Revista Brasileira de Farmacognosia 25 (2015) 164–169

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Acute genotoxicity analysis *in vivo* of the aqueous extract of *Maytenus guyanensis* Amazonian chichuá

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ARTICLE INFO

Article history:
Received 21 January 2015
Accepted 9 March 2015
Available online 30 March 2015

Keywords:
Mutagenicity
Maytenus guyanensis
Western Amazon

ABSTRACT

The species *Maytenus guyanensis* Klotzsch ex Reissek, Celastraceae, present a wide variety of possible pharmacological activities and its roots and stems are used by popular medicine in the western Amazon rainforest. Few studies have demonstrated the genotoxic safety of the popular use of this species, and owing to this, the present study aimed to perform an analysis of the acute genotoxicity *in vivo* of the aqueous extract of *M. guyanensis*. Male and female mice from *Mus musculus* species, of weights ranging from 20 to 40 g, organized in eight groups with different treatments were used. The aqueous extracts of the bark of *M. guyanensis* were administered orally by gavage with 0.1 ml of the test substance *per* 10 g of the animal, followed by performance of comet assay in peripheral blood, PCE/NCE correlation and occurrence of micronuclei in the bone marrow. It was found that the aqueous extract of *M. guyanensis*, with ten times higher concentration than those used in ethnopharmacology, did not present genotoxic effect and, moreover, it has antigenotoxic action in mice treated acutely. Further studies regarding bioaccumulation and chronic effects of this species are suggested, in order to improve the understanding of its mechanism of action, ensuring the efficacy and safety of its utilization and developing phytotherapics and drugs.

Introduction

Brazil possesses almost 19% of the world's flora, of which the Amazon Forest is one of the most rich and diversified area on the planet; however, roughly 99% of the medicinal plants do not have its efficacy and pharmacological safety proven (Giulietti et al., 2005; Fão et al., 2012; Meneguetti et al., 2014), making necessary a phytochemical and pharmacological approach (Andrade et al., 2007), which may represent a great economic potential to be explored by the pharmaceutical industry (Cechinel-Filho and Rosendo, 1998).

The popular medicinal species *Maytenus guyanensis* Klotzsch ex Reissek belongs to Celastraceae botanical family and is a small endemic tree, popularly known as "chichuá", "xixuá", "chuchauasi", "chuchuhuashu", "chuchuasi", "chuchasa" or "tonipulmon" (Revilla, 2001; Prata, 2007; Prata and Mendonca, 2009). The species present a wide variety of possible pharmacological activities, wherein its roots and stems are used popularly as analgesic, muscle relaxant, wound healing, insecticide, immunosuppressive, anti-inflammatory, anti-ulcerogenic, anti-rheumatism, anti-diarrheal, antibacterial, antifungal, anti-helminthic, antiprotozoal, antitumor, aphrodisiac and gynecologically active (Revilla, 2002; Borrás, 2003; Macari et al., 2006; Fonseca et al., 2007).

Phytochemical studies of species from the genus *Maytenus* have demonstrated presence of several chemical groups, in which we can contrast the quinone-methide triterpenoids that presented several biological activities, such as tripanocide (Duarte et al.,

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http://dx.doi.org/10.1016/j.bjp.2015.03.003
0102-695X/

2002), anti-helminthic (Mena-Rejón et al., 2007), cytotoxic and antitumor (Morita et al., 2008). Other metabolites identified within this genus were: tannins, flavonoids and alkaloids (Santos-Oliveira et al., 2009; Gonzalez et al., 1996; Chavez et al., 1998).

