Food Research International 71 (2015) 68–82

Contents lists available at ScienceDirect

Food Research International

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

Chemical characterization and chemo-protective activity of cranberry phenolic powders in a model cell culture. Response of the antioxidant defenses and regulation of signaling pathways

María Angeles Martín [a], Sonia Ramos [a], Raquel Mateos [a], Jannie P.J. Marais [b], Laura Bravo-Clemente [a], Christina Khoo [b], Luis Goya [a,*]

[a] Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, 28040 Madrid, Spain
[b] Ocean Spray Cranberries Inc., One Ocean Spray Drive, Lakeville–Middleboro, MA 02349, USA

ARTICLE INFO

Article history:
Received 22 December 2014
Received in revised form 19 February 2015
Accepted 28 February 2015
Available online 8 March 2015

Keywords:
Biomarkers of oxidative damage
HepG2 cells
Natural bioactive compounds
Plant antioxidants
Polyphenols
Oxidative stress

ABSTRACT

Oxidative stress and reactive oxygen species (ROS)-mediated cell damage are implicated in various chronic pathologies. Emerging studies show that polyphenols may act by increasing endogenous antioxidant defense potential. Cranberry has one of the highest polyphenol content among commonly consumed fruits. In this study, the hepato-protective activity of a cranberry juice (CJ) and cranberry extract (CE) powders against oxidative stress was screened using HepG2 cells, looking at ROS production, intracellular non-enzymatic and enzymatic antioxidant defenses by reduced glutathione concentration (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR) activity and lipid peroxidation biomarker malondialdehyde (MDA). Involvement of major protein kinase signaling pathways was also evaluated. Both powders in basal conditions did not affect cell viability but decreased ROS production and increased GPx activity, conditions that may place the cells in favorable conditions against oxidative stress. Powder pre-treatment of HepG2 cells for 20 h significantly reduced cell damage induced by 400 μM *tert*-butylhydroperoxide (t-BOOH) for 2 h. Both powders (5–50 μg/ml) reduced *t*-BOOH-induced increase of MDA by 20% (CJ) and 25% (CE), and significantly reduced over-activated GPx and GR. CE, with a significantly higher amount of polyphenols than CJ, prevented a reduction in GSH and significantly reduced ROS production. CJ reversed the *t*-BOOH-induced increase in phospho-c-Jun N-terminal kinase. This study demonstrates that cranberry polyphenols may help protect liver cells against oxidative insult by modulating GSH concentration, ROS and MDA generation, antioxidant enzyme activity and cell signaling pathways.

1. Introduction

Oxidative stress and reactive oxygen species (ROS)-mediated cell damage have been implicated in the development of various human chronic pathologies such as cardiovascular disease, certain cancers and a number of neurodegenerative diseases (Sohal, Mockett, & Orr, 2002). Epidemiological studies have related a diet rich in fruits and vegetables to the prevention of chronic degenerative diseases linked to oxidative stress (Cassidy, Huang, Rice, Rimm, & Tworoger, 2014; Ramos, 2008; Wedick et al., 2012).

Fruits, including berries, are one of the most important food sources of phenolic compounds in our diets (Caillet, Côté, Doyon, Sylvain, & Lacroix, 2011). The North American cranberry (*Vaccinium macrocarpon* Ait. Ericaceae) is of growing public interest as a functional food because of potential health benefits linked to the elevated content of bioactive phytochemicals in the fruit. In fact, cranberry ranks high among fruits in both antioxidant quality and quantity (Caillet et al., 2011; He & Liu, 2006; Vinson, Su, Zubik, & Bose, 2001) because of its substantial flavonoid and phenolic acid content. Cranberries, in powder, extract or juice, have been reported to be beneficial for the treatment of urinary tract infections (Blumberg et al., 2013; Pérez-López et al. 2009; Wang et al., 2012), obesity, insulin resistance and intestinal inflammation (Anhe et al., 2014), management of hyperglycemia (Da Silva Pinto, Ghaedian, Rahul Shinde, & Shetty, 2010; Kim, Kim, & Kwak, 2014), diabetes and hypertension (Apostolidis, Kwon, & Shetty, 2006), and hypercholesterolemia (Kim et al., 2014) and have shown antiproliferative activity in cancer cells (He & Liu, 2006; Seeram et al., 2006).

