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Protective effect and induction of DNA repair by *Myrciaria cauliflora* seed extract and pedunculagin on cyclophosphamide-induced genotoxicity

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ABSTRACT

Ellagitannins are well-known antioxidants in medicinal plants, foods, and edible fruits, particularly in *Myrciaria cauliflora* (jabuticaba). Thus, this study aimed to evaluate the protective effects of jabuticaba seed extract (JSE) and pedunculagin using in vivo micronucleus test and comet assay in mouse bone marrow cells, in combination with cyclophosphamide (CP), a bioreductive alkylating agent. The ellagitannin composition of JSE was determined by HPLC/PDA, with castalagin, vescalagin, and pedunculagin as the main compounds (124.4, 45.5, and 15.6 mg/g dw, respectively). Results from pre- and co- treatments with JSE or pedunculagin clearly showed their protective action against CP-induced micronuclei and DNA damage. The effects of both tannins in post-treatments with CP suggested they influence DNA repair systems. These findings indicate that JSE and pedunculagin possess chemopreventive as well as DNA repair-inducing properties.

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1. Introduction

Vegetable tannins are plant-derived natural products that may occur in almost any part of a plant – seeds, leaves, fruits, root, bark, and wood [1]. It is believed that the regular consumption of fruits and vegetables with high levels of polyphenols is beneficial to human health because of their strong antioxidant and anti-inflammatory activities, which reduce the risk of age-related degenerations and diseases [2].

Tannins are oligomeric and polymeric polyphenols with a wide range of chemical structures, generally classified into two major groups depending on the structure of the monomer: (1) proanthocyanidins (condensed tannins) formed through the condensation of flavan-3-ols (catechins), (2) gallo- and ellagitannins (hydrolysable tannins), which are polyesters with a sugar moiety (mainly D-glucose) and organic acids, gallic acid, and hexahydroxydiphenic acid [2].

Ellagitannins possess many biological properties, including antioxidant, anticancer and anti-inflammatory activities [3–6]. Several species belonging to the Myrtaceae family are a rich source of ellagitannins [7], among them *Myrciaria cauliflora* (Mart.) O. Berg., a Brazilian tree that bears an edible, purplish-black sweet fruit known as jabuticaba [8]. It has a slightly acid taste and is consumed *in natura* or processed as jam, juice, liqueur, and wine [9]. The sun-dried jabuticaba peel is used in popular medicine as a treatment for hemoptysis, cough, bronchitis, asthma, diarrhea, and dysentery, as well as a rinse for chronic throat inflammation [10]. Several phenolic compounds, such as flavonoids, anthocyanins, depsides, and phenolic acids have been previously isolated from the jabuticaba fruit [9]. Seven ellagitannins were detected and tentatively identified by LC-TOF-MS, and only two of them, isooenothein C and oenothein C, were isolated from the fruits [11,12].

Since there is an increasing interest in ellagitannins' beneficial effects on human health and on preventing age-related diseases, the investigation of their antigenotoxic potential may provide information to subsidize the development of new chemopreventive compounds [1,2].

Many tests assess the genotoxicity and antigenotoxicity of compounds, among them the micronucleus (MN) test and the comet

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assay in mouse bone marrow cells, both widely used and accepted by international regulatory agencies to evaluate new chemicals and pharmaceuticals [13]. The MN test assesses compounds' clastogenic and aneugenic actions, as well as their cytotoxic potential [14]. The comet assay is a suitable method for detecting a wide range of DNA breaks in eukaryotic cells, for it measures single- and double-strand breaks, incomplete excision repair sites, and alkali-labile sites [15,16].

Thus, the aim of this study was to evaluate the genotoxic and cytotoxic activities of the jabuticaba seed extract (JSE) via the MN test and the comet assay in mouse bone marrow cells. In addition, both tests assessed the chemopreventive effects of JSE and pedunculagin isolated from the jabuticaba seed against cyclophosphamide-induced micronuclei and DNA damage, using pre-, co-, and post-treatments.

2. Materials and methods

2.1. General experimental information

Column chromatography was run using Diaion HP-20 (Supelco) or Sephadex LH-20 (Sigma-Aldrich). Analytical TLC was carried out with Silica gel 60 F_{254} (Merck) plates, using formic acid-ethyl formiate-toluene (1:7:1) as the mobile phase. TLC spots were visualized by spraying plates with a 1% ethanolic solution of ferric chloride in HCl (0.1%) and UV light. All NMR experiments were recorded on a Bruker Avance III 500 spectrometer operating at 500.13 MHz for ^{1}H and 125 MHz for ^{13}C, using TMS as internal reference. ESI-TOF MS spectra were recorded on a Bruker microTOF instrument.

2.2. Chemicals

Cyclophosphamide (CP) was purchased from Hera Medicamentos (Belo Horizonte, Brazil). Fetal calf serum was obtained from Laborclin (Campinas, Brazil), and dibasic sodium phosphate, monobasic sodium phosphate and Giemsa were purchased from Doles (Goiânia, Brazil). Agarose normal melting, agarose low melting, phosphate buffered saline (PBS), Triton X-100, dimethyl sulfoxide (DMSO), Stock Lysis solution, Tris-HCl buffer and ethidium bromide, reagents used in the comet assay, were purchased from Genética Brasil (Brasília, Brazil) and Life Technologies (São Paulo, Brazil).