Phytochemical studies of leaves, stem barks and roots of *M. guyanensis* lead to isolation and identification of ten terpenoids, wherein five are friedelane: friedeline, friedelol, 16β-friedeline, 29-hydroxifriedeline and 3,7-friedelodione; one of them is a β-amerine oleanane; one of them is a α-amerine ursane, and three are friedo-nor-oleanane (quinine-methides): tingenone, 22-hydroxy-tingenone and 22-hidroxi-pristimerine. In addition, two steroids: β-sitosterol and sitostenone, one sesquiterpene alkaloid named *N,N*-dimethylserine (Sousa et al., 1986; Facundo et al., 2015) and one flavonoid: 4-methyl-epigalocatequine (Macari et al., 2004) were also identified; however, few studies have demonstrated the genotoxic safety of the popular use of this species, and owing to it, the present study aimed to perform an analysis of the acute genotoxicity *in vivo* of the aqueous extract of *M. guyanensis*.

Materials and methods

Collection and identification of botanical material

The barks of *Maytenus guyanensis* Klotzsch ex Reissek, Celastraceae, were collected in February 2008 at the Adolpho Ducke's Forest Reserve, located at the km 26, AM-010 road (Manaus-Itacoatiara – Lat 02°53′ S, Long 59°58′ W). The identification of the species was done at the Herbário do Instituto Nacional de Pesquisa da Amazônia (INPA), Exsiccate n° 188.485.

Extract preparation

Barks from *M. guyanensis* were grinded to improve solvent contact area. The extract was prepared by infusion with distilled water for 10 min at 80 °C, at the following concentrations: 3.85 mg/ml, value normally used by populations and ten, twenty and fifty times more concentrated (Camparoto et al., 2002; Meneguetti et al., 2014).

Animals' treatment

The tests were performed from January to March 2014, at Laboratory de Genetic e Toxicology Applied from Centro academic Lutheran de Ji-Paraná (CEULJI/ULBRA), in Ji-Paraná city, Rondônia, Brazil. All of the experiments were approved by Ethics Committee on Animal Use (CEUA) from Oswaldo Cruz Foundation – RO (Fiocruz-RO), protocol number 2013/12.

Mus musculus's male and female mice were used, acquired from CEULJI/ULBRA vivarium, with weight ranging from 20 to 40 g. The aqueous extracts from barks of *M. guyanensis* were administered *via* oral gavage, two amounts at 48 and 24 h, respectively, before the test began. The animals were weighed before the dosage and the volume administered was calculated as 0.1 ml of the testing substance *per* 10 g of the animal.

The animals were divided into eight groups, each one containing eight animals, four were male and four female, and they were maintained in environments with controlled temperature (25 °C), with cycles of 12 h of light and 12 h of darkness, in polyethylene cages, with access to water and food.

The following groups were organized as follows:

G1 – negative control (CN), where only H_2O was administered;
G2 to G5 – treated with aqueous extracts of *M. guyanensis* in concentrations 3.85 mg/ml, 38.5 mg/ml, 77 mg/ml and 192 mg/ml, respectively;
G6 and G7 – treated, respectively, with 3.85 mg/ml and 38.5 mg/ml, with addition of 2.0 mg cyclophosphamide *per* 100 g of animal weight, treated *via* intraperitoneal injection, 24 h before the application of the first dose of the aqueous extract of *M. guyanensis*. These concentrations were chosen, based on a study carried out by Meneguetti et al. (2014), in which these same concentrations presented anticytotoxic and anti-mutagenic action in *in vitro* studies;
G8 – positive control (CP), just 2.0 mg of cyclophosphamide was administered per 100 g of mice weight (Magalhães et al., 2010), *via* intraperitoneal injection, 24 h before the application of the first dose of the aqueous extract of *M. guyanensis*, being also treated with 0.1 ml of H_2O per 10 g of the animal, *via* gavage, at the same periods as too much groups.

Comet assay

The genotoxicity and acute antigenotoxicity analysis were done by comet assay, from the method described by Singh et al. (1988) and reviewed by Tice et al. (2000). The experiment was performed in blood cells from the animals submitted to the treatment with the aqueous extract of *M. guyanensis*. The peripheral blood of animals was collected after the decapitation with a guillotine, wherein two slides per animal were prepared.

