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Chemopreventive effect and angiogenic activity of punicalagin isolated from leaves of *Lafoensia pacari* A. St.-Hil.

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ABSTRACT

Punicalagin is the major ellagitannin constituent from leaves of *Lafoensia pacari*, a Brazilian medicinal plant widely used for the treatment of peptic ulcer and wound healing. Genotoxic, cytotoxic, antigenotoxic, and anticytotoxic effects of punicalagin were assessed using micronucleus (MN) test and comet assay in mice. Due to the extensive use of *L. pacari* in the wound healing process, we also assessed the angiogenic activity of punicalagin using the chick chorioallantoic membrane (CAM) angiogenic assay. The highest dose of punicalagin (50 mg/kg) showed significant cytotoxic effect by MN test and in the co-treatment with cyclophosphamide (CPA), this cytotoxicity was enhanced. Co-treatment, pre-treatment and post-treatment of punicalagin with CPA led to a significant reduction in the number of DNA breaks and in the frequency of CPA-induced MN, indicating antigenotoxic effect. Using the CAM model, punicalagin exhibited angiogenic activity in all doses mainly at the lowest concentration (12.5 μg/μL). Therefore, these findings indicate an effective chemopreventive role of punicalagin and a high capacity to induce DNA repair. Also, the angiogenic activity presented by punicalagin in this study could contribute for the processes of tissue repairing and wound healing.

1. Introduction

Lafoensia pacari A. St.-Hil., Lythraceae, is a medicinal plant known in Brazil as dedaleiro or pacari. Its leaves and stem bark are used in folk medicine as wound healing, antipyretic, antidiarrhoeal, as well as in the treatment of gastritis, ulcers, and cancer (Solon et al., 2000; Mundo and Duarte, 2007). Several pharmacological studies involving extracts from pacari have shown antioxidant, antigenotoxic, anti-inflammatory, analgesic, antiulcer, antimicrobial, and antidepressant-like activities (Solon et al., 2000; Rogerio et al., 2003, 2006, 2008a, 2008b, 2010; Lima et al., 2006, 2013; Matos et al., 2008; Galdino et al., 2009; Silva-Júnior et al., 2010; Nascimento et al., 2011; Pereira et al., 2011; Tamashiro-Filho et al., 2012).

Among the active molecules already isolated from the Lythraceae family, the ellagitannins have attracted a lot of attention in recent years because of their beneficial properties to human health. These molecules belong to a group of hydrolysable tannins which have been found only in dicotyledoneous angiosperms (Vivas et al., 2004; Lipińska et al., 2014). Punicalagin, a hydrolysable tannin that contains in its structure gallagyl and HHDP groups linked to a glucose moiety (Fig. 1), is abundant in pomegranate fruits (*Punica granatum* L.) and in species of the genus *Terminalia* (Quideau, 2009). A remarkable number of studies has already confirmed a wide range of biological activities for this compound, such as anti-inflammatory, antimicrobial, antidiabetic and antioxidant (Reddy et al., 2007; Endo et al., 2010; Lee et al., 2010; Aqil et al., 2012; Yang et al., 2012; Banihani et al., 2013).

Studies *in vitro* have already confirmed the antigenotoxic activity of punicalagin, however, antigenotoxicity studies *in vivo* were not performed yet with this compound. Chen et al. (2000) revealed that pre-treatment with punicalagin prevented gene mutations and DNA strand breaks induced by bleomycin in Chinese hamster ovary cells. Zahin et al. (2014) showed that punicalagin is capable to significantly reduce benzo[*a*]pyrene-induced DNA adducts using rat liver microsomal proteins *in vitro*. Furthermore, the same study demonstrated that punicalagin protected the DNA of *Salmonella typhimurium* against the

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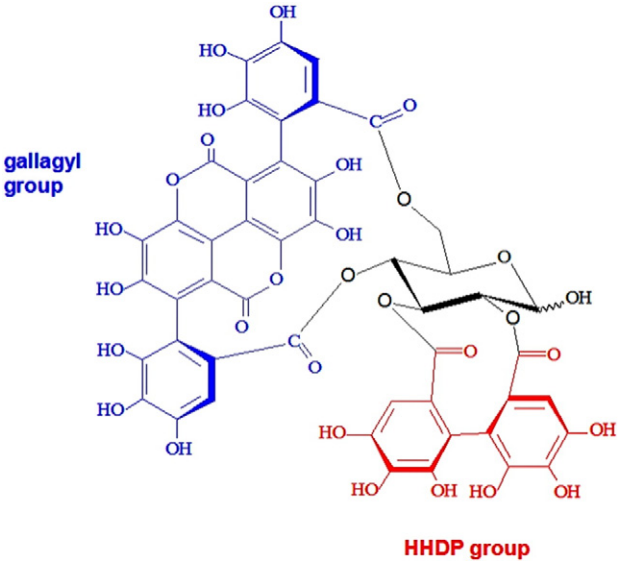

Fig. 1. Chemical structure of punicalagin.

mutagenic actions of sodium azide, methyl methanesulfonate, benzo[*a*]pyrene and 2-aminoflourine.

While several reports have described the beneficial effects of tannins and related polyphenols, other studies have revealed that some products from tannin hydrolysis, such as ellagic and gallic acids, exhibit genotoxic action and contribute to the formation of DNA breaks (Labieniec and Gabryelak, 2003).