2.3. Plant material and preparation of JSE

M. cauliflora ripe fruits were collected in Jabuticabal Farm (S 16°49′53″, W 49°14′45″), Goiás State, Brazil, in October 2011. Fresh seeds (1.8 kg) were homogenized in a blender with water, then were exhaustively extracted with 50% acetone, using an overhead stirrer apparatus at room temperature. The acetone was removed under reduced pressure and the suspended aqueous extract was filtered. Following, a liquid–liquid extraction with ethyl acetate (14 × 50 mL) was carried out. The combined organic phase was evaporated to yield an ethyl acetate extract (0.63 g). The aqueous layer was freeze-dried to yield a 57.1 g extract (JSE).

2.4. Isolation and identification of pedunculagin

Part of JSE (52.1 g) was dissolved in methanol (2.0 L) to separate soluble (21.5 g) and insoluble (7.1 g) methanolic extracts. The soluble methanolic extract was subjected to Diaion HP-20 column chromatography (200 g) and eluted with a decreasing polarity gradient of H_2O/MeOH. Seven main fractions were combined, and fraction JSM 4 (1.1 g) was subjected to Sephadex LH-20 CC (eluting with an increasing polarity gradient of $CHCl_3$/EtOH followed by EtOH/MeOH) to give pedunculagin as pure compound (321 mg).

2.5. Quantification of ellagitannins in JSE and isolation of castalagin/vescalagin (HPLC/PDA)

The chromatographic system used was a Shimadzu LC-10AVP with two LC-10ADvp solvent delivery units (Shimadzu Corp., Japan) connected to an SPD-10AVvp ultraviolet photodiode array detector. Chromatographic separations were performed using a LiChrospher 100 RP-18 (5 μm), 25 cm × 0.4 cm i.d. (Merck Millipore, Billerica, MA, USA). The mobile phase consisted of acetonitrile (solvent A) and 0.01 M H_3PO_4: 0.01 M KH_2PO_4 (solvent B) with the following gradient profile: 0–15 min, 7–10% A in B; 15–40 min, 10–50% A in B, then followed by a 5 min re-equilibration. Analyses were conducted using a 1.0 mL/min flow rate, detector wavelength at 216 nm, and sample injection volume of 20 μL.

Identification of compounds was made by comparing their RT values and UV spectra against those of standards (pedunculagin, vescalagin and castalagin). Vescalagin and castalagin were purified from JSE by semi-preparative HPLC (Shim-pack PREP-ODS(H) column, 25 × 2 cm, 5 μm, Shimadzu Corp., Japan, and injection volume of 2 mL) using the same eluent gradients as the analytical program. Quantification was based on the measured integration area applying the calibration equation of the corresponding standard. The concentrations used for the calibration were 2.5–100, 1–264 and 4–936 μg/mL for pedunculagin, vescalagin and castalagin, respectively.

2.6. Spectral data of the isolated compounds

Pedunculagin (**1**) is a mixture of α-anomer and β-anomer. Light brown amorphous powder, ESI-TOF MS: *m/z* 783.0671 [M-H]$^-$ (calc. for $C_{34}H_{23}O_{22}$, 783.0686). ^{1}H NMR (acetone-d_6, 500 MHz), δ: 3.79 (1H, dd, *J* = 13, 2 Hz, H-6α), 3.85 (1H, dd, *J* = 13, 1 Hz, H-6β), 4.22 (1H, dd, *J* = 10, 6 Hz, H-5β), 4.61 (1H, dd, *J* = 10, 7 Hz, H-5α), 4.86 (1H, dd, *J* = 9, 8 Hz, H-2β), 5.06 (1H, d, *J* = 8 Hz, H-1β), 5.07 (1H, dd, *J* = 10, 4 Hz, H-2α), 5.08 (1H, t, *J* = 10 Hz, H-4α), 5.08 (1H, t, *J* = 10 Hz, H-4β), 5.24 (1H, dd, *J* = 10, 9 Hz, H-3β), 5.26 (1H, dd, *J* = 13, 7 Hz, H-6α), 5.30 (1H, dd, *J* = 13, 6 Hz, H-6β), 5.46 (1H, d, *J* = 4, H-1α), 5.47 (1H, t, *J* = 10 Hz, H-3α), 6.33 and 6.52 (2H, s, HHDP-6″/6″β), 6.34 and 6.57 (2H, s, HHDP-6″/6″α), 6.60 and 6.67 (2H, s, HHDP-6″/6″β), 6.61 and 6.66 (2H, s, HHDP-6″/6″α). ^{13}C NMR (acetone-d_6, 125 MHz), δ: 63.6 (2C, C-6α/β), 67.5 (C-5α), 69.7 (C-4β), 69.9 (C-4α), 72.6 (C-5β), 75.6 (C-2α), 75.9 (C-3α), 77.7 (C-3β), 78.5 (C-2β), 91.8 (C-1α), 95.4 (C-1β), 107.3, 107.4, 107.6, 107.7, 107.8, 107.9, 108.4 and 108.5 (8C, HHDP-6″/6″α/β).