These and other health benefits, including reduced risks of cancer and cardiovascular disease, are believed to be due to the presence of various polyphenolic compounds, including flavonoids such as flavonols, flavanols (monomeric and polymeric) and anthocyanins, and phenolic acids such as coumaric and chlorogenic, as well as the synergistic effects among them (Chu & Liu, 2005; Heinonen, 2007; Seeram, Adams, Hardy, & Heber, 2004). In vitro evidence has shown the antioxidant potential of cranberry phenolic compounds (Borges, Degeneve, Mullen, & Crozier, 2010) while the antioxidant and chemo-protective

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

http://dx.doi.org/10.1016/j.foodres.2015.02.022
0963-9969/

properties of individual food flavonoids or polyphenolic extracts have been widely reported in cultured cells (Ramos, 2008), animal models (Mukhatar & Ahmad, 2000) and humans (Ahn et al., 2003; Bettuzzi, Rizzi, & Belloni, 2007). A recent publication also showed the bioavailability and bioactivity of cranberry phenolics from consuming cranberry juice (McKay, Chen, Zampariello, & Blumberg, 2015).

The protective effect of these compounds is related to their function in sequestering ROS and/or maintaining the cell components in their correct redox state, but emerging findings indicate that natural compounds may also act by increasing the endogenous antioxidant defense potential through regulation of cell signaling pathways (Martín et al., 2010a; Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005; Ramos, 2008). It is known that mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinase (PI3K/AKT) are enhanced in response to treatments with dietary compounds (Chen & Kong, 2004; Ramos, 2008). MAPKs family includes members such as extracellular-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK) and p38. These MAPKs and PI3K/AKT pathways are transducers of a multitude of extracellular stimuli by phosphorylating and activating downstream transcription factors enabling the cell to respond to stress by increasing or decreasing the expression of critical genes (Chen & Kong, 2004; Ramos, 2008). Several studies have shown that chlorogenic acid (Granado-Serrano et al., 2007), epicatechin (Granado-Serrano et al., 2007; Granado-Serrano et al., 2010) and quercetin (Granado-Serrano, Martín, Bravo, Goya, & Ramos, 2012) induce a varied set of antioxidant mechanisms in diverse organs or cultured cells through the regulation of protein kinases involved in cell signaling pathways.

The liver is particularly susceptible to toxic and oxidative abuse since the portal vein brings blood to this organ after intestinal absorption. The absorbed drugs and xenobiotics in a concentrated form can cause ROS- and free radical-mediated damage that may result in inflammatory and fibrotic stress (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006). Therefore, studies dealing with the effect of antioxidants at a physiological level in liver of live animals and at a cellular level in cultured hepatic cells are necessary. Human liver HepG2 is widely used for biochemical and nutritional studies as a cell culture model in human hepatocytes since they retain their morphology and most of their function in culture (Brandon et al., 2006; Goya, Martín, Ramos, Mateos, & Bravo, 2009). Previous cell culture studies have demonstrated that several flavonoids (Kanazawa, Uehara, Yanahitani, & Hashimoto, 2006) and hydroxycinnamic acids (Mateos, Goya, & Bravo, 2006) are absorbed and metabolized by HepG2 cells. These cells are able to express main antioxidant defense enzymes such as catalase, glutathione peroxidase (GPx), glutathione reductase (GR) on par with or better than that of primary human hepatocytes (Lee et al., 2002; Mersch-Sundermann, Knasmuller, Wu, Darroudi, & Kassie, 2004). Thus, in this study, HepG2 cells were utilized to screen for hepato-protective activity of two cranberry powders against an oxidative challenge induced by the potent oxidant *tert*-butyl hydroperoxide (*t*-BOOH). In addition, the involvement of PI3K/AKT and MAPK proteins in the protective response to oxidative stress in these cells was also investigated.