The samples in cellular suspension were mixed with a thin layer of agar "low melting" 0.75% and laid upon layers pre-covered with normal agarose at 1.5%. These slides were plunged in a lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10 with addition of 1% Triton X-100 and 10% DMSO at the time of use), for 96 h at 4 °C. The lysis enables the migration of the DNA fragments that were brought about after incubation of the slides in alkaline tampon (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min and, subsequently, an electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min in a DNA electrophoresis vat. The slides were neutralized after electrophoresis with three tampons at 0.4 M, pH 7.5 and colored with silver nitrate.

The analysis of the cells was performed randomly by visual evaluation, totaling 100 cells/slide, wherein two parameters were considered: Damage Index (0–400) and Damage Frequency (0–100%).

Correlation PCE/NCE and occurrence of micronuclei in bone marrow

Phosphate tampon

The solutions A and B were prepared separately. For the solution A, 27.6 g of anhydride potassium phosphate (KH_2PO_4) was added *per* liter of distilled water. In the solution B was added 35.6 g of sodium dibasic phosphate ($NA_2PO_4{\cdot}7H_2O$) *per* liter of distilled water. After, 16.5 ml of the solution A was added to 46 ml of the solution B and 37.5 ml of distilled water was added, filling with 100 ml with the phosphate tampon with pH = 5.8 (Silva et al., 2011).

Bone medulla gathering and preparation of the microscopic slides

After the animals were sacrificed by guillotine decapitation, the femurs were withdrawn, cleaned and the two ends removed with a surgical scissor. The bone marrow was extracted with a histological needle directly upon the slide with 10 μl of fetal bovine serum, and with a curved histological needle the marrow was homogenized with serum, wherein a smear of each femur was made, and two slides per animal were prepared.

The slides were dried in an incubator at 37 °C, colored with a mixture of Giemsa (10%) and phosphate tampon (pH = 5.8), washed with distilled water, dried at room temperature and marked with a numeric code, for a "blind" analysis. The coloration was used to

