 7 | 0 | 0 | 7 |
| | $M_5$ | 0 | 100 | 0 | 0 | 0 | 0 |
| | $M_6$ | 0 | 100 | 0 | 0 | 0 | 0 |
| | Mean ± SD | 1.33 ± 2.80 | | | | | 1.33 ± 2.80 |
| *M. robusta extract* (250 mg/kg) | $M_1$ | 15 | 85 | 10 | 5 | 0 | 20 |
| | $M_2$ | 12 | 88 | 8 | 4 | 0 | 16 |
| | $M_3$ | 13 | 87 | 10 | 3 | 0 | 16 |
| | $M_4$ | 15 | 85 | 11 | 4 | 0 | 19 |
| | $M_5$ | 21 | 79 | 15 | 6 | 0 | 27 |
| | $M_6$ | 9 | 91 | 8 | 1 | 0 | 10 |
| | Mean ± SD | 14.16 ± 4.02[a] | | | | | 18.00 ± 5.62[b] |
| *M. robusta extract* (500 mg/kg) | $M_1$ | 16 | 84 | 13 | 3 | 0 | 19 |
| | $M_2$ | 14 | 86 | 10 | 4 | 0 | 18 |
| | $M_3$ | 8 | 92 | 7 | 1 | 0 | 9 |
| | $M_4$ | 14 | 86 | 11 | 3 | 0 | 17 |
| | $M_5$ | 10 | 90 | 9 | 1 | 0 | 11 |
| | $M_6$ | 15 | 85 | 12 | 3 | 0 | 18 |
| | Mean ± SD | 11.16 ± 4.21[a] | | | | | 15.33 ± 4.22[b] |
| N-nitroso-N-ethylurea (50 mg/kg) | $M_1$ | 33 | 67 | 27 | 6 | 0 | 39 |
| | $M_2$ | 21 | 79 | 13 | 7 | 1 | 30 |
| | $M_3$ | 22 | 78 | 13 | 7 | 2 | 33 |
| | $M_4$ | 30 | 70 | 14 | 10 | 6 | 52 |
| | $M_5$ | 22 | 78 | 15 | 7 | 0 | 29 |
| | $M_6$ | 35 | 65 | 30 | 5 | 0 | 40 |
| | Mean ± SD | 27.16 ± 6.24[a] | | | | | 37.16 ± 8.56[a] |

[a]Significantly different from the vehicle control ($P < 0.001$). [b]Significantly different from the vehicle control ($P < 0.01$). [c]Total number of damaged cells (classes 1 + 2 + 3).

**Table 2.** Number of micronucleated polychromatic erythrocytes (MNPCE) observed in the bone marrow cells of male Swiss mice (M) treated with a *Maytenus robusta* hydroalcoholic extract, and respective controls.

| Treatments | Number of MNPCE per animal | | | | | | MNPCE (Mean ± SD) | PCE/NCE (Mean ± SD) |
|---|---|---|---|---|---|---|---|---|
| | $M_1$ | $M_2$ | $M_3$ | $M_4$ | $M_5$ | $M_6$ | | |
| Vehicle control (Tween) | 4 | 7 | 6 | 3 | 7 | 5 | 5.33 ± 1.63 | 1.09 ± 0.05 |
| *M. robusta* (50 mg/kg) | 0 | 3 | 2 | 5 | 4 | 3 | 2.83 ± 1.72 | 1.22 ± 0.23 |
| *M. robusta* (250 mg/kg) | 6 | 5 | 2 | 0 | 7 | 1 | 3.50 ± 2.88 | 1.29 ± 0.15 |
| *M. robusta* (500 mg/kg) | 6 | 1 | 1 | 2 | 0 | 1 | 1.83 ± 2.13 | 1.51 ± 0.12[b] |
| N-nitroso-N-ethylurea (50 mg/kg) | 17 | 19 | 12 | 15 | 16 | 19 | 16.33 ± 2.13[a] | 1.24 ± 0.23[c] |

Two thousand cells were analyzed per group. NCE = normochromatic erythrocyte; SD = standard deviation. [a]Significantly different from the vehicle control ($P < 0.001$). [b]Significantly different from the vehicle control ($P < 0.01$). [c]Significantly different from the vehicle control ($P < 0.05$).

Table 2 shows the micronucleus test results obtained for male Swiss mice treated with *M. robusta* extract: the mean number of MNPCEs in untreated controls and treated ani-

mals. The positive mutagen ENU induced a statistically significant increase in MNPCE. The clastogenicity test revealed no increase in the mean number of MNPCE at any extract doses. These data suggest that *M. robusta* extract had no clastogenic effect on the bone marrow cells of mice. We also analyzed the cytotoxicity of *M. robusta* extract by calculating the PCE:NCE ratio in the bone marrow of mice to obtain a cytotoxicity index (Gollapudi and McFadden, 1995). In normal bone marrow, the PCE:NCE ratio is generally about 1:1 (Schmid, 1975). The tested extract increased hematopoiesis: a statiscally significant increase in PCE:NCE ratio occurred at the higher extract dose tested (Table 2).

No previous results have been obtained from genetic toxicological studies performed on other *Maytenus* species. However, another species of the Celastraceae family, *A. populnea*, also displays anti-ulcer activity (Andrade et al., 2006), shares some of the compounds present in *M. robusta* extract, and has displayed similar genotoxic effects. *A. populnea* hydroalcoholic leaf extract evaluated using the micronucleus test and chromosome aberration assays induced no clastogenic effects in the bone marrow cells of Wistar rats (Pugliesi et al., 2007). A chloroform fraction of barkwood extract induces an increase (although not a statistically significant one) in the average amount of DNA damage in peripheral blood cells of Swiss mice but also showed no clastogenic effects in the bone marrow and peripheral blood cells of the rodents (Ribeiro et al., 2009).

The results obtained through our studies demonstrate that the hydroalcoholic extract of the aerial parts of *M. robusta* is genotoxic but not clastogenic at the concentrations intended to be evaluated for possible clinical efficacy. Further studies to investigate the genetic toxicity of isolated compounds of this plant species should provide a better understanding of the genotoxic mechanisms described herein.

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