Some vegetable tannins also have shown angiogenic effects, such as Gemin A and 5-desgalloylstachyurin (Gu et al., 2006). The chick embryo chorioallantoic membrane (CAM) assay has been widely used as an *in vivo* model to study the angiogenic activity of various agents (Almeida et al., 2014; Manjunathan and Ragunathan, 2015; Rabhi et al., 2015). Drug toxicity on chick embryos can be evaluated by embryo death or adverse effects on CAM, including inflammation, angiogenesis, necrosis, and thickening of the chorioallantoic membrane (Vargas et al., 2007). Angiogenesis plays a critical role in many normal physiological processes involving the growth of new blood vessels from pre-existing vessels, being essential for organ growth and repair (Carmeliet, 2005). There is a wide range of clinical applications for substances with angiogenic activity, such as increase of myocardial vascularization after infarction, repair of the central nervous system following trauma or ischemia and wound healing.

Therefore, the present study performed the micronucleus (MN) test and the comet assay in three different treatments (co-, pre- and post-treatment) to assess the protective effects of punicalagin against DNA damage induced by cyclophosphamide (CPA) in mice. These same tests were used to assess the cytotoxic and genotoxic effects of punicalagin in mice. Moreover, we also evaluated the angiogenic activity of punicalagin by the CAM assay.

2. Materials and methods

2.1. Chemicals

Cyclophosphamide was purchased from Hera Medicamentos (Belo Horizonte, Brazil), whereas dexamethasone ($C_{22}H_{29}FO_5$) and dye solutions hematoxylin-eosin and Giemsa were purchased from Aché Laboratórios Farmacêuticos S.A. (Anápolis, Brazil). Regederm® gel-cream was purchased from Netfarma (Aparecida de Goiânia, Brazil), fetal calf serum was obtained from Laborclin (Campinas, Brazil), and dibasic sodium phosphate, monobasic sodium phosphate, formaldehyde 3.7% solution, and paraffin were purchased from Doles (Goiânia, Brazil). Agarose normal melting, agarose low melting, Triton X-100, dimethyl sulfoxide (DMSO), Stock Lysis solution (distilled water, NaCl, EDTA, TRIS, NaOH, sodium lauryl sarcosinate), Tris-HCl buffer and ethidium bromide were purchased from Genética Brasil (Brasília, Brazil) and Life Technologies (São Paulo, Brazil).

Column chromatography was run using Diaion HP-20 (Supelco) and analytical TLC was carried out with Silica gel 60 F_{254} (Merck) plates. HPLC/UV was performed on a Shimadzu instrument equipped with a diode array detector and 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: 0–20 min, 8–18% A in B; 20–35 min, 18–50% A in B. Analyses were conducted using a 1.0 mL/min flow rate, 260 nm detector wavelength, and 20 μL sample injection volume. Optical rotation was measured with a Perkin-Elmer ADP 400 polarimeter. All NMR experiments were recorded on a Bruker Avance III 500 spectrometer operating at 500.13 MHz for ^1H and 125 MHz for ^{13}C, using TMS as internal reference. ESI-TOF MS spectra were recorded on a Bruker microTOF.

2.2. Plant material and isolation of punicalagin

Leaves from *L. pacari* were collected in the city of Caldazinha (S 16° 39′ 54.5″; W 49° 00′ 03.9″; 1100 m), Goiás State, Brazil, in December 2011 and identified by Professor J. R. Paula from the School of Pharmacy of Universidade Federal de Goiás (UFG). A voucher specimen (UFG-47581) was deposited at the UFG Herbarium.

Dried and grounded leaves of *L. pacari* (250 g) were submitted to ultrasound extraction (30-minute cycles) with 50% acetone (26 × 500 mL) at room temperature. The acetone was evaporated under reduced pressure at 35 °C in the dark and the suspended aqueous extract was filtered to remove fats and chlorophylls. Ethyl acetate (10 × 100 mL) was used in a liquid-liquid extraction and the combined organic phase was evaporated to generate an ethyl acetate extract (19.2 g). The aqueous layer was lyophilized to yield a 74.7 g extract; part of it (45.1 g) was dissolved in methanol (400 mL) to separate soluble (44.2 g) and insoluble (0.9 g) methanolic extracts. Part of the soluble methanolic extract (20 g) was subjected to Diaion HP-20 open column chromatography (200 g), having been eluted with a gradient system of H_2O/MeOH with decreasing polarity. Twelve main fractions (M1–M12) were combined following TLC analysis using formic acid-ethyl formiate-toluene (1:7:1) as the mobile phase. Visualization of TLC spots was performed by spraying a 1% ethanolic solution of ferric chloride in HCl (0.1%) and under UV light.

All 12 fractions were further analyzed by HPLC/UV (Supporting information section). Fractions M2–M7, which were eluted with H_2O, MeOH 20%, and MeOH 40% in Diaion HP-20 column chromatography, consisted of pure punicalagin (13.5 g). Structure elucidation of punicalagin was determined by spectroscopic methods (ESI-TOF MS, 1D and 2D NMR – Supporting information section) and compared with findings from the literature (Doig et al., 1990; Kraszni et al., 2013).

