Toxicology and Applied Pharmacology 310 (2016) 1–8

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

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

Cristiene Costa Carneiro [a,*], Suzana da Costa Santos [b], Ruy de Souza Lino Jr. [c], Maria Teresa Freitas Bara [d], Beatriz Abdallah Chaibub [d], Paulo Roberto de Melo Reis [e], Dwight Assis Chaves [e], Antônio Jorge Ribeiro da Silva [f], Luana Santos Silva [a], Daniela de Melo e Silva [a], Lee Chen-Chen [a]

[a] Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74001970 Goiânia, GO, Brazil
[b] Instituto de Química, Universidade Federal de Goiás, 74001970 Goiânia, GO, Brazil
[c] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, 74605050 Goiânia, GO, Brazil
[d] Faculdade de Farmácia, Universidade Federal de Goiás, 74605170 Goiânia, GO, Brazil
[e] Laboratório de Estudos Experimentais e Biotecnológicos, Pontifícia Universidade Católica de Goiás, 74605010 Goiânia, GO, Brazil
[f] Núcleo de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, 21941590 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Article history:
Received 23 June 2016
Revised 8 August 2016
Accepted 16 August 2016
Available online 18 August 2016

Keywords:
Punicalagin
Antigenotoxicity
Anticytotoxicity
Chemoprevention
DNA repair
Angiogenesis

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

* Corresponding author at: Departamento de Genética, Instituto de Ciências Biológicas, C.P. 131, Campus Samambaia, Goiânia, GO 74001-970, Brazil.
E-mail address: profacristiene@gmail.com (C.C. Carneiro).

http://dx.doi.org/10.1016/j.taap.2016.08.015
0041-008X/

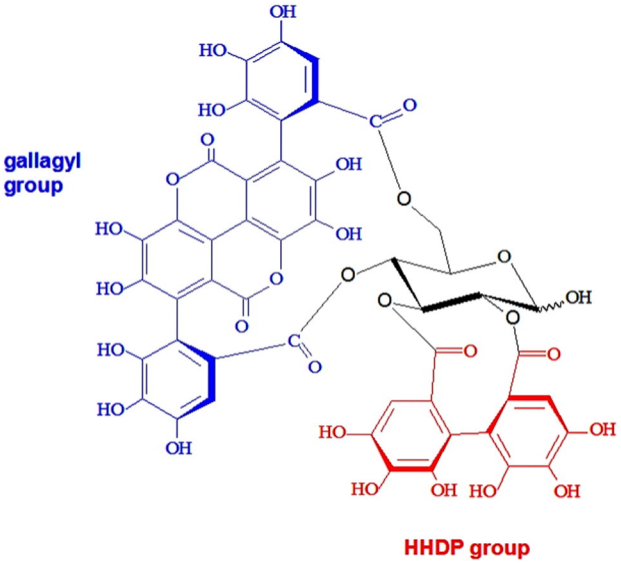

Fig. 1. Chemical structure of punicalagin.

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