Castalagin (**2**) white amorphous powder, ESI-TOF MS: *m/z* 933.0631 [M-H]$^-$ (calc. for $C_{41}H_{25}O_{26}$, 933.0640). ^{1}H NMR (acetone-d_6, 500 MHz), δ: 4.01 (1H, d, *J* = 13 Hz, H-6), 5.03 (1H, dd, *J* = 7.0, 1.4 Hz, H-3), 5.04 (1H, dd, *J* = 4.7, 1.4 Hz, H-2), 5.10 (1H, dd, *J* = 13, 2.6 Hz, H-6), 5.24 (1H, dd, *J* = 7.4, 7.0 Hz, H-4), 5.62 (1H, ddd, 7.4, 2.6, 1.0 Hz, H-5), 5.74 (1H, d, *J* = 4.7 Hz, H-1), 6.64 (1H, s, H-6′, HHDP B), 6.79 and 6.91 (2H, s, H-6′ HHDP A and Flavogalloyl C). ^{13}C NMR (acetone-d_6, 125 MHz), δ: 64.5 (C-6), 65.6 (C-3), 66.7 (C-1), 68.6 (C-4), 70.4 (C-5), 73.3 (C-2).

Vescalagin (**3**) white amorphous powder, ESI-TOF MS: *m/z* 933.0562 [M-H]$^-$ (calc. for $C_{41}H_{25}O_{26}$, 933.0642). ^{1}H NMR (acetone-d_6, 500 MHz), δ: 4.01 (1H, d, *J* = 13 Hz, H-6), 4.58 (1H, dd, *J* = 7.0, 1.5 Hz, H-3), 4.92 (1H, d, *J* = 2.3 Hz, H-1), 5.10 (1H, dd, *J* = 13, 2.6 Hz, H-6), 5.22 (1H, dd, *J* = 7.4, 7.0 Hz, H-4), 5.24 (1H, dd, *J* = 2.3, 1.5 Hz, H-2), 5.65 (1H, ddd, 7.4, 2.6, 1.0 Hz, H-5), 6.63 (1H, s, H-6′, HHDP B), 6.79 and 6.79 (2H, s, H-6′ HHDP A and Flavogalloyl C). ^{13}C NMR (acetone-d_6, 125 MHz), δ: 64.5 (C-6), 64.6 (C-1), 67.7 (C-3), 68.6 (C-4), 70.4 (C-5), 77.0 (C-2).

2.7. Animal testing

2.7.1. General

This study was approved by the Ethics Committee on Animal Use of the Federal University of the Goiás, Goiânia (protocol no. 115/14). The experiments followed national and international standards of management and experimentation with animals [17,18]. Healthy, young, male adult outbred mice (*Mus musculus*, Swiss Webster), between 7 and 12 weeks old, weighing 30–40 g, obtained from the animal facilities of the same university, were randomly allocated to treatment groups. All animals were brought to the laboratory five days before the experiments and housed in polyethylene cages (40 cm × 30 cm × 16 cm), lined with wood shavings, in groups of five animals at 25 ± 2 °C and 50 ± 10% relative humidity, with a 12-h light/dark natural cycle. Standard food pellets and water were provided *ad libitum*.

2.7.2. Protocol in vivo

The mice were randomized into control and experimental groups, divided in thirteen groups of five animals each and weighed before chemical administration. Diluted JSE and pedunculagin, and the negative control (water) were given by gavage, whereas CP was administrated intraperitoneally (i.p.). Doses of JSE (30 and 300 mg/kg) were based on a previous micronucleus test performed with jabuticaba peel extract [31]. The lowest dose was selected for pedunculagin (30 mg/kg) to avoid acute toxicity in mice during pre-treatment. Animals in group 1 received 0.15 mL of water that was used as negative control, and animals in group 2 received 50 mg/kg of CP according to body weight (bw) by single administration and served as positive control.

2.7.2.1. Treatment and co-treatment. Aanimals in group 3 were treated with 300 mg/kg of JSE in order to evaluate the genotoxicity. Animals in groups 4 and 5 were respectively treated with JSE at doses 30 and 300 mg/kg concomitant with CP. Animals in group 11 were treated with 30 mg/kg of pedunculagin concomitant with CP.

2.7.2.2. Treatment and pre-treatment. Animals in group 6 received only JSE at 300 mg/kg for five days to evaluate the genotoxicity. Animals in groups 7, 8 and 12 were treated for five days with 30 and 300 mg/kg JSE and 30 mg/kg of pedunculagin, respectively, followed by CP 2 h after the final feeding.

2.7.2.3. Post-treatment. Animals in group 9, 10 and 13 were firstly treated with CP, then after 2 h they received doses of JSE at 30 and 300 mg/kg, and pedunculagin at 30 mg/kg

All groups of animals treated with CP were euthanized by cervical dislocation 24 h after administration of CP while the animals that received JSE alone were euthanized 24 h after the its last administration. Both femurs were immediately removed and processed for the micronucleus test and comet assay. Bone marrow cells from both femurs were flushed using fetal calf serum, centrifuged at 300 x *g* for 5 min, and the supernatant was discarded. The pellet was resuspended in a drop of calf serum and used to prepare the slides. The slides were air-dried and fixed with absolute methanol for 5 min at room temperature. The slides were stained with Giemsa, dibasic sodium phosphate, and monobasic sodium phosphate.