2.3. Animal testing preparation for in vivo protocols

This study was approved by the UFG Animal Research Ethics Committee (CEUA/UFG protocol number 061/13). Following the methods described by Silva et al. (2015), healthy, young male adult (8–12 weeks) outbred mice (*Mus musculus* – Swiss Webster), weighing 25–30 g, obtained from the university's animal facilities, were taken to the laboratory five days prior to experiments and housed in plastic cages (24 ± 2 °C; humidity, 55 ± 10%; light-dark natural cycle, 12 h). Standard food pellets (appropriate commercial rodent diet Labina, Ecibra Ltda, Santo Amaro, SP, Brazil) and water were provided *ad libitum*.

Animals were randomized into control and experimental groups, divided into ten groups of five each and weighed before chemical administration. All treatments involved intraperitoneal (i.p.) administration of

punicalagin. Animals in group 1 received the same volume of water (0.3 mL) i.p. and served as negative control, whereas animals in group 2 received 50 mg/kg of CPA according to body weight (bw) by single i.p. administration and served as positive control. In the *co-treatment*, animals in groups 4, 5, and 6 were respectively treated with 12.5, 25, and 50 mg/kg of punicalagin bw i.p. concomitant with CPA administration i.p., and animals in group 3 received 50 mg/kg of punicalagin bw i.p. on its own. For the *pre-treatment*, animals in groups 8 and 9 received punicalagin i.p. at 12.5 and 25 mg/kg bw, respectively, for five days, followed by CPA, 2 h after the final feeding. Animals in group 7 received punicalagin at 25 mg/kg bw i.p. on its own for five days. In *post-treatment*, animals in group 10 were treated with CPA, and after 6 h and 12 h they received punicalagin at 12.5 mg/kg bw i.p., totalizing a final dose of 25 mg/kg bw.

We had already administrated a dose of 50 mg/kg in the pre-treatment, however, at third day of treatment this dose was lethal for 40% of the animals.

All animals treated with CPA were euthanized by cervical dislocation 24 h after administration; animals which received only punicalagin were sacrificed 24 h after the last administration. Bone marrow cells from both femurs were flushed using fetal calf serum, and following centrifugation (300*g*, 5 min) they were used to prepare MN test slides and the comet assay.

2.3.1. Micronucleus test. The MN assay was performed according to methods described by von Ledebur and Schmid (1973). As previously mentioned, mouse bone marrow cells were smeared on glass slides, coded for blind analysis, air-dried, and fixed with absolute methanol for 5 min at room temperature. Smears were stained with Giemsa, dibasic sodium phosphate, and monobasic sodium phosphate. Three slides were prepared per animal and 2000 polychromatic erythrocytes (PCE) were counted to determine micronucleated polychromatic erythrocytes (MNPCE) frequency. Genotoxicity and antigenotoxicity were assessed by frequency of MNPCE, whereas cytotoxicity and anticytotoxicity were evaluated by the polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio. Slides were analyzed through an optical microscope (Olympus BH-2 10 × 100, Tokyo, Japan).

Genotoxic and antigenotoxic activities were assessed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test, and cytotoxicity and anticytotoxicity were assessed by the qui-square (χ^2) test. Results were considered statistically significant if $p < 0.05$.

2.3.2. Comet assay. The comet assay was performed using the alkaline method with few modifications, as described by Singh et al. (1988). Briefly, 10 μL of mouse bone marrow cells, previously diluted in fetal calf serum, were suspended in 120 μL of 0.5% (*w*/*v*) low melting point agarose and layered over a frosted microscopic slide previously coated with a layer of 1.5% normal melting agarose. Slides were then immersed in a lysing solution of pH 10 (Triton X-100, DMSO, and Stock Lysis Solution) and left overnight. Slides were then transferred to a horizontal electrophoresis chamber containing an alkaline solution (300 mM NaOH, pH > 13) at 4 °C for 20 min, for unwinding of the DNA. Using the same alkaline solution, electrophoresis was performed for 30 min (300 mA, 25 V). Slides were then washed three times with neutralizing buffer (0.4 M Tris-HCl buffer, pH 7.5), stained with ethidium bromide (final concentration 0.02 mg/mL), examined under an Axioplan-Imaging fluorescent microscope, and subjected to image analysis using the TriTek CometScore™ software (version 1.5). The DNA damage was quantified by Olive tail moment (OTM) measurement, calculated by multiplying the total intensity of fluorescence in the comet tail by the tail length measured from the center of the comet head. The intensity of fluorescence is proportional to DNA breaks. A total of 100 nucleoids per animal were analyzed.

For statistical analysis was considered the average and standard deviation of OTM from each treated group and performed the Student-*t*-test to compare the treated groups and their respective control groups. Results were considered statistically significant if p was <0.05.

