Food Research International 49 (2012) 728–738

Contents lists available at SciVerse ScienceDirect

Food Research International

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

Chemo-protective activity and characterization of phenolic extracts from *Corema album*

Antonio J. León-González [a], Raquel Mateos [b], Sonia Ramos [b], M. Ángeles Martín [b], Beatriz Sarriá [b], Carmen Martín-Cordero [a], Miguel López-Lázaro [a], Laura Bravo [b], Luis Goya [b,*]

[a] Departamento de Farmacología, Facultad de Farmacia, Universidad de Sevilla, Prof. García González 2, 41012 Sevilla Spain
[b] Departamento de Metabolismo y Nutrición, Instituto de Ciencia y Tecnología de Alimentos y Nutrición — ICTAN — CSIC, José Antonio Novais, 10; 28040 Madrid Spain

ARTICLE INFO

Article history:
Received 5 July 2012
Accepted 14 September 2012

Keywords:
Antioxidant defenses
Biomarkers of oxidative damage
Chlorogenic acid
Hydroxycinnamic acids
Natural bioactive compounds
Oxidative stress

ABSTRACT

There is currently substantial interest in the cyto-protective effects of natural compounds against oxidative stress and in studying of the defense mechanisms involved. *Corema album* fruit is an edible berry consumed along the Atlantic littoral of the Iberian Peninsula. The aim of this study was to characterize the phenolic composition and evaluate the chemo-protective effects against oxidative stress of three phenolic extracts from this fruit on liver cells.

Characterization of phenolic compounds, achieved by liquid chromatography and diode-array, mass spectrometry and electrospray ionization-time of flight-mass spectrometry detection, showed a main fraction of hydroxycinnamic acids. Liver HepG2 cells were treated with 1–40 μg/mL of the extracts and exposed to oxidative stress chemically induced. Cell viability, reactive oxygen species (ROS), reduced glutathione (GSH), antioxidant enzymes and biomarkers of oxidative damage were evaluated.

Treatment of HepG2 cells with the extracts partially prevented ROS increase, GSH depletion, antioxidant enzymes over-activity and oxidative damage to proteins and lipids induced by stress. The results support the traditional use of *C. album* as a medicinal plant and suggest that inclusion of its berries in the diet would contribute to the protection afforded by fruits, vegetables and plant-derived beverages against oxidative stress related diseases.

1. Introduction

Oxidative stress is the main cause of liver diseases and plant extracts with antioxidant properties has received extensive attention as possible therapeutic and preventive agents which counteract the production of free radicals and reactive oxygen species (ROS) and thus combat oxidative stress.

Plant polyphenols have gained increasing interest because of their numerous biological effects such as free-radical scavenging, metal chelation, modulation of enzymatic activity, inhibition of cellular proliferation and altering signal transduction pathways (Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). Epidemiological studies have also highlighted the association between the consumption of polyphenol-rich foods and the prevention of degenerative human diseases such as cardiovascular diseases, cancer and other degenerative disorders (Manach, Scalbert, Morand, Remesy, & Jiménez, 2004).

Corema album D. Don (Ericaceae) fruit is a wild edible berry traditionally consumed along the Atlantic littoral of the Iberian Peninsula. Berries of *C. album* have been used in popular medicine as antipyretic and are offered in the south of Spain as appetizers. Unpublished results have indicated that the main phenolic fraction in *C. album* is composed of hydroxycinnamic acids. The major hydroxycinnamic acids, *p*-coumaric, caffeic, ferulic and sinapinic acids, are ubiquitously found in fruits, vegetables, cereals and also in high concentrations in tea, mate (Bravo, Goya, & Lecumberri, 2007) and particularly in coffee, averaging 160 mg per cup (Williamson, Dionisi, & Renouf, 2011). The interest in these compounds is related to their antioxidant activity, which may have some health beneficial effects *in vivo*. The extent of their protective effect *in vivo* depends on their bioavailability for intestinal absorption, metabolism, and subsequent interaction with target tissues. In this line, different studies have shown that hydroxycinnamic acids are extensively absorbed in cultured cells (Mateos, Goya, & Bravo, 2006), rats (Azuma et al., 2000; Lafay et al., 2006) and humans (Nardini, Cirillo, Natella, & Scaccini, 2002; Olthof, Hollman, & Katan, 2001).