2.7.3. Micronucleus test

The micronucleus test was carried out according to MacGregor et al. [19]. Slides were analyzed through optical microscope (Olympus BH-2 10 × 100, Tokyo, Japan), and 2000 polychromatic erythrocytes (PCE) were counted per animal to determine the frequency of micronucleated polychromatic erythrocytes (MNPCEs). Genotoxicity and antigenotoxicity were assessed by the frequency of MNPCEs, whereas cytotoxicity and anticytotoxicity were evaluated by the ratio between PCE and normochromatic erythrocytes (NCE), determined from a total of 2000 counted erythrocytes (immature and mature).

2.7.4. Comet assay

The alkaline single cell gel electrophoresis (SCGE) assay, also known as comet assay was carried out under alkaline conditions using the method described by Attia et al. [20] and Singh et al. [21]. Fifteen μl of bone marrow cells were suspended in 120 μL of 0.5% low melting point agarose, then layered on slides previously coated with 1.5% normal melting point agarose. Slides were then kept in cuvettes (protected from light) containing cold lysis solution (Triton X-100, DMSO and Stock Lysis Solution) for 4 h. Electrophoresis was carried out for 30 min (25 V, 300 mA). Following electrophoresis, the slides were placed in a staining tray, covered with a neutralizing buffer (0.4 M, Tris-HCl, pH 7.5), and kept in the dark for 5 min. For analysis, slides were stained with 20 μL of ethidium bromide solution (0.02 mg/mL) and covered with a cover slip. One hundred nucleoids were analyzed per sample by a fluorescence microscopy system Axioplan-Imaging® using the Isis software, with a 510–560 nm excitation filter and a 590 nm barrier filter, at an increase of 200 X. The TriTek CometScore™ software (version 1.3) was used to assess genomic damage. In this software, pixel intensity provides values corresponding to genomic damage estimates, which are given as arbitrary units. Nucleoids with completely fragmented heads were not taken into account. Among seventeen parameters provided by the software, the percentage of DNA in the tail was selected.

2.8. Statistical analysis

Initially, tests of normality and homoscedasticity were applied to data. In the MN test, genotoxic and antigenotoxic activities were assessed by one-way ANOVA followed by Tukey's post-hoc test, and cytotoxicity and anticytotoxicity were analyzed by $\chi 2$ test, using the SigmaStat 3.2 software.

The percentage reduction of MNPCEs induced by CP was calculated according to Alves et al. [3]:

$$\text{Reduction}\,(\%) = \left[\frac{A-B}{A-C}\right] \text{x}100$$

– A: corresponds to the MNPCE mean observed in the treatment with CP (positive control);– B: corresponds to the MNPCE mean observed in the treatments (JSE/pedunculagin + CP);– C: corresponds to the MNPCE mean in the negative control.

Statistical tests were used to compare the treatments and control groups and results were considered statistically significant if p was < 0.05.

3. Results

3.1. Chemical characterization of JSE

The methanol fraction obtained from JSE was submitted to column chromatography over Diaion HP 20 and Sephadex LH-20 to yield pedunculagin (**1**). Castalagin (**2**) and vescalagin (**3**) were isolated from JSE by semi-preparative HPLC. Structural elucidation of pure compounds was determined by spectroscopic methods (ESI-TOF MS, 1D, and 2D NMR) and by comparison with data from the literature [22–24].

The HPLC chromatographic profile of JSE revealed the presence of two major compounds in the extract: castalagin and vescalagin. Quantification was based on standard curves obtained from the HPLC analysis, and results are presented in Table 1 as mg of

Table 1
Quantitative analysis of JSE by HPLC.

Compound	Retention time (min)	PDA – λ max (nm)	Concentration (mg/g JSE dw)	Concentration (mg/100 g fruit fw)
Vescalagin	7.0	200, 221	45.50	28.7
Pedunculagin	8.2 and 13.7	196, 219	15.60	9.8
Castalagin	10.2	200, 221	124.40	78.4

compound/g of JSE dry weight (dw) and as mg of compound/100 g of whole fruit fresh weight (fw).

3.2. *Mouse bone marrow MN test*

Results for the frequencies of MNPCE, PCE/NCE, and% MNPCE reduction for all treatments with JSE are shown in Table 2. In the genotoxicity analysis, the treatment with JSE 300 mg/kg alone had no effect on MNPCEs compared to the negative control ($p < 0.05$); the same finding was observed for the five-day treatment with JSE alone. Both treatments failed to show genotoxic action of JSE on mouse bone marrow cells. As regards cytotoxicity, only JSE's five-day treatment and pre-treatment significantly reduced the PCEs/NCEs ratio ($p < 0.05$), hence demonstrating a cytotoxic effect.

In the antigenotoxicity evaluation, pre-, co-, and post-treatments of JSE doses of 30 and 300 mg/kg with CP showed significant decreases ($p < 0.001$) in MNPCE frequency compared to the positive control. These treatments presented MNPCE reductions equal to or higher than 86% in all groups treated with CP (groups 4, 5, 7, 8, 9, and 10). In addition, anticytotoxic action was also observed in all three treatments, as PCE/NCE ratios were significantly different from the positive control ($p < 0.001$).