2.4. Angiogenic activity/chick embryo chorioallantoic membrane (CAM) assay

The CAM model was used to assess punicalagin's angiogenic activity according to a methodology adapted from Melo-Reis et al. (2010). Sixty fertilized chicken eggs (*Gallus domesticus*) were incubated at 37 °C in a humidified atmosphere (60–70% relative humidity). On the fifth day of incubation, a circular hole was opened in the large end of the eggshell, the thin white membrane on the CAM was removed, and the eggs were returned to the incubator. On the thirteenth day of incubation, the eggs were divided into six treatment groups of 10 eggs each: (1) Punicalagin at 12.5 μg/μL; (2) Punicalagin at 25 μg/μL; (3) Punicalagin at 50 μg/μL; (4) water (vehicle control); (5) dexamethasone at 4 μg/μL (angiogenesis inhibitor); and (6) Regederm® gel-cream (angiogenesis inductor). Filter paper discs containing each of these solutions in the indicated concentrations were placed on top of the CAM under sterile conditions, and after 72 h the angiogenic response was evaluated. CAM were fixed in formaldehyde solution (3.7%) for 5 min, cut with curved blunt scissors and maintained in Petri plates with formaldehyde solution. Analysis and quantification of newly-formed vascular net in CAM were made through captured images by a digital camera (Nikon Coolpix L810 16.1 megapixels). The percentage area of vascularization from each treatment was determined using programs GIMP for Windows (version 2.8) and Image J (version 1.28). The amount of selected pixels is proportional to the level of vascularization from the captured image field. In order to analyze the angiogenic activity of punicalagin, all treated groups were compared by ANOVA followed by Tukey's test. Results were considered statistically significant if $p < 0.05$.

2.5. Histology of the CAM blood vessels

The membranes were fixed in formaldehyde solution at 10% for 24 h and later immersed in paraffin. The CAM were then cut from paraffin blocks, stained with hematoxylin-eosin (HE) and examined under a light microscope. Different parameters were assessed: chorionic and allantoic epithelial layer integrity, presence of inflammatory elements, neovascularization, fibroblasts, and necrosis. Results were visually classified according to intensity and the data was transformed into quantitative variables through the assignment of the following scores: absent (0), discrete (1), moderate (2), and intense (3).

To analyze histological parameters of CAM, all treated groups were compared by ANOVA followed by Tukey's test. Results were considered statistically significant if $p < 0.05$.

3. Results

3.1. Micronucleus test

Data on the frequencies of MNPCE and ratio of PCE/NCE in the bone marrow of mice that were treated alone or pre-, co- or post-treated with punicalagin and cyclophosphamide are presented in Table 1.

3.1.1. Co-treatment. Punicalagin 50 mg/kg alone had no effect on the frequency of MNPCE in comparison to the negative control ($p > 0.05$), but caused a significant reduction of PCE/NCE ratio ($p < 0.05$). All doses of punicalagin co-treated with CPA (12.5, 25, and 50 mg/kg) were able to significantly reduce the DNA damage induced by the positive control ($p < 0.05$). The lower doses (12.5 and 25 mg/kg) were able to protect the mice bone marrow cells from the cytotoxic effect induced by CPA ($p < 0.05$). In the other hand, the highest dose (50 mg/kg) showed a significant decrease of PCE/NCE ratio in comparison to the positive control ($p < 0.05$).

Table 1
Effect of various treatments using punicalagin in the frequency of micronucleated polychromatic erythrocytes (MNPCE) and in the polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) ratio in the bone marrow of mice.

Groups	Treatments (mg/kg bw i.p.)	MNPCE/2000 PCE (mean ± SD)	PCE/NCE (mean ± SD)
1	Negative control (H_2O)	4.20 ± 0.45	1.03 ± 0.03
2	Positive control (CPA)	27.2 ± 1.30	0.71 ± 0.02
Co-treatment			
3	Punicalagin (50)	2.8 ± 0.44	0.92 ± 0.01**
4	Punicalagin (12.5) + CPA	18.6 ± 1.51*	0.86 ± 0.05*
5	Punicalagin (25) + CPA	12.8 ± 0.83*	0.85 ± 0.04*
6	Punicalagin (50) + CPA	10 ± 1*	0.61 ± 0.01*
Pre-treatment			
7	Punicalagin (25)	2.8 ± 0.83	1.06 ± 0.03
8	Punicalagin (12.5) + CPA	11.4 ± 1.34*	0.93 ± 0.01*
9	Punicalagin (25) + CPA	7.6 ± 0.54*	1.21 ± 0.04*
Post-treatment			
10	Punicalagin (12.5) + CPA	3.2 ± 0.83*	0.97 ± 0.02*

CPA: cyclophosphamide at 50 mg/kg body weight (bw). All values are means ± standard deviation (SD) from five mice. Punicalagin and CPA were diluted in physiological saline and i.p. injected. Animals in group 1 received the same water volume (0.3 mL) i.p. and served as negative control, whereas animals in group 2 received 50 mg/kg CPA and served as positive control. Animals in group 3 received 50 mg/kg punicalagin on its own, and animals in groups 4, 5, and 6 were respectively treated with 12.5, 25, and 50 mg/kg punicalagin concomitant with CPA. Animals in group 7 received punicalagin at 25 mg/kg on its own for five days, and animals in groups 8 and 9 received punicalagin at 12.5 and 25 mg/kg, respectively, for five days, followed by CPA, 2 h after the final feeding. Finally, animals in group 10 were treated with CPA, and after 6 h and 12 h they received punicalagin at 12.5 mg/kg. Animal groups treated with punicalagin alone (3 and 7) were compared with the negative control, and those co-, pre- or post-treated with CPA (4, 5, 6, 8, 9, and 10) were compared with the positive control. A total of 2000 PCE were scored per animal for the MN test. ANOVA, Tukey's test and qui-square.

* In comparison with the positive control ($p < 0.05$).