On the other hand, the absorbed drugs and xenobiotics can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic processes (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006). The liver is particularly susceptible to toxic and oxidative insults since the portal vein brings blood to this organ after intestinal absorption. Therefore, studies dealing with the effects of antioxidants at a cellular level in cultured hepatic cells are essential. Human HepG2, a well differentiated transformed cell line, is a reliable model for cultured hepatocyte-type cells used for biochemical, pharmacological and nutritional studies since

* Corresponding author. Tel.: +34 91 544 56 07; fax: +34 91 549 36 27.
E-mail address: luisgoya@ictan.csic.es (L. Goya).

0963-9969/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodres.2012.09.016

they retain hepatocyte morphology and most of its function in culture (Alía, Ramos, Mateos, Bravo, & Goya, 2006). Different studies have demonstrated that hydroxycinnamic acids (Mateos et al., 2006), flavonoids (Kanazawa, Uehara, Yanagitani, & Hashimoto, 2006) and olive oil phenols, hydroxytyrosol and hydroxytyrosyl acetate (Mateos, Goya, & Bravo, 2005) are absorbed and metabolized by cultured HepG2 cells. In this study, the main phenolic compounds in three different extracts from *C. album* were characterized and quantified, and their hepato-protective activity against an oxidative stress chemically induced by a potent prooxidant, *tert*-butylhydroperoxide (*t*-BOOH) in human HepG2 was tested in HepG2 cells.

2. Materials and methods

2.1. Reagents

Formic acid and HPLC grade methanol were obtained from Panreac (Barcelona, Spain). Chlorogenic acid, rutin, hesperetin, resveratrol and *p*-hydroxybenzoic acid, *tert*-butylhydroperoxide (*t*-BOOH), *o*-phthalaldehyde (OPT), glutathione reductase (GR), reduced (GSH) and oxidized glutathione, nicotine adenine dinucleotide (reduced) (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,4-dinitrophenylhydrazone (DNPH), gentamycin, penicillin G, streptomycin, β-mercaptoethanol and EDTA were purchased from Sigma-Aldrich (Madrid, Spain). Cyanidin 3-*O*-glucoside chloride, delphinidin 3-*O*-glucoside chloride and cyanidin 3-*O*-arabinoside chloride were acquired from Extrasynthese (Lyon, France). The Bradford reagent was from BioRad Laboratories (Madrid, Spain). Other reagents were of analytical or chromatographic quality.

2.2. Extraction of phenolic compounds from C. album

Wild *C. album* berries were harvested in Huelva (Spain) 37° 04′ 10.15″ N–6° 41′15.45″ W, in September 2009, and identified by Dr. Mari Cruz Diaz Barradas, from the Department of Plant Biology and Ecology, University of Seville. *C. album* fruits not showing any physical damage were selected, washed under running tap water and blot dried. Ripe fruits were lyophilized and freeze-dried samples were ground and stored at −20 °C until further analysis. Three different phenolic compounds extraction methods were applied: 'acetone extract' (A) was obtained by homogenizing 100 g of lyophilized *C. album* with 100 mL of acetone/formic acid/water (70:0.5:29.5, v/v/v) using ultrasonic equipment for 45 min at room temperature. The final extract was lyophilized and resulting a 4.79% yield with respect to fresh fruit. 'Ethyl acetate extract' (EA) was obtained by homogenizing 100 g of lyophilized fruit with 100 mL of ethyl acetate using ultrasonic equipment for 45 min at room temperature. The final extract was evaporated under vacuum producing a 0.29% yield with respect to fresh fruit. 'Water extract' (W) was obtained by homogenizing 300 g of frozen ripe fruit with 300 mL of water using ultrasonic equipment for 45 min at room temperature. The final extract was lyophilized and resulting a 4.5% yield with respect to fresh fruit. Finally, 10 mg/mL stock solutions of A and W in water and EA in ethanol were prepared to characterize their phenolic composition by HPLC and for cell treatment.