Results of the antigenotoxic and anticytotoxic effects of pedunculagin are shown in Table 3. Pre-, co-, and post-treatments with pedunculagin and CP exhibited a significant reduction in MNPCE frequency compared to the positive control ($p<0.001$). This compound induced MNPCE reductions of 99%, 94%, and 102% in the co-, pre-, and post-treatments, respectively. In co- and pre-treatments, pedunculagin was able to protect mouse bone marrow cells from the CP-induced cytotoxic effect. In contrast, during post-treatment the PCE/NCE ratio was significantly reduced ($p<0.001$), demonstrating a synergistic cytotoxic effect of pedunculagin with CP.

3.3. *Comet assay*

Results of the comet assay, which was applied to verify the levels of primary DNA damage in mouse bone marrow cells treated with JSE, with or without CP, are shown in Figs. 1 and 2. The JSE dose of 300 mg/kg alone did not present any significant difference (p □ 0.05) compared to the negative control. On the other hand, pre-treatment for five days with JSE alone significantly decreased the extent of DNA damage verified by the comet assay (DNA in tail) compared to the respective negative control ($p < 0.001$).

In the antigenotoxic evaluation, JSE and pedunculagin were able to significantly reduce CP-induced DNA damage in all treatments tested by the comet assay — co-, pre-, and post-treatments — in comparison with animals treated with CP alone ($p < 0.05$) (Figs. 2 and 3).

4. Discussion

Exotic tropical fruits have received considerable attention from researchers in the last few decades because of their potential use in preventing degenerative diseases. They have in common high polyphenol contents, viewed as having the capacity to scavenge oxidatively-generated free radicals. Jabuticaba, for instance, a member of the Myrtaceae family, is an edible fruit with several medicinal applications and high polyphenol content, including phenolic acids, flavonoids, anthocyanins, depsides, galloyl esters, and ellagitannins [8,9,11]. Most studies on jabuticaba have been conducted on the whole fruit or on peels and have focused mainly on flavonoid and anthocyanin compounds [9]. Seeds and peels are co-products generated during the jabuticaba's juice and wine processing and are discarded, even though they contain most of the fruit's bioactive compounds [25]. There are clear differences in the chemical composition of the fruit's seeds and peels, as has been demonstrated by previous reports in which seeds displayed the highest concentrations of ellagitannins and proanthocyanidins and the greatest antioxidant capacity, whereas skin exhibited the highest amounts of anthocyanins and quercetin derivatives [26].

In this study, the ellagitannins isolated from JSE are composed mainly of castalagin and vescalagin, *C*-glycosidic ellagitannins with an open-chain glucose core; and, in lower amounts, of pedunculagin, a simple ellagitannin with $^4C_1$-glucopyranose core and two hexahydroxydiphenoyl (HHDP) units (Table 1, Fig. 1). Both types of ellagitannins possess the capacity to complex with metal ions, proteins/enzymes, and basic compounds, in addition to their antioxidant and antitumor activities [1,2].

In the MN test and the comet assay, JSE alone in both co- and pre-treatments did not induce an increase in the MNPCE frequency or in DNA in the tail (Table 2), showing no genotoxic effects. Similarly, previous studies have reported the absence of genotoxic action in several hydrolysable tannins and their hydrolyzed products e.g. ellagic acid, gallic acid, and propyl gallate using in vitro Ames test and in vivo MN test [27,28].

Mild cytotoxic effects of JSE were observed on animals treated for five days; it is noteworthy that the same treatment resulted in a lower percentage of DNA in the tail than the negative control (Fig. 2). Ellagitannins present in JSE have already shown selective cytotoxicity against tumor cell lines [29,30], and oenothein B, a dimeric ellagitannin, also significantly decreased the PCEs/NCEs ratio by oral and i.p. routes in the MN test on mouse bone marrow cells [27].

Contrary to our results, Leite-Legatti et al. [31] previously reported no cytotoxic effects on mouse bone marrow cells for the 15-day treatment with jabuticaba peel extract at 300 mg/kg bw. Differently from the seeds, jabuticaba peel revealed the presence of two anthocyanins as major compounds: delphinidin 3-glucoside and cyanidin 3-glucoside [31].

Given that the ageing of the world population has led to an increase in age-related degenerations and diseases such as cancer, much effort has been made to develop new antitumor drugs, and the identification of dietary constituents with chemopreventive properties becomes an alternative to preventing these diseases. Antigenotoxic and antimutagenic compounds can be used as cancer chemopreventive agents against clastogens. The potential protective agent may act through different mechanisms, e.g. chemical or enzymatic inactivation of mutagens, inhibition of metabolic activation of promutagens, scavenging of free radicals produced by mutagens, modification of repair system activity, direct interaction with clastogen, and thus prevention of its interaction with DNA [32]. Several ellagitannins and their hydrolyzed product, ellagic acid, exhibit antimutagenic activity on Trp-P-1, Trp-P-2 (mutagens in burned meat) and MNNG (N-methyl-N0-nitro-N-nitrosoguanidine), as well as against direct-acting mutagens 3-hydroxyamino-1-methyl-5H-

Table 2
Mean values of MNPCE frequency, PCE/NCE ratio and reduction rate with two doses of the JSE in mice bone marrow cells for genotoxic, antigenotoxic, cytotoxic and anticytotoxic evaluation.