** In comparison with the negative control ($p < 0.05$).

3.1.2. Pre-treatment. Animals treated with punicalagin alone at 25 mg/kg for five days did not show significant difference in the MNPCE frequency or PCE/NCE ratio compared to the negative control ($p > 0.05$). A significant reduction of MNPCE was observed at doses 12.5 and 25 mg/kg in comparison to the animals treated with CPA alone ($p < 0.05$). Moreover, these doses were able to significantly increase the PCE/NCE ratio when compared to the positive control group ($p < 0.05$). Also, a dose of 50 mg/kg was performed in the pre-treatment, however, it was lethal for 40% of the animals at third day.

3.1.3. Post-treatment. The animals treated twice with 12.5 mg/kg showed a significant reduction ($p < 0.05$) in the MNPCE frequency, revealing the highest protection against CPA-induced DNA damage in the present study. Also, in this same dose was observed a significant increase in the PCE/NCE ratio in comparison to the positive control ($p < 0.05$).

3.2. Comet assay

Fig. 2 shows the genotoxic and protective effects of punicalagin against CPA-induced DNA breaks. Animals treated once with 50 mg/kg or treated for five days at dose 25 mg/kg did not show increase in the OTM values in comparison to the negative control ($p > 0.05$). In addition, punicalagin was able to significantly reduce the number of CPA-induced DNA breaks in the co-, pre- and post-treatment ($p > 0.05$).

3.3. Chick embryo chorioallantoic membrane (CAM) assay

The results of the angiogenic activity of punicalagin are presented in Fig. 3, which shows images of different CAM treated with punicalagin and controls, as well as their vascularization percentages. In the groups treated with punicalagin (12.5 and 25 μg/μL) and regederm, more blood vessels were formed compared to the negative and inhibitor controls ($p < 0.05$). However, in the concentration of 50 μg/μL punicalagin did not show significant increase in the vascularization percentage compared to the negative ($p < 0.05$).

3.4. CAM histological analysis

Following the CAM image analysis, membranes were submitted to histological analysis (Fig. 4 and Table 2). In the angiogenesis analysis, results showed an increase in the number of blood vessels for all treatments with punicalagin in comparison to the negative control and the inhibitor agent ($p < 0.05$). The presence of fibroblasts for CAM treated with punicalagin was significantly higher than groups treated with water and inhibitor ($p < 0.05$). A significant dose-dependent increase of inflammatory cells and necrosis (karyorrhexis) were observed in all treatments with punicalagin in comparison to the negative control and the inhibitor agent ($p < 0.05$).

4. Discussion

Punicalagin was isolated for the first time from *L. pacari* leaves. The amount obtained, 197 mg g^{-1} of dried leaf, is greater than the average obtained from pomegranate husk (82.4 mg g^{-1}) (Lu et al., 2008), which shows how *L. pacari* leaves emerge as a new source of this important natural product.

Our results with punicalagin showed no genotoxic effects in micronucleus test and comet assay in mice. These findings are in accordance

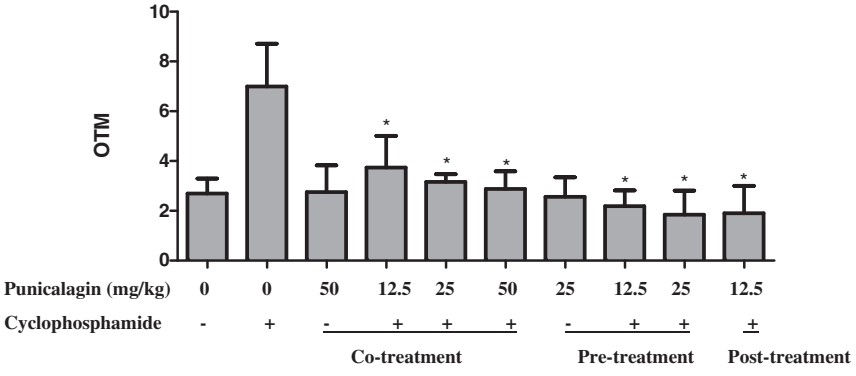

Fig. 2. Punicalagin's genotoxic evaluation and its protective effects against cyclophosphamide-induced DNA breaks in mouse bone marrow cells using the comet assay. For the comet assay, the same groups of animals treated for the micronucleus test were used. OTM: Olive tail moment. All results are means ± standard deviation (SD) from five mice. A total of 100 nucleoids per animal were analyzed. All groups treated with punicalagin alone were compared with the negative control (water), and those co-, pre- or post-treated with cyclophosphamide (CPA) were compared with the positive control (CPA). Punicalagin and CPA were diluted in physiological saline solution and i.p. injected. Student-*t*-test. *In comparison with the positive control ($p < 0.05$).