2. Methods and materials

2.1. Reagents

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). Benzoic acid, salicylic acid, protocatechuic acid, gallic acid, vanillic acid, *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, ellagic acid, quinic acid, galacturonic acid, catechin, epicatechin, quercetin, 4-dimethylaminocinnamaldehyde (DMAC) and Sephadex LH-20 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercitrin, hyperoside, myricetin, myricetrin, procyanidin A2, cyaniding-3-galactoside and peonidin-3-glucoside were purchased from Indofine Chemical Company, Inc. (Hillsborough Township, NJ, USA). Cyanidin-3-glucoside was purchased from ChromaDex (Irvine, CA, USA). Citric acid, malic acid, glucose and fructose were purchased from Fisher Scientific (Waltham, MA, USA). Anti-AKT and antiphospho-Ser473-AKT (p-AKT), anti-ERK1/2 and antiphospho-ERK1/2 (p-ERKs) recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK1/2, and antiphospho-JNK1/2 (p-JNKs) recognizing phosphorylated Thr183/Tyr185 of JNK1/2 and anti-ß-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Bradford reagent was from BioRad Laboratories (Madrid, Spain). All reagents were of analytical or chromatographic quality.

2.2. Cranberry powder preparation

Cranberry extracts were obtained from Ocean Spray Cranberries (MA, USA). Cranberry juice (CJ) powder was prepared from the juice of mature berries of the commonly cultivated cranberry plant (*Vaccinium macrocarpon*). Cranberry juice processing consists of the milling and pressing of the berries after a hot (50 °C for 1 h) commercial pectinase maceration. CJ was then prepared by spray drying cranberry concentrate with magnesium hydroxide as the carrier and tri-calcium phosphate as an anti-caking agent. The powder is fine, free-flowing and rosy red in color, and contains approximately 90% cranberry solids. The cranberry extract (CE) powder is a water-soluble, phenolic-rich extract of cranberry utilizing a proprietary resin separation process. CE was standardized to 55% proanthocyanidin (PAC) content on a dry weight basis as analyzed by the 4-dimethylaminocinnamaldehyde (DMAC) method. Finally, 10 mg/mL stock solutions of CJ and CE were prepared in distilled water for cell treatment.

2.3. Chemical characterization of cranberry powders

2.3.1. HPLC-MS/MS analysis of individual phenolics, flavonols and flavanols

Individual phenolic acids, flavonols and flavanols in the cranberry extracts were analyzed by HPLC-MS/MS (Borges et al., 2010) after the powders were dissolved in water (400 mg cranberry juice powder in 25 mL distilled water and 100 mg cranberry extract powder in 25 mL of distilled water). HPLC analyses were made with an Agilent 1260 infinity series LC system (Agilent technologies, Palo Alto, CA, USA) equipped with a binary pump, autosampler and coupled with a QTRAP 4000 mass spectrometer (AB Sciex, Framingham, MA, USA). Separation was carried out on a Phenomenex Kinetex 2.6 u C18, 100 Å 150 × 3.0 mm column (Torrance, CA, USA). Gradient elution was conducted, using: 0.1% formic acid/deionized water (phase A) and 0.1% formic acid/acetonitrile (phase B) at a constant flow of 0.5 mL/min using the following gradient: 0 – 4 min, isocratic of 5% B; 4 – 26 min, linear gradient from 5% B to 45% B; 26 – 30 min linear gradient from 45% B to 100% B; and 30 – 35 min, isocratic of 100% B. The injection volume was 15 μL and column temperature set at 40 °C. All the analyses were performed using the Turbo V ionization source in negative ion mode with the following settings: curtain gas (CUR) 25 (arbitrary units), collision gas (CAD) high, ion spray voltage (IS) −4000 V, ion source gas 1 (GS1) and 2 (GS2) 60 (arbitrary units). The drying gas (N2) was heated to 600 °C. The declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized for all the compounds using standard material (Table 1). Compounds were identified by comparing their retention time, MS and MS/MS fragmentation spectra with those obtained from pure standard solutions. Compounds were quantified by multi reaction monitor (MRM) experiments, considering one MS/MS transition for each compound.

Table 1
List of phenolic acids, flavonols and flavanols identified from cranberry juice powder and cranberry extract powder as well as MS/MS parameter to optimized MRM quantification conditions.