Group	Treatments (mg/kg bw)	MNPCE/2000 PCE (Mean ± SD)	MNPCE Reduction (%)	PCE/NCE (Mean ± SD)
1	Negative Control[1]	4.2 ± 0.45	–	1.03 ± 0.03
2	Positive Control[2]	27.2 ± 1.3[a]	–	0.71 ± 0.02[a]
	Treatment and co-treatment			
3	JSE 300	4.8 ± 0.83	–	1.16 ± 0.04
4	JSE 30 + CP	4.8 ± 0.83[b]	97%	0.91 ± 0.32[b]
5	JSE 300 + CP	6.0 ± 1.41[b]	92%	0.91 ± 0.27[b]
	Treatment and pre-treatment			
6	JSE 300	5.4 ± 1.14	–	0.80 ± 0.21[a]
7	JSE 30 + CP	6.0 ± 0.70[b]	92%	1.01 ± 0.11[b]
8	JSE 300 + CP	7.4 ± 1.14[b]	86%	1.07 ± 0.10[b]
	Post-treatment			
9	JSE 30 + CP	4.4 ± 0.89[b]	99%	0.90 ± 0.13[b]
10	JSE 300 + CP	5.0 ± 1.00[b]	97%	0.83 ± 0.15[b]

CP: cyclophosphamide. All values are means ± standard deviation (SD) from five mice. Group 1 – distilled water 0.1 mL/10 g body weight (bw) by gavage (negative control), Group 2 – 50 mg/kg CP bw (positive control). Group 3 – 300 mg/kg JSE, Groups 4 and 5 – 30 and 300 mg/kg JSE, respectively, concomitant with CP. Group 6 – JSE at 300 mg/kg during five days, Groups 7 and 8 – JSE at 30 and 300 mg/kg, respectively, for five days, followed by CP, 2 h after the final feeding. Groups 9 and 10 – CP, and after 2 h – JSE at 30 and 300 mg/kg, respectively. Groups 3 and 6 were compared with negative control; Groups 4, 5, 7, 8, 9, and 10 were compared with positive control. A total of 2000 PCEs were scored per animal for the MN test. JSE and CP were diluted in saline solution and administrated by gavage and intraperitoneally (i.p.), respectively. ANOVA, Tukey's test and qui-square.

[a] Significant difference from the negative control (water) ($p < 0.05$).
[b] Significant difference from the positive control (CP) ($p < 0.001$).

Table 3
Mean values of MNPCE frequency, PCE/NCE ratio and reduction rate with one dose of the pedunculagin (Ped) in mice bone marrow cells for antigenotoxic and anticytotoxic evaluation.

Group	Treatments (mg/kg bw)	MNPCE/2000 PCE (Mean ± SD)	MNPCE Reduction (%)	PCE/NCE (Mean ± SD)
1	Negative Control[1]	4.2 ± 0.45	–	1.03 ± 0.03
2	Positive Control[2]	27.2 ± 1.30[a]	–	0.71 ± 0.02[a]
	Co-treatment			
11	Ped 30 + CP	4.4 ± 0.54[b]	99%	0.91 ± 0.34[b]
	Pre-treatment			
12	Ped 30 + CP	5.6 ± 0.84[b]	94%	0.90 ± 0.30[b]
	Post-treatment			
13	Ped 30 + CP	3.8 ± 0.83[b]	102%	0.59 ± 0.26[b]

CP: cyclophosphamide. All values are means ± standard deviation (SD) from five mice. Group 1 – distilled water 0.1 mL/10 g body weight (bw) by gavage (negative control), Group 2 – 50 mg/kg bw of CP (positive control). Group 11 – 30 mg/kg pedunculagin (Ped) concomitant with CP. Group 12 – Ped at 30 mg/kg, for five days, followed by CP, 2 h after the final feeding. Group 13 – CP, and after 2 h – Ped at 30 mg/kg. Groups 11, 12 and 13 were compared with positive control. A total of 2000 PCEs were scored per animal for the MN test. Ped and CP were diluted in saline solution and administrated by gavage and i.p., respectively. ANOVA, Tukey's test and qui-square.

[a] Significant difference from the negative control (water) ($p < 0.05$).
[b] Significant difference from the positive control (CP) ($p < 0.001$).