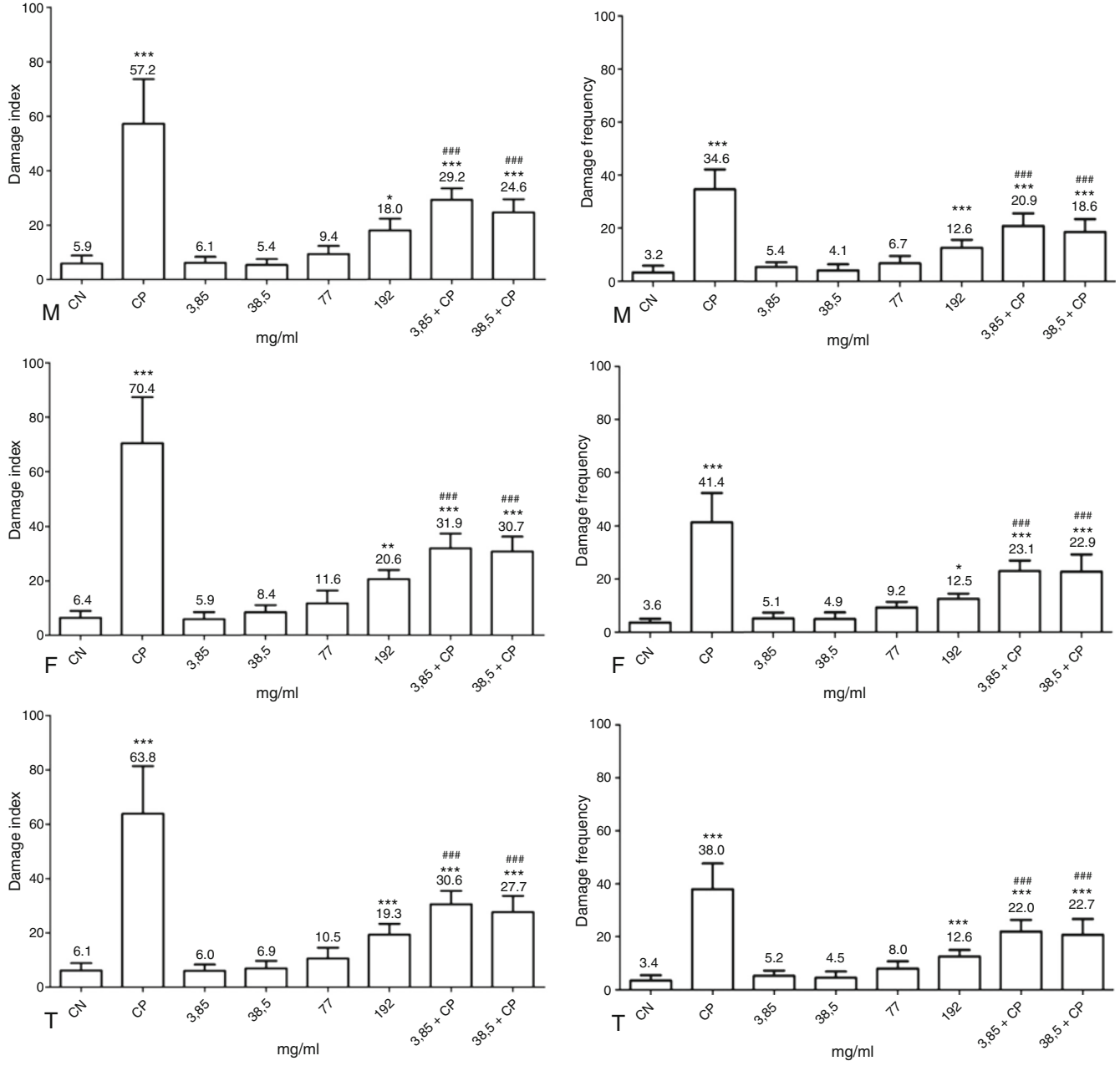

Fig. 1. Genotoxic and antigenotoxic acute effects evaluated in damage index and damage frequency by the comet test in peripheral blood of mice, which underwent the treatment with aqueous extract of *M. guyanensis*. M: Males; F: Females; T: Total – Male and Female. Statistically significant for genotoxicity $^{*}(p<0.05)$, $^{**}(p<0.01)$ and $^{***}(p<0.001)$ when compared to CN. Statistically significant for antigenotoxicity $^{\#}(p<0.05)$, $^{\#\#}(p<0.01)$ and $^{\#\#\#}(p<0.001)$ when compared to CP.

differentiate light blue polychromatic erythrocytes (PCE) and red normochromatic erythrocytes (NCE) (Ribeiro et al., 2003).

Analyses of slides

The zig-zag model was used to determine simultaneously the amount of micronuclei of each 1000 PCE and the relationship between PCE and NCE in 1000 erythroid cells per slide.

This relationship was established to avoid determination of false-negatives, demonstrating whether there was cytotoxicity or cellular depression (Shahrim et al., 2006). Afterwards, the count of only micronuclei in PCE was continued until the count of 1000 cells. The count of micronuclei occurred only in PCE, due to its indication for organisms with acute exposition analysis (Villela et al., 2003), the same as used in the present study.

Statistical analysis

The variance analysis was done by the ANOVA test, using Turkey's test as a counterproof, affecting the comparison of the means of different treatments with the control groups. For this purpose, the software Graph pad Prism 5.0 was used.

Results

Comet assay

The acute genotoxicity results demonstrated by the index and frequency of damage performed by the comet assay can be observed in Fig. 1. The 3.85 mg/ml, 38.5 mg/ml and 77 mg/ml concentrations did not present genotoxic effects, unlike the 192 mg/ml, that presented significant genotoxicity relative to CN.