Peak	Compound	RT (min)	MS/MS parameters (scan type: MRM)				
			$[M-H]^-$ (Q1 mass)	Fragment ions (Q3 mass)	De-clustering potential (DP)	Collision energy (CE)	Collision cell exit potential (CXP)
1	Gallic acid	2.3	168.931	97.2	−75	−24	−15
				124.929	−75	−26	−19
2	Protocatechuic acid	4.1	152.601	91.052	−90	−36	−1
				108.781	−90	−22	−3
3	Catechin	9.6	288.930	108.818	−80	−36	−5
				122.924	−80	−42	−7
4	Vanillic acid	10.1	166.881	107.864	−55	−26	−5
				123.176	−55	−18	−9
5	Chlorogenic acid	10.5	352.997	84.972	−80	−54	−13
				190.922	−80	−22	−13
6	Caffeic acid	10.6	178.856	89.028	−80	−44	−1
				134.919	−80	−24	−7
7	Epicatechin	12.1	288.915	108.922	−85	−34	−15
				244.859	−85	−22	−13
8	p-Coumaric acid	13.4	162.845	116.951	−65	−40	−21
				119.002	−65	−20	−19
9	Benzoic acid	14.7	120.822	76.976	−50	−14	−17
				91.525	−50	−36	−1
10	Ferulic acid	14.8	192.829	133.932	−75	−22	−1
				177.932	−75	−20	−7
11	Salicylic acid	15.3	136.694	64.946	−45	−40	−7
				92.878	−45	−26	−11
12	Ellagic acid	15.4	300.892	116.756	−130	−60	−17
				144.894	−130	−52	−19
13	Myricetrin	15.5	463.132	270.821	−355	−56	−19
				315.900	−355	−36	−15
14	Hyperoside	15.8	463.017	242.915	−230	−74	−1
				300.027	−230	−40	−1
15	Quercitrin	17.2	446.904	254.996	−150	−58	−13
				300.505	−150	−30	−21
16	Myricetin	17.7	316.939	106.898	−105	−50	−15
				179.066	−105	−30	−1
17	t-Cinnamic acid	19.8	146.807	77.057	−45	−30	−11
				103.034	−45	−18	−1
18	Quercetin	20.4	301.012	151.013	−150	−30	−11
				178.881	−150	−26	−1

2.3.2. HPLC analysis of anthocyanins

HPLC analyses (Brown & Shipley, 2011) were made with an Agilent 1100 series LC system (Agilent technologies, Palo Alto, CA, USA) equipped with a binary pump, autosampler and a diode array detector (DAD). Separation was carried out with a Phenomenex Kinetex 2.6 u XB-C18, 100 Å 50 × 4.6 mm column (Torrance, CA, USA). Gradient elution was conducted, using 99:1, deionized water: 85% phosphoric acid (phase A) and 50:49:1, deionized water:acetonitrile:85% phosphoric acid (phase B) at a constant flow of 1.5 mL/min using the following gradient: 0 – 7.8 min, linear gradient from 12% B to 30% B; 7.8 – 11.4 min, linear gradient, from 30% B to 40% B; 11.4 – 13.2 min, linear gradient from 40% B to 70% B; and 13.2 – 14.2 min, isocratic of 70% B. The injection volume was 5 μL, column temperature set at 30 °C and data were collected at 520 nm (DAD). Cyanidin-3-galactoside, cyanidin-3-glucoside, and peonidin-3-glucoside were used as external standards and anthocyanins are quantified by the integration of the peak areas comparable to the external standards. Cyanidin-3-arabinoside is quantified using cyanidin-3-galactoside. Peonidin-3-galactoside and peonidin-3-arabinoside are quantified using peonidin-3-glucoside. Stock standard solutions of cyanidin-3-galactoside, cyanidin-3-glucoside, and peonidin-3-glucoside were prepared by dissolving 1 – 5 mg of individual compounds in a 5% phosphoric acid/water solution. The stock solutions were further diluted with the 5% phosphoric acid solution to a final concentration 20 – 40 μg/mL.

2.3.3. Determination of PAC content by two different methods

2.3.3.1. DMAC assay using the procyanidin A2 standard (BL-DMAC method). A Biotek Precision XS microplate (Biotek, Winooski, VT, USA) was used to dispense and dilute the blank solution, procyanidin A2 standard and samples into the wells of a 96-well plate, based on the previously published DMAC protocol (Prior et al., 2010). After addition of the DMAC solution to the 96-well plate, a Biotek Synergy 2 Multi-Mode Microplate Reader was used to record the absorbance at 640 nm over a time period of 35 min for each well. The maximum absorbance readings were used for the calculations, and the amount of PACs was calculated as milligrams (mg) of procyanidin A2 equivalents per gram (g) of powder.