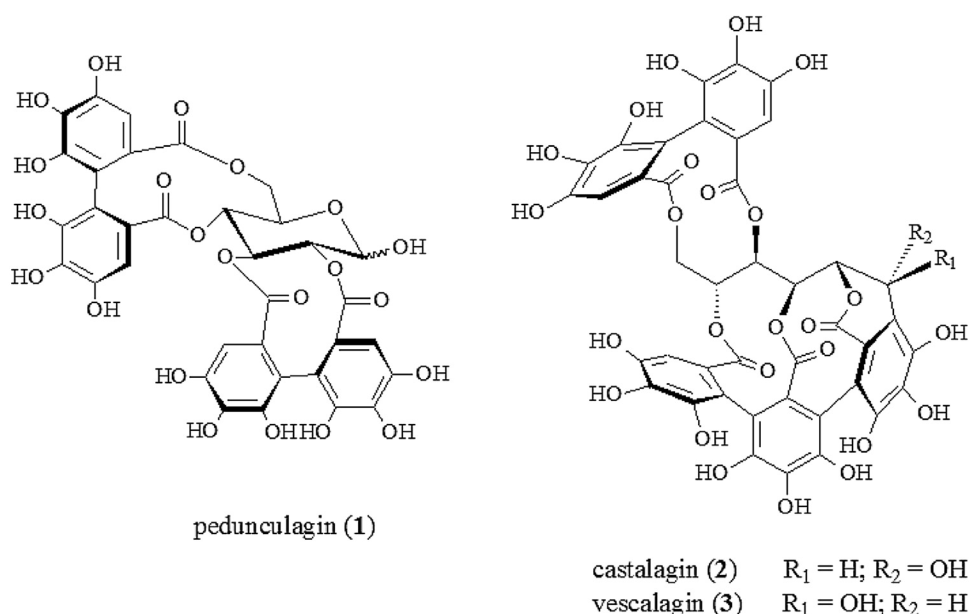

Fig. 1. Chemical structures of ellagitannins isolated from jabuticaba seed extract (JSE).

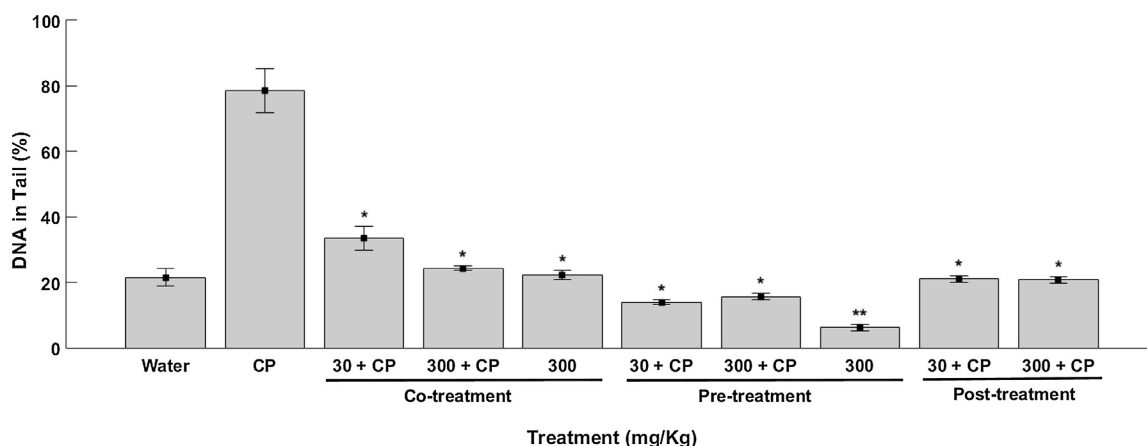

Fig. 2. Assessment of JSE's genotoxic and antigenotoxic activities in mouse bone marrow cells using the comet assay estimated by the parameter percentage of DNA in the tail. The comet assay involved the same groups of animals treated for the MN test. All results are means ± standard deviation (SD) from five mice. A total of 100 nucleoids per animal were analyzed. All groups treated with JSE alone were compared with the negative control (water), and those co-, pre-, or post-treated with CP were compared with the positive control (CP). Student-t test. ** $p < 0.001$ compared to the negative control, * $p < 0.05$ compared to the positive control.

pyrido[4,3-*b*]indole (*N*-OH-Trp-P-2), (±)-7b,8a-dihydroxy-9a,10a-epoxy-7,8,9,10-tetra-hydrobenzo-[a]pyrene (B[a]P diol epoxide), and mitomycin C to various extents [27,28,32,33].

Chemotherapeutic agent CP was used in the present study to assess antigenotoxic and anticytotoxic effects of JSE and pedunculagin in mouse bone marrow cells via the MN test and the comet assay. It is an alkylating agent that exerts its cytotoxic effects through chemically reactive metabolites phosphoramide mustard, which is associated with therapeutic effects, and acrolein, which is linked to toxic side effects. Acrolein has been described as being mutagenic to mammalian cells, interfering with the antioxidant defense system and producing cellular toxicity through the generation of reactive oxygen species (ROS) [34].

Pre-, co-, and post-treatment in mice with JSE at all doses and pedunculagin at 30 mg/kg bw led to a significant decrease in the frequency of CP-induced MNs and percentage of DNA in the tail, which confirmed the antigenotoxic properties of these ellagitannins. These results are in accordance with studies performed with pedunculagin using the Ames test, which showed antimutagenic activity against mutagens MNNG, Trp- P-1(+S9), Trp-P-2 (+S9), and *N*-OH-Trp-P-2 (-S9) [33]. Moreover, experiments with outer bark (cork) extracts of *Quercus suber* L., which are rich in castalagin and vescalagin, and UV radiation on L929 fibroblast cell culture led to intracellular reduction of ROS and prevention of DNA fragmentation. Thus, significant UV-induced cell death was avoided by pre-incubation with cork extracts [35].