In the analysis of the antigenotoxic potential, 3.85 mg/ml and 38.5 mg/ml concentrations demonstrated significant decrease in both the damage index and the damage frequency relative to the CP group.

Correlation PCE/NCE

The data corresponding to the correlation PCE/NCE influenced by the aqueous extract of *M. guyanensis* are organized in Table 1. The correlation PCE/NCE of the 3.85 mg/ml and 18.5 mg/ml concentrations did not present significant difference relative to CN. This result was not observed in the 77 mg/ml and 192 mg/ml concentrations, that decreased the correlation, demonstrating the occurrence of cytotoxicity (Shahrim et al., 2006; Silva et al., 2011), that is normally confirmed due to the dose-response effect (Krishna and Hyashi, 2000) and the likely explanation is that the frequency of cell depression (Nesslany et al., 2004) can induce to apoptosis (Ouanes et al., 2003), due to it being subjected to balance regulation between the activation and suppression of cell death in certain situations (Kaufmann and Hengartner, 2001).

The 3.85 mg/ml and 38.5 mg/ml concentrations that did not present cytotoxity, also demonstrated anticytotoxic action, increasing the PCE/NCE correlation, significantly reversing the damage caused by cyclophosphamide.

Micronuclei in bone marrow

The mean of micronuclei for each 1000 cells of mice bone marrow subjected to treatment with different concentrations of the aqueous extract of *M. guyanensis*, can be visualized in Fig. 2.

The 3.85 mg/ml, 38.5 mg/ml and 77 mg/ml concentrations did not present mutagenic effects in male and female mice, however, when the total mean was assessed, the 77 mg/ml concentration showed to be mutagenic relative to CN, which was also observed in the 192 mg/ml concentration in male, female and total mean.

The 3.85 mg/ml and 38.5 mg/ml concentrations demonstrated antigenotoxic effects again, acting as antimutagenic, decreasing significantly the number of micronuclei relative to CP.