2.3.3.2. DMAC assay using cranberry PAC standards. Sephadex LH-20 column separation followed by the absorbance measurement of the DMAC colorimetric reaction was used to determine PAC content in the cranberry powders (Cunningham, Vannozzi, O'Shea, & Turk, 2002). An aqueous sample was loaded onto pre-hydrated Sephadex LH-20 polyprep column. For a dry weight basis determination, 0.500–1.0 g of the sample was weighted and dispensed onto the LH-20 column. Distilled water (10 mL), then 25% EtOH (10 mL) solution was used to elute off the sugars, organic acids, anthocyanins, and flavonols. These eluents were discarded. The column was then washed sequentially with 70% acetone solution (2.5 mL + 2.5 mL) and combined eluent collected in a 15 mL tube. 1 mL of this eluent

was added to a DMAC reaction tube/cuvette. A reaction blank was also prepared by adding 1 mL of a 70% acetone solution to DMAC reaction tube/cuvette. Freshly prepared DMAC reagent (3 mL) was added to each DMAC reaction tube/cuvette (including reagent blank). These tubes were mixed by vortex and the absorbance was recorded after 5 min at 640 nm on a Hatch DR2500 spectrophotometer (Loveland, CO, USA). The blank solution was used to zero the spectrophotometer before the samples were recorded. When needed, the sample concentration was adjusted by dilution to ensure an optimum absorption of 0.1–1. The PAC concentrations were calculated by utilizing the recorded absorbance, and it was calculated on dry weight basis. The calculations for the powders were as follows:

$$\mu g\ PA/g\ DWB = ((ABS/RF) \times (EV/VA) \times (1/(W \times DF \times B))) / PR$$

ABS	Absorbance at 640 nm
RF	Response factor (0.0085 for CJ and 0.0079 for CE)
EV	Final elution volume (5 mL)
VA	Volume analyzed (1 mL)
W	Weight on column (≈0.5 g)
DF	Dilution factor
B	Percent dry weight (≈95%)
PR	Percent recovery (90%)

The response factor was calculated by the absorbance reading (1.13 cm path length) at 640 nm when the DMAC reagent reacted with a known concentration (1 μg/mL) of purified cranberry PAC solution. The recovery was calculated by the absorbance of the DMAC reaction from a purified PAC sample with and without the Sephadex LH-20 separation step. After the determination of the response factors, a known PAC solution was used as a quality control sample.

2.3.4. HPLC analysis of sugars

Samples were prepared by solubilizing 1 g of the cranberry powder in 50 mL of water. The mixtures were applied on Bio-Rex 5 anionic resin columns (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to separate the acids and phenolic compounds from the sugars. The resin columns were prepared by weighing 1 g of 100–200 mesh Bio-Rex 5 resin into plastic prep columns and pre-conditioned with 5 mL of distilled water. The cranberry samples (0.5 mL) were then absorbed onto the resin columns and eluted with deionized water (2 × 1 mL) to collect the sugars. HPLC analyses of the eluents were carried out on an Agilent 1100 series LC system (Agilent technologies, Palo Alto, CA, USA) equipped with a quaternary pump and autosampler. Separation was carried out on a Bio-Rad Aminex HPX-87C carbohydrate analysis column (Bio-Rad, CA, USA) and the sugars were measured with refractive index detection. Sugar standards comprising out of fructose, dextrose and sucrose (5, 20, 50 and 100 g/L, respectively) were used to calibrate the instrument and to quantify the samples.

2.3.5. Organic acids

Analyses were performed on a Dionex ICS-2100 Ion Chromatography system (Thermo Scientific, Sunnyvale, CA, USA) equipped with an EG40 Eluent Generator, an ASRS 300, 4 mm suppressor, and a CR-ATC. The separation of the organic acids was carried out on a RFIC™ IonPac® AS11-HC, Analytical, 4 × 250 mm ion exchange column, connected with a RFIC™ IonPac® AG11-HC, 4 × 50 mm guard column and measured by suppressed conductivity detection. A stock standard containing citric, isocitric, fumaric, galacturonic, malic, tartaric, and quinic acids is prepared in deionized water to a concentration of 0.5%. The stock standard is then diluted to prepare working standards of 50, 25, and 5 mg/L, which are used to calibrate the instrument.

2.3.6. Total phenolics by Folin–Ciocalteu method

This colorimetric assay, based on a redox reaction, was used to determine the total phenolic content in the cranberry powder samples (Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez, 2013). The Folin–Ciocalteu reagent (FCR) was purchased from Sigma Aldrich (St. Louis, MO, USA), and is measured as gallic acid equivalence (GAE).