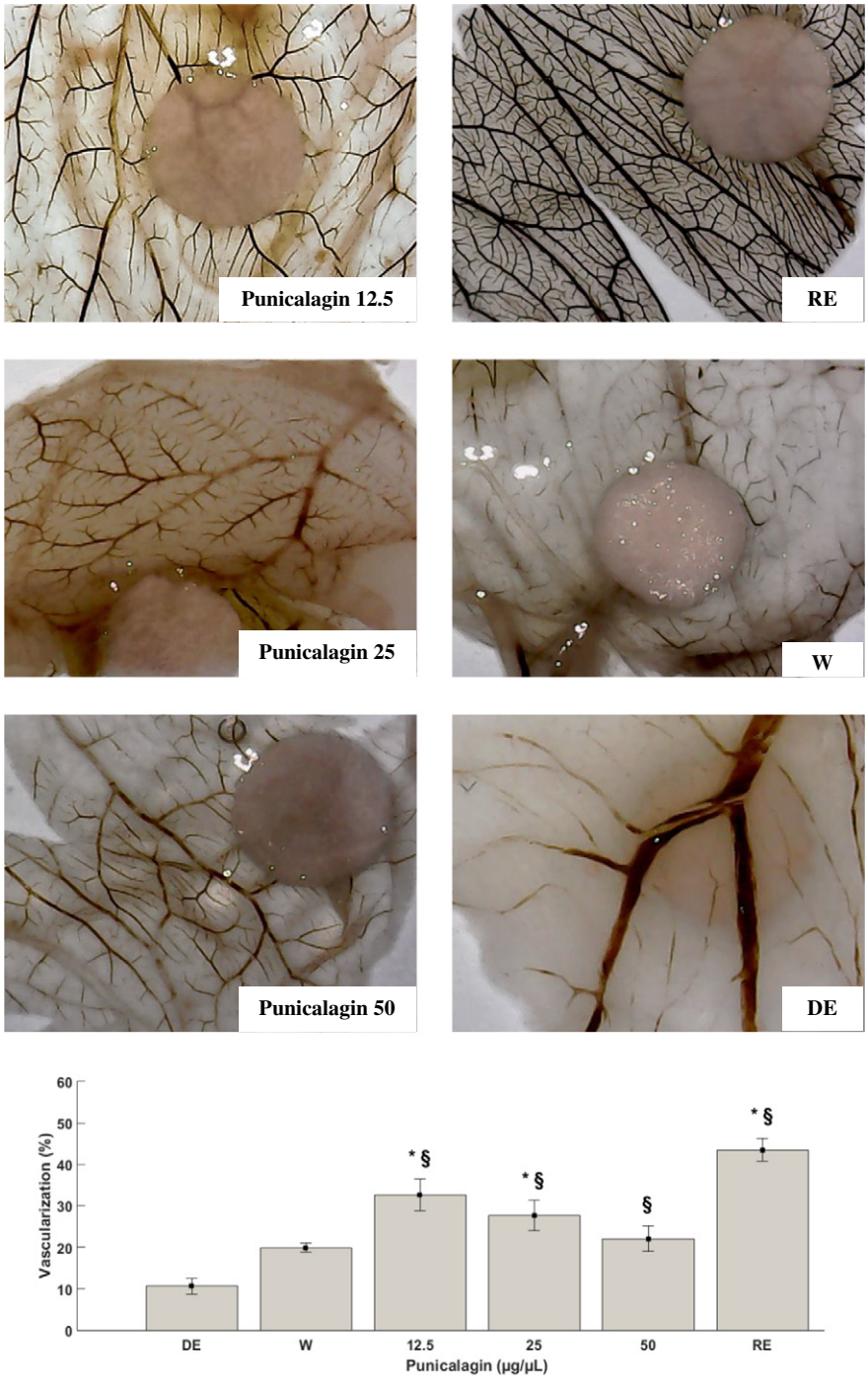

Fig. 3. Photomicrograph of different chick embryo chorioallantoic membranes (CAM) after 72 h of treatments with W (water 3 μL, negative control), RE (Regederm® 30 μg/μL, angiogenesis inductor), DE (dexamethasone 4 μg/μL, angiogenesis inhibitor), Punicalagin 12.5 (Punicalagin 12.5 μg/μL), Punicalagin 25 (Punicalagin 25 μg/μL) and Punicalagin 50 (Punicalagin 50 μg/μL). Ten CAM were obtained per treatment group and considered for the vascularization percentage means, as presented in the graph above. $^{*}p < 0.05$ in comparison to the negative control. $^{\S}p < 0.05$ in comparison to DE control.

to previous study with *Salmonella typhimurium* strains in Ames mutagenicity test (Zahin et al., 2014). Tannins are known for their ability to form complexes with molecules such as proteins, polysaccharides, metals and DNA (Haslam, 1998; Andrade Jr. et al., 2006; Moilanen et al., 2016). Kulkarni et al. (2007) revealed that punicalagin has a high affinity with bovine serum albumin and metal ions, but, it has a very weak and nonspecific binding with DNA.

The MN test is able to detect cytotoxic effects by PCE/NCE ratio. When the proliferation of bone marrow cells is affected by a toxic agent, the number of immature erythrocytes (PCE) decreases in relation to that of mature erythrocytes (NCE), leading to a decrease in the PCE/NCE ratio (Hayashi et al., 2000). In our results the animals treated only with punicalagin at 50 mg/kg exhibited a mild cytotoxic activity, but in the co-treatment this same dose was able to enhance the cytotoxic effects of CPA. Also, the dose of 50 mg/kg was lethal in the pre-treatment, which could be related to its cumulative effects. Substances with a cytotoxic profile are recommended for cancer therapy, especially if they do not cause DNA damage in normal cells, and punicalagin presented this profile in the present study. Larrosa et al. (2006) reported that punicalagin and its main hydrolysis product, ellagic acid, induced