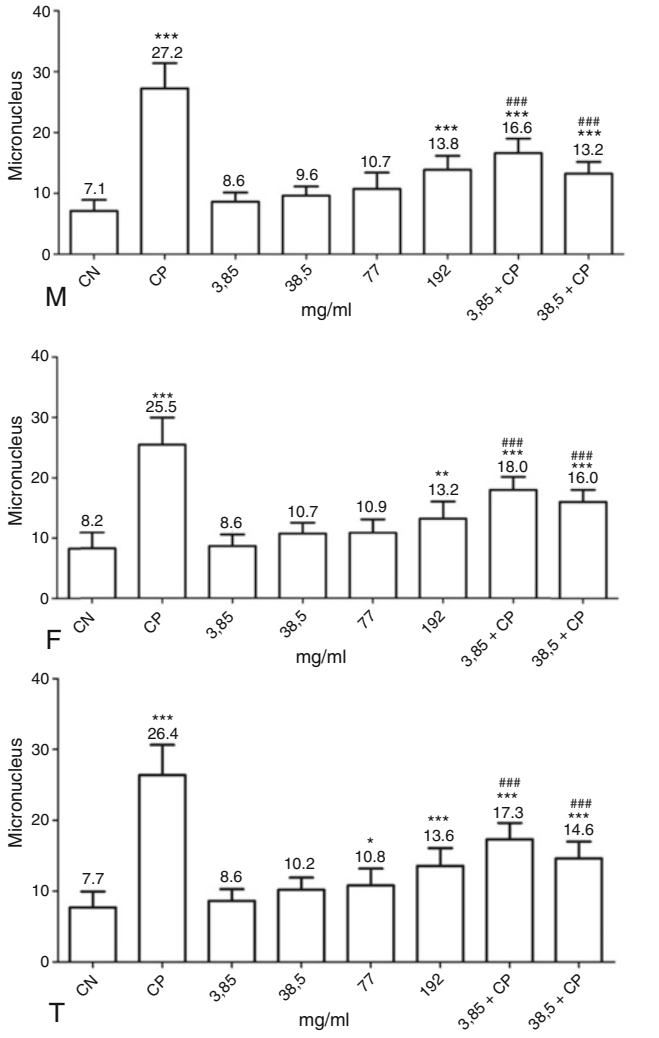

Fig. 2. Mutagenic effect evaluated by the mean of the number of micronuclei in mice bone marrow cells, subjected to treatment with aqueous extract of *M. guyanensis*. M: Males; F: Females; T: Total – Male and Female. Statistically significant for mutagenicity $^{*}(p<0.05)$, $^{**}(p<0.01)$ and $^{***}(p<0.001)$ when compared to CN. Statistically significant for antigenotoxicity $^{\#}(p<0.05)$, $^{\#\#}(p<0.01)$ and $^{\#\#\#}(p<0.001)$ when compared to CP.

Discussion

The genotoxic safety and antigenotoxic action of 3.85 mg/ml and 38.5 mg/ml concentrations of the aqueous extract of *M. guyanensis* was already seen in a previous study using *Allium cepa*, where these same concentrations demonstrated anticytotoxic and antimutagenic action relative to meristems germination, mitotic indexes and micronuclei formation (Meneguetti et al., 2014), being also in agreement with Hurtado (2013), in which fractions of *M. guyanensis* showed anti-proliferative activity within a wide period of time, not presenting clastogenic or aneugenic actions, where this latter was also observed in a study carried out by Mendes et al. (2012), demonstrating that the ethanolic extract of *M. rigida* does not induce chromosomal abnormalities.

The antimutagenic action was also observed by *Salmonela/Microsome* test (Ames Test) in which the hydroalcoholic extract of the bark of *M. krukovii* presented exhibits inhibitory activity against the T98 and T100 lines (Bruni et al., 2006).

Previous studies have demonstrated that the quinone-methide triterpenes are one of the main causes of the antigenotoxic action found in some species of the genus *Maytenus*, acting *in vitro* against tumor cells and against experimental tumors, in which the species *Maytenus ilicifolia* presented inhibitory activities against different sarcomas and neoplastic cells (Santana et al., 1971; Santos-Oliveira et al., 2009), strengthening the results found in the 3.85 mg/ml and 38.5 mg/ml concentrations of the aqueous extract of *M. guyanensis*, that probably occurred due to the presence of quinone-methide triterpenes identified previously in this species (Facundo et al., 2015). Similar results were observed with the tingenone-quinone methide (maitenine) *in vitro* tests that demonstrated inhibition of Leuk-P 138, CA 9KB and V79 neoplastic cell lines (Kupchan and Karim, 1976).

Studies performed in humans with other triterpenes isolated from *Maytenus* ssp. demonstrated the decrease of wounds on the base of the tongue and pharynx by approximately 50%, caused by epidermoid carcinomas and 40% of the lymphoepithelioma with invasion to the orbit, besides positive results in 2 of the 7 patients with uterine epidermoid carcinoma (Santana et al., 1971).

In another clinical series, the triterpene maitenine "tingenone analog" (Morita et al., 2008; Gullo et al., 2012; Facundo et al., 2015), also isolated from the genus *Maytenus*, potentiated other anti-cancerogenic substances of natural sources in eleven patients with advanced basal-cell carcinoma, in which all of the patients presented clinical recovery, with reduction of wound size by more than 50% (Melo et al., 1974; Corsino et al., 2000).

The anti-ulcerogenic effects occurred mainly due to the antioxidant activities of catequine derivatives found in some species of the genus *Maytenus*, and these derivatives act in a more effective

Table 1
Mean ± standard deviation of the number of Polychromatic Erythrocytes (PCE), Normochromatic Erythrocytes (NCE) and of the PCE/NCE ratio in mice bone marrow cells, which underwent the treatment with aqueous extract of *Maytenus guyanensis*.