In contrast to the present results, pre-treatment on mice with jabuticaba peel extract at 30, 100, and 300 mg/kg bw had not been able to reduce CP-induced chromosomal damage in immature erythrocytes (MNPCE), hence showing no antigenotoxic properties for jabuticaba peel [31]. These results confirm that the protective action against CP-induced DNA damage is mainly due to the ellagitannins in the seeds, which act as scavengers against physiological radicals ROO$^{\bullet}$, OH$^{\bullet}$ and $O_2{}^{---\bullet}$produced by acrolein, the CP metabolite [36]. Another mechanism for antioxidant activity is tannins' capacity to complex with metal ions, such as Fe^{3+} and Fe^{2+}, which stabilize these ions' pro-oxidative activity and hence prevent OH$^{\bullet}$ production by Fenton reaction [37].

Post-treatments with JSE and pedunculagin showed significant effects in the recovery of DNA damage, suggesting that these ellagitannins have a stimulatory action on DNA repair systems. Reduction in the percentage of DNA in the tail, detected by the comet assay, indicates repair of the initial CP-induced DNA damage, such as adduct formation and DNA single-strand breaks. This rapid repair avoided the outcome of mis-repaired DNA lesions expressed as chromosome damage, i.e. micronuclei, as confirmed by the high

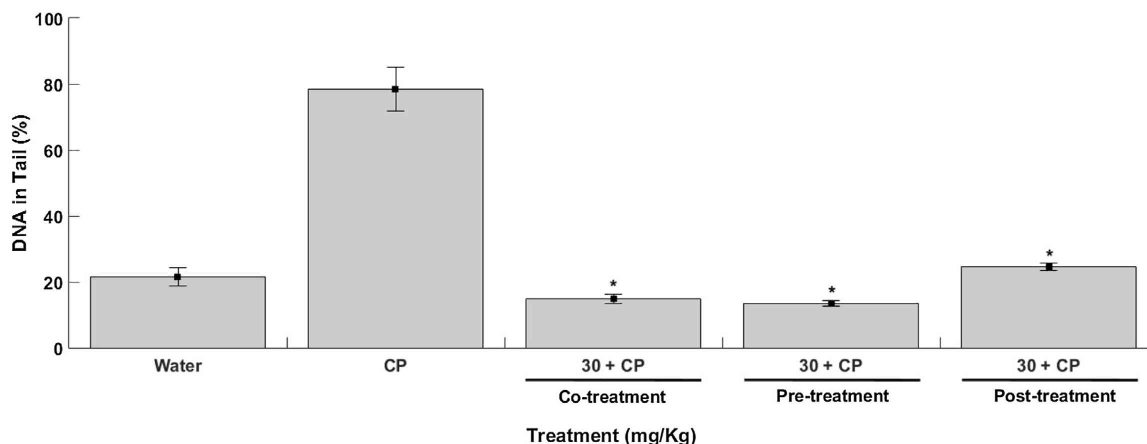

Fig. 3. Assessment of the genotoxic and antigenotoxic activities of pedunculagin in mouse bone marrow cells using the comet assay estimated by the parameter percentage of DNA in the tail. The comet assay involved the same groups of animals treated for the MN test. All results are means ± standard deviation (SD) from five mice. A total of 100 nucleoids per animal were analyzed. All groups treated with pedunculagin and CP were compared with the positive control (CP). Student-t test. * $p < 0.01$ compared to the positive control.

percentages of reduction of CP-induced MNPCEs (Tables 2 and 3), hence demonstrating an increased efficiency of DNA repair systems in the presence of JSE and pedunculagin. Similarly, ellagic acid, a hydrolyzed product of ellagitannins, helped in the recovery of MNNG-induced DNA damage by accelerating DNA repair efficiency in damaged Chinese hamster lung fibroblast cells [38]. Another mechanism could be the interaction of ellagitannins with the hepatic microsomal cytochrome P450 before the metabolic activation of CP, which would prevent the formation of mutagenic metabolite acrolein [34].

As regards the degree of anticytotoxicity observed in post-treatments, there were strong differences between *C*-ellagitannin isomers castalagin and vescalagin, major compounds in JSE and pedunculagin. *C*-ellagitannins counter-attacked CP-induced cytotoxic effects, whereas pedunculagin, a simple ellagitannin with $^4C_1$-glucopyranose core, significantly reduced the PCE/NCE ratio, demonstrating its synergistic cytotoxic effect with CP.

Ellagitannins may differ in biological activities due to their chemical structures, as seen in previous studies in which pedunculagin showed greater cytotoxicity against solid tumor cancer cells, such as human hepatocellular and breast carcinoma cells Hep-G2 and MCF-7, as well as human HCT-116 colon cancer cells, when compared to castalagin [36].

The lowest dose of JSE (30 mg/kg) applied in this work is equivalent to human consumption of 4.75 g/kg bw of fresh jabuticaba fruit, which corresponds to 332.5 g of jabuticaba for a person weighing 70 kg. Frequent consumption of this fruit can have positive effects on human health and could be considered a preventive dietary supplement against cancer.

5. Conclusion

Castalagin, vescalagin, and pedunculagin were the major compounds isolated from jabuticaba seeds. These ellagitannins mixed in JSE showed no genotoxic activity via the MN test and the comet assay. Antigenotoxic effects were induced by pre- and co-treatments of JSE or pedunculagin with CP. However, the most noteworthy finding by the present study is that these compounds not only protect DNA from injury, but also help to repair DNA damage by accelerating DNA repair efficiency in damaged cells.

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Conflict of interest statement

None declared.

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