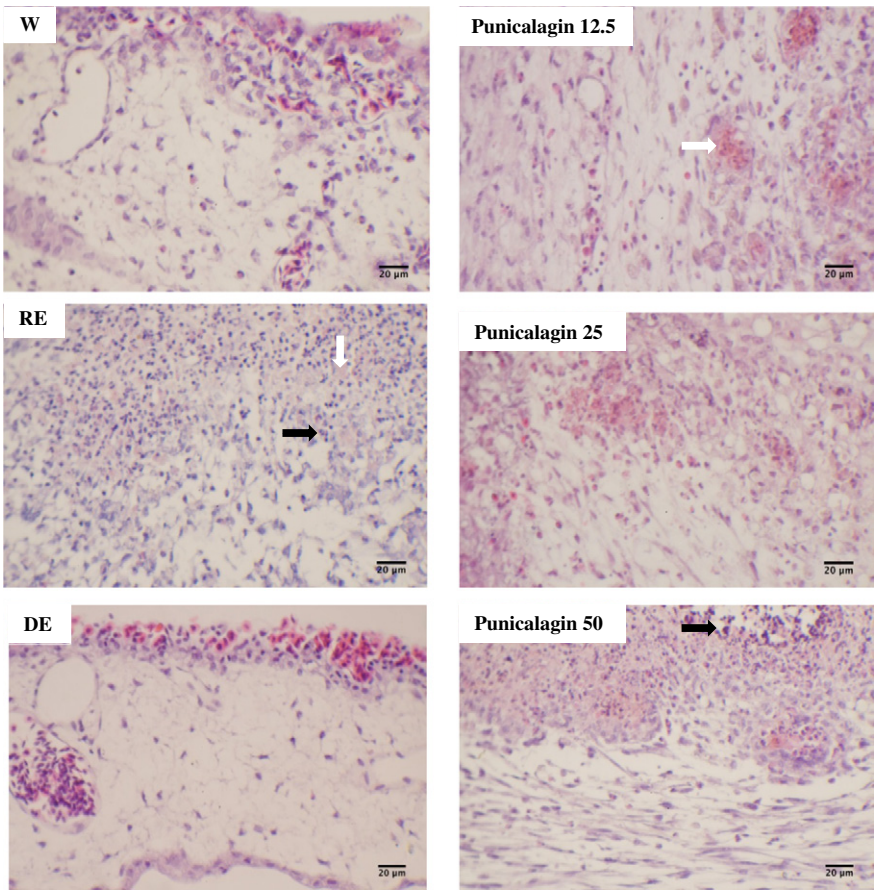

Fig. 4. Photomicrography of chick embryo chorioallantoic membranes (CAM) stained with hematoxylin-eosin (HE), obtained from different treatment groups: W (water 3 μL, negative control), RE (Regederm® 30 μg/μL, angiogenesis inductor), DE (dexamethasone 4 μg/μL, angiogenesis inhibitor), Punicalagin 12.5 (Punicalagin 12.5 μg/μL), Punicalagin 25 (Punicalagin 25 μg/μL) and Punicalagin 50 (Punicalagin 50 μg/μL). Five CAM were stained with HE and used for histological analysis. White arrows indicate karyorrhexis, and black arrows indicate inflammatory elements.

apoptosis by chromatin condensation and activation of caspases 3 and 9 only in human colon cancer cells but not in normal colon cells. Using the MN test in mouse bone marrow, Silva et al. (2014) demonstrated that the ellagitannin oenothein B induced significant decrease in the PCE/NCE ratio without presenting any mutagenic effect.

The chemotherapeutic agent CPA was used in our study to assess the antigenotoxic and anticytotoxic effects of punicalagin. CPA is a DNA alkylating agent which exerts its cytotoxic effects through chemically reactive metabolites that alkylate DNA, producing cross-links. Moreover, it induces the generation of oxidative stress mediated by redox balance disruption and, together with its metabolite acrolein, causes the inactivation of microsomal enzymes and leads to increased reactive oxygen species (ROS) and lipid peroxidation (Chakraborty et al., 2009; Rehman et al., 2012). In the present study punicalagin exhibited strong antigenotoxic effect during co-treatment and pre-treatment. In previous studies, punicalagin had already shown antimutagenic and anticarcinogenic activities which might be related to its antioxidant properties (Wang et al., 2013; Zahin et al., 2014). Several studies reported the capacity of this ellagitannin to inhibit lipid peroxidation and to scavenge reactive species like O_2^-, H_2O_2, ^-OH, NO_2^- and N_3^-, decreasing the oxidative stress and minimizing the genotoxic effects of some mutagens, including CPA (Chen et al., 2000; Kulkarni et al., 2007; Aqil et al., 2012; Fouad et al., 2016; Rao et al., 2016; Xu et al., 2016). Also, current studies *in vitro* and *in vivo* showed that punicalagin attenuated oxidative damage by inducing Nrf2 translocation to the nucleus, which up-regulated the activity and expression of several antioxidant enzymes

Table 2
Histological analysis of chick embryo chorioallantoic membranes (CAM). Media ± standard deviation (SD) of histological parameters classified in a 0–3 scale (obtained from five membranes in each treatment).