Treatment (mg/ml)	Gender	*N*	(PCE)	(NCE)	Correlation (PCE/NCE)
CN	Male	4	2571 ± 8.2	2429 ± 8.2	1.06 ± 0.07
	Female	4	2616 ± 5.8	2384 ± 5.8	1.10 ± 0.05
	Total	8	2594 ± 7.5	2406 ± 7.5	1.08 ± 0.06
CP	Male	4	1835 ± 13.6	3165 ± 13.6	0.58 ± 0.07^{***M}
	Female	4	1737 ± 4.7	3262 ± 4.7	0.53 ± 0.02^{***F}
	Total	8	1786 ± 11.3	3214 ± 11.3	0.56 ± 0.06^{***T}
3.85	Male	4	2622 ± 8.1	2377 ± 8.1	1.10 ± 0.07
	Female	4	2581 ± 6.7	2419 ± 6.7	1.07 ± 0.06
	Total	8	2602 ± 7.8	2398 ± 7.8	1.08 ± 0.07
38.5	Male	4	2526 ± 5.8	2474 ± 5.8	1.02 ± 0.05
	Female	4	2541 ± 7.3	2459 ± 7.3	1.03 ± 0.06
	Total	8	2534 ± 6.6	2466 ± 6.6	1.03 ± 0.05
77	Male	4	2362 ± 13.2	2637 ± 13.2	0.90 ± 0.09^{**M}
	Female	4	2381 ± 11.3	2619 ± 11.3	0.91 ± 0.08^{***F}
	Total	8	2372 ± 12.3	2628 ± 12.3	0.90 ± 0.09^{***T}
192	Male	4	2161 ± 11.2	2839 ± 11.2	0.76 ± 0.07^{***M}
	Female	4	2165 ± 9.4	2835 ± 9.4	0.77 ± 0.06^{***F}
	Total	8	2163 ± 10.4	2837 ± 10.4	0.76 ± 0.07^{***T}
3.85 + CP	Male	4	2155 ± 6.6	2845 ± 6.6	0.76 ± 0.04$^{***M\#\#\#M}$
	Female	4	2194 ± 7.4	2806 ± 7.4	0.78 ± 0.05$^{***F\#\#\#F}$
	Total	8	2174 ± 7.3	2826 ± 7.3	0.77 ± 0.04$^{***T\#\#\#T}$
38.5 + CP	Male	4	2212 ± 8.6	2787 ± 8.6	0.79 ± 0.05$^{***M\#\#\#M}$
	Female	4	2221 ± 7.9	2779 ± 7.9	0.80 ± 0.05$^{***F\#\#\#F}$
	Total	8	2217 ± 8.2	2783 ± 8.2	0.80 ± 0.05$^{***T\#\#\#T}$

Statistically significant for genotoxicity: $^{*}M(p<0.05)$, $^{**}M(p<0.01)$ and $^{***}M(p<0.001)$ when compared to male CN. $^{*}F(p<0.05)$, $^{**}F(p<0.01)$ and $^{***}F(p<0.001)$ when compared to female CN. $^{*}T(p<0.05)$, $^{**}T(p<0.01)$ and $^{***}T(p<0.001)$ when compared to total CN. Statistically significant for antigenotoxicity: $^{\#}M(p<0.05)$, $^{\#\#}M(p<0.01)$ and $^{\#\#\#}M(p<0.001)$ when compared to male CP. $^{\#}F(p<0.05)$, $^{\#\#}F(p<0.01)$ and $^{\#\#\#}F(p<0.001)$ when compared to female CP. $^{\#}T(p<0.05)$, $^{\#\#}T(p<0.01)$ and $^{\#\#\#}T(p<0.001)$ when compared to total CP.

way on the wound of the digestive tract, inhibiting the damage of mucosa cells by free radicals generated by the very digestive process. This effect is also related to an anti-mutagenic action, protecting against genotoxic agents that may induce the malignant transformation of the intestinal mucosa cells (Krul et al., 2001).