2.4. Cell culture and treatment

Human HepG2 cells within passage number 85–95 were grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in DMEM F-12 medium from Biowhitaker (Cultek, Madrid, Spain), supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. Plates were changed to FBS-free medium the day before the assay. Two different types of experiments were designed for this study: A) experiment of plain treatment of cells with CJ and CE for 20 h to evaluate the direct effect of the extracts and B) experiment of pre-treatment of cells with CJ and CE for 20 h before submitting the cells to an oxidative stress by the potent pro-oxidant *t*-BOOH to evaluate a protective effect of the extracts against an oxidative insult. For the first experiment, direct effect by plain treatment with the samples, the different concentrations of CJ and CE (0.5, 1, 5 and 10 μg/mL) were dissolved in serum-free culture medium and added to the cell plates for 20 h; then the cells were collected and/or assayed. For the second experiment, to evaluate the protective effect of CJ and CE against pro-oxidant-induced toxicity, same concentrations as above of CJ and CE were diluted in serum-free culture medium and added to the cell plates for 20 h; then the medium was discarded and fresh medium containing 400 μM *t*-BOOH was added for 2 h. The concentrations of CJ and CE used in this study were selected according to the literature (see Discussion) and our previous studies with other plant or fruit extracts (León-González et al., 2012; Martín et al., 2010a; Tan et al., 2014).

2.5. Assay of cell viability by the lactate dehydrogenase (LDH) leakage assay

Cellular viability was estimated by the assay of LDH leakage from the cell (Alía, Ramos, Mateos, Bravo, & Goya, 2006). HepG2 cells were seeded at 10^6 cells/plate in 60 mm plates, treated as above and culture medium was collected and cells scraped in PBS. Cells were first sonicated to ensure breaking down the cell membrane to release the total amount of LDH; then, after centrifugation (1000 *g*, 15 min) to clear up the cell sample, 10 μL was placed into a well of a 96 multiwell for the assay. In the same manner, 10 μL of each culture medium was also deposited into a well of a 96-well multiwell. The LDH leakage percentage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content. LDH activity was determined by the disappearance of NADH used by the enzyme to reduce pyruvate to lactate.

2.6. Assay of ROS

In the experiment of direct treatment, cells cultured at 10^4 cells/plate in 24-well multiwell plates were treated for 20 h with the different concentrations of CJ and CE, dissolved in distilled water and then in serum-free culture medium, the DCFH-DA probe was added for 30 min, then they were washed twice with PBS before fluorescence was measured. In experiment of protection, the different concentrations of CJ and CE were added to the cell plates for 20 h, the DCFH-DA probe added for 30 min, then the cell plates were washed twice with PBS and new serum-free medium containing 400 μM *t*-BOOH was added to all cultures except controls for 2 h. Cellular ROS were quantified by the DCFH-DA assay using a microplate reader at excitation wavelength of 485 nm and emission wavelength of 530 nm (Alía et al., 2006). By quantifying fluorescence, a fair estimation of the overall oxygen species generated under the different conditions was obtained.

2.7. Assay of antioxidant defenses

2.7.1. Assay of non-enzymatic defense GSH

The content of GSH was quantified by a fluorometric assay (Alía et al., 2006). The method takes advantage of the reaction of GSH with OPT at pH 8.0. After the different treatments, the culture medium was removed and cells were detached and homogenized by ultrasound with 5% trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells for 30 min at 1000 *g*, 50 μL of the clear supernatant was transferred to a 96 multiwell plate for the assay. Fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The results of the samples were referred to those of a standard curve of GSH.

2.7.2. Assay of enzymatic defenses GPx and GR

Cells were collected in PBS and centrifuged at low speed (300 *g*) for 5 min to pellet. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at 3000 *g* for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity is based on the oxidation of GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance of NADPH by GR (Alía et al., 2006). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (Alía et al., 2006). Protein was measured by the Bradford reagent.