Treatments (μg/μL)	Angiogenesis	Presence of inflammatory cells	Presence of fibroblasts	Presence of necrosis	Thickening in chorioallantoic membrane
H_2O	0.6 ± 0.5	0.6 ± 0.5	0.4 ± 0.5	0.0 ± 0.0	0.0 ± 0.0
Regederm	2.6 ± 0.5	2.6 ± 0.5	2.6 ± 0.5	2.6 ± 0.5	1.0 ± 0.0
Dexamethasone	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PUNI 12.5	2.4 ± 0.5*,***	1.8 ± 0.4*,***	2.2 ± 0.4*,***	1.2 ± 0.4*,**,***	0.8 ± 0.8
PUNI 25	1.4 ± 0.9***	2.0 ± 0.7*,***	2.0 ± 1.0*,***	2.0 ± 1.0*,***	0.4 ± 0.5
PUNI 50	1.8 ± 1.0***	2.4 ± 0.5*,***	1.6 ± 0.8***	2.0 ± 0.7*,***	0.6 ± 0.8

ANOVA, Tukey's test.
* In comparison with water ($p < 0.05$).
** In comparison with Regederm ($p < 0.05$).
*** In comparison with dexamethasone ($p < 0.05$).

(hemeoxygenase, glutathione, superoxide dismutase and catalase) (Rao et al., 2016; Xu et al., 2016). Nrf2 is a transcription factor that regulates the expression of antioxidant proteins and protect against oxidative damage triggered by toxic agents chemical, physical or biological. Under physiological conditions, Nrf2 is kept in the cytoplasm binding to protein Keap1. In response to intracellular oxidative stress, Nrf2 disconnects from Keap1 and is translocated to the nucleus where it binds to DNA and initiates transcription of antioxidative genes (Moi et al., 1994; Scannevin et al., 2012).

Like several other tannins, punicalagin is capable of chelate iron ions, which could inhibit the Fenton reaction and decrease the oxidative stress on DNA (Kulkarni et al., 2007; Aloqbi et al., 2016). Furthermore, the tannins' high affinity with compounds such as proteins and alkaloids suggest that punicalagin could also form complexes with many mutagens, including CPA, inducing antigenotoxic action (Okuda et al., 1984; Chen et al., 2000; Zahin et al., 2014).

Other remarkable observation in our work is the recovering effect of DNA damage presented by punicalagin at post-treatment. Exposing mice to punicalagin after the CPA treatment caused a significant reduction in MNPCE and in the OTM, as well as an increase in the PCE/NCE ratio. Similar results were obtained when Chinese hamster lung fibroblast cells, human lymphocytes, and Hep-G2 cells underwent post-treatment with tannic acid and its related compounds (gallic acid, ellagic acid, and propyl gallate) after mutagens MNNG, TRP-P-2, PhIP, and H_2O_2 (Sanyal et al., 1997; Chakraborty et al., 2004; Wu et al., 2004). However, further studies are required in order to better understand the punicalagin's action mechanisms in the DNA repair process.

In our study using the CAM model, the results showed that punicalagin exhibited angiogenic activity in fertilized chicken eggs. It is known that many angiogenic factors such as VEGF, MMP2, MMP9, TNF, IL-6, and IL-8, are activated by inflammatory cells (Izumi-Nagai et al., 2007; Wiegand and Hipler, 2008; Manjunathan and Ragunathan, 2015). Of the known angiogenic factors, VEGF family members are the major mediators of the regulatory machinery that controls angiogenesis during development and in pathological conditions (Folkman, 1995). Gu et al. (2006) reported that tannins isolated from *Geum japonicum* up-regulated the expression of VEGFb and VEGFc, which contributed to early revascularization on rats' infarcted myocardium. Also, tannin extracts from *Terminalia chebula* Fructus Retz. induced wound healing effect by enhance the inflammation and the expression of VEGFa on rat wounds, which promoted increase in the amount of newly formed capillaries (Li et al., 2011). Some studies stated that the release of VEGF may be related to Nrf2 activation (Kuang et al., 2013; Wiesner et al., 2013), thus, the angiogenic activity of punicalagin possibly is due to Nrf2 activation with consequent release of VEGF.

Mundo and Duarte (2007) reported that leaf infusion and topical application of stem bark from *L. pacari* are used in folk medicine for the treatment of gastric ulcers and wound healing. Our results indicated that punicalagin could be applied in therapy of gastric ulcers and wound healing due to its angiogenic activity. Another important aspect of our work was the necrosis induction by punicalagin and regederm. The literature states that necrosis may develop to full healing due to the proliferation of inflammatory cells and angiogenesis. However, enhanced necrotic processes prevent complete tissue regeneration (Elmore, 2007; Nikoletopoulou et al., 2013). Therefore, our results suggest that the topical use of punicalagin and regederm for wound healing purposes should be done at lower concentrations.

5. Conclusion

In summary, genotoxic effects were not observed in punicalagin *via* MN and comet tests. The highest dose (50 mg/kg) showed a significant cytotoxic effect on mouse bone marrow cells, especially when co-administered with CPA. Co-treatment and pre-treatment of punicalagin with CPA resulted in significant OTM reduction and in the frequency of CPA-induced MN, indicating that this tannin presents an effective chemopreventive role. In the present study, post-treatment with punicalagin at 12.5 mg/kg yielded the largest protection against CPA-induced DNA damage, showing in this way an evidence of punicalagin has a high capacity to induce DNA repair mechanism. Using the CAM model, punicalagin exhibited angiogenic activity in fertilized chicken eggs, which was confirmed by the histological analysis of membranes. Therefore, these findings support the possible use of punicalagin in chemoprevention and in the treatment of gastric ulcers and wound healing.

Conflict of interest

All the authors of this manuscript state that they have no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

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