The antioxidant action was already reported for the species *M. guyanensis* (Macari et al., 2006), for *M. procumbens* (Momtaz et al., 2013) and for *ilicifolia* (Macari et al., 2006; Negri et al., 2009) in which the lipid peroxidation is inhibited (Santos-Oliveira et al., 2009) and shows chelant activity of heavy metals, besides acting on different free radicals (Ho et al., 1992; Melo et al., 2001).

In this study, the concentration of 77 mg/ml of the aqueous extract of *M. guyanensis* did not present genotoxic action for the index and frequency of damage evaluated by the comet assay, or for the formation of micronuclei on the bone marrow of male and female mice. On the other hand, it presented genotoxic effects in the total mean of the micronuclei test and also in the ratio PCE/NCE. The negative results for the concentration of 77 mg/ml in the comet assay might have occurred due to analyses of only the acute effects, in which further studies are suggested for a better comprehension of the chronic effects caused by the ingestion of infusions of aqueous extracts of *M. guyanensis*; according to Bode and Dong (2014) several natural compounds found in various plants consumed by traditional population are potential carcinogens or tumor promoters and its use for long periods of time should be avoided.

In the 192 mg/ml concentration the data demonstrated genotoxic effects in all of the tests performed, being in agreement with a study done by Meneguetti et al. (2014), in which the 77 mg/ml and 192 mg/ml concentrations presented cytotoxic and mutagenic actions.

It is important to highlight that 77 mg/ml and 192 mg/ml concentrations are, respectively, twenty- and fifty-fold more concentrated than the concentration normally used by the population: 3.85 mg/ml (Camparoto et al., 2002), bringing a certain tranquility relative to the use of this species in the ethnopharmacology.

This genotoxicity found in high concentrations was not evidenced in other studies using plants of the same genus, in which the chronic toxicology of the infusion of *M. ilicifolia* was tested in rats and mice with doses ranging from 20 to 40 fold higher than that commonly used by humans, not causing changes in weight, behavior, temperature and in the biochemical parameters of the serum and hematological parameters (Carlini and Frochtengarten, 1988).

In other toxicologic studies, this time acute, performed in mice and rats by infusions and lyophilized extract of *M. ilicifolia*, and toxic effects were not evidenced in doses until 1600-fold higher than the doses used by humans (Santos-Oliveira et al., 2009).

In human beings, a study of clinical toxicology (Phase I trial), administered during 14 days, the double of the dosage of *M. ilicifolia* used in ethnopharmacology, did not observe abnormal results that could be attributed to the plant use. Only symptoms like dry mouth, nausea, and gastralgia in a few volunteers were recorded, with recovery during the study, demonstrating thus that *M. ilicifolia* is not toxic for humans when used in the same way as popular medicine (Carlini and Frochtengarten, 1988).

It was found that aqueous extract of *M. guyanensis* in concentrations up to tenfold higher than the concentration used in ethnopharmacology does not present genotoxic effects and, moreover, it has antigenotoxic actions in mice treated acutely. Further studies of bioaccumulation and chronic effects of this species are suggested in order to improve the understanding of its action mechanisms, ensuring the efficacy and safety of its utilization and development of phytotherapics and drugs.

Authors' contribution

DUOM (doctoral student) participated in all stages of the study, including statistical analysis and writing of the article. RAL (doctoral student), GMP and JBF (graduate student), participated in the preparation of the aqueous extract and article writing. FCS (doctoral student) and RCP contributed in the genotoxic analysis (treatment of animals, comet assay, correlation pce/nce and micronucleus analysis); JSLTM and VAF contributed to supervision and writing the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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