2.8. Assay of lipid peroxidation biomarker malondialdehyde (MDA)

HepG2 cells were seeded at 2×10^6 per plate and after the treatments suspended in PBS, pooled two plates in one and centrifuged at 300 *g* for 10 min to precipitate cells. Cellular MDA was analyzed in supernatants by high-performance liquid chromatography (HPLC) as its DNPH derivative. A stock solution of MDA obtained by hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) in sulphuric acid, was used as standard at the concentration range of 0.1–20 nmol/mL (Mateos, Goya, & Bravo, 2004). An Agilent 1100 Series HPLC-DAD was used and MDA values were expressed as nmol of MDA/mg protein. Total protein concentrations in the samples were estimated by the Bradford reagent.

2.9. Assay of signaling pathways

2.9.1. Preparation of cell lysates

To detect AKT, p-AKT, ERK1/2, p-ERKs, JNK1/2 and p-JNKs, cells were lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM 1,4-dithiothreitol (DTT), 0.1% Triton X-100, 200 mM ß-glycerolphosphate, 0.1 mM Na_3VO_4, 2.5 μg/mL leupeptin, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at −80 °C until used for Western blot analyses.

2.9.2. Protein determination by Western blotting

Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by ß-actin and bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

2.10. Statistics

Statistical analysis of data obtained from cell culture studies was performed as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. Means sharing the same letter are not significantly different from each other while means that have different letter are significantly dissimilar from each other. A SPSS version 21.0 program was used.

3. Results

3.1. Identification and quantification of polyphenols in cranberry powders

High levels of varied phenolic constituents were detected in the two cranberry powders. HPLC chromatograms of phenolic acid, flavonol and flavanol content of CJ and CE at 280 nm and MRM targeted analysis by LC/MS/MS quantification are shown in Fig. 1. Fig. 2 depicts HPLC chromatograms of the anthocyanin content from CJ powder (Fig. 2A) and CE powder (Fig. 2B) at 520 nm. Table 1 shows the list of compounds identified along with their retention time (RT) and UV characteristics of the chromatographic peaks, $[M - H]^-$ and their corresponding fragment ions of each polyphenol. The flavonols, flavanols and phenolic acids included in the LC/MS/MS method were elected based on compounds identified previously from cranberry and availability of commercial standards. Eighteen phenolic acids, flavonols and flavanols were detected and quantified in each powder (Table 2). Results indicated that CJ contained high amount of organic acids (with low antioxidant capacity) and low (but still significant) quantities of anthocyanins, phenolic acids, flavonols and flavanols (with high antioxidant potential). On the contrary, the amount of organic acids in CE was low to none existing but the quantity of highly bioactive compounds such as phenolic acids, flavonols and flavanols was much higher comparing to CJ. More specific, components with a high antioxidant capacity such as chlorogenic acid, epicatechin and quercetin were found in remarkable amounts in CE. Table 1 also includes the total phenolic content measured by the Folin–Ciocalteu reagent (FCR) and reported as gallic acid equivalence (GAE). The reagent does not only measure phenols, but will also react with any reducing substance and therefore values are different from the LC/MS/MS method.

3.2. Direct effect of CJ and CE

LDH leakage to the culture medium indicative of cell damage remained unaltered after treatment for 20 h with both CJ and CE powders (up to 50 μg/mL) (Fig. 3A). Since no harm in cell integrity was observed, it can be assumed that the range of concentrations finally selected (0.5–50 μg/mL) can be safely used to study the potential protective effect of CJ and CE against a condition of oxidative stress in cultured cells. HepG2 cells treated for 20 h (Fig. 3B) with 25–50 μg/mL CJ generated ROS levels that were clearly below those of control non-stressed cells, whereas those treated with CE showed a dose-dependent decrease of ROS production. Treatment of HepG2 cells with increasing concentrations of CJ for 20 h did not affect GSH concentration (Fig. 4A) whereas the same treatment with CE provoked a significant decrease in GSH levels (Fig. 4A). Cell treatment with all four concentrations of CJ and CE for 20 h resulted in a significant increase in the GPx activity (Fig. 4B) whereas no significant changes in GR activity could be observed after any treatment except for slight reduction with the highest dose of CE (Fig. 4C).

3.3. Protective effect of CJ and CE against a chemically-induced oxidative stress

The potential protective effect of CJ and CE against the *t*-BOOH-induced oxidative stress was initially evaluated by determining cell viability, as measured by LDH leakage. Fig. 5A shows that incubation of HepG2 cells with 400 μM *t*-BOOH for 2 h increased cell death up to 40%. However, pre-treatment for 20 h of HepG2 cultures with all the doses of CJ