2.3. HPLC analysis

Phenolic composition of extracts was analyzed using an Agilent 1100 liquid chromatographic system equipped with an autosampler, quaternary pump and diode-array (DAD) detector. A 250 mm × 4.6 mm i.d., 5-μm particle size Nucleosil 120 RP-18 column (Teknokroma) preceded by a ODS precolumn was used. Elution was performed at a flow rate of 1.0 mL/min, using as mobile phase mixture of 1% (v/v) formic acid in deionized water (solvent A) and methanol (solvent B). The solvent gradient changed from 90% A to 85% A in 5 min, to 70% A in 15 min, to 50% A in 15 min, to 30% A in 7 min, to 10% A in 3 min maintained for 5 min and to 90% A in 10 min. Chromatograms were acquired at 280 nm to register hydroxybenzoic acids, flavanones and stilbenes. Wavelengths 320, 360 and 520 nm were selected to monitor hydroxycinnamic acids, flavonols and anthocyanins, respectively. *p*-hydroxybenzoic acid, hesperetin, resveratrol, chlorogenic acid, rutin and cyanidin 3-*O*-glucoside were used to quantify hydroxybenzoic acids, flavanones, stilbenes, hydroxycinnamic acids, flavonols and anthocyanins, respectively.

2.4. LC–MS analysis

LC–MS measurements were performed on an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a DAD detector and a quadrupole mass spectrometer (Agilent Technologies). Chromatographic conditions (eluents, column, flow rate, gradient, etc.) were as described above. Eluent flow was split 8:1 between the DAD detector and the MS ion source. The MS was fitted to an atmospheric pressure electrospray ionization (ESI) source, operated in negative ion mode. The electrospray capillary voltage was set to 3000 V, with a nebulizing gas (nitrogen) flow rate of 12 L/h and a drying gas temperature of 300 °C. Mass spectrometry data were acquired in scan mode (mass range m/z 100–900) at a scan rate of 1.5 s.

2.5. HPLC–ESI-QTOF analysis

The chromatography was performed on an Agilent 1200 series LC system coupled to an Agilent 6530A accurate-mass quadrupole time-of-flight (Q-ToF) with ESI-Jet Stream Technology (Agilent Technologies). A 250 mm × 4.6 mm i.d., 5 μm particle size Nucleosil 120 RP-18 column (Teknokroma) preceded by an ODS precolumn was used. Each sample (20 μL) was injected and separated isocratically by using a mobile phase consisting of water and acetonitrile, both containing 0.1% formic acid, at a flow rate of 0.4 mL/min. The Q-ToF acquisition conditions were as follows: 2 GHz, mass range between 100 and 1000 m/z, negative polarity, drying gas volume and temperature 8 L/min and 350 °C, sheath gas volume and temperature 11 L/min and 325 °C, nebulizer pressure 45 psi, cap voltage 3500 V, nozzle voltage 1000 V, and fragmentor voltage 75 V. Data acquisition and qualitative analysis were performed by using MassHunter Workstation Software.

2.6. Cell culture and treatment

Human hepatic HepG2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in DMEM F-12 medium from Biowhittaker (Lonza, Madrid, Spain), supplemented with 2.5% fetal bovine serum (FBS) and 50 mg/L each of gentamicin, penicillin and streptomycin. The different concentrations of the three extracts (1, 5, 10, 20 and 40 μg/mL) were dissolved in serum-free culture medium and added to the cell plates for 20 h except in the ROS assay. In the experiments to evaluate the protective role of the compounds against an oxidative insult, cells were pre-treated with the same concentrations of the compounds for 20 h, then the medium was discarded and fresh medium containing 400 μM *t*-BOOH was added for 3 h, after which the cell cultures were processed for each assay.

2.7. Evaluation of cell viability, ROS production and antioxidant defenses

Cellular damage was evaluated by lactate dehydrogenase (LDH) leakage (Alía et al., 2006). Cells were seeded (2×10^5 cells per plate) in 60 mm plates, grown for 20 h with the different treatments and then the cell culture medium was collected and the cells were scraped off in phosphate buffer saline (PBS). LDH leakage was estimated from the ratio between the LDH activities in the culture medium and the total activity, culture medium plus intracellular. Cellular ROS were quantified by the dichlorofluorescein assay using a microplate reader (Alía et al., 2006). Cells were seeded in 24-well plates (2×10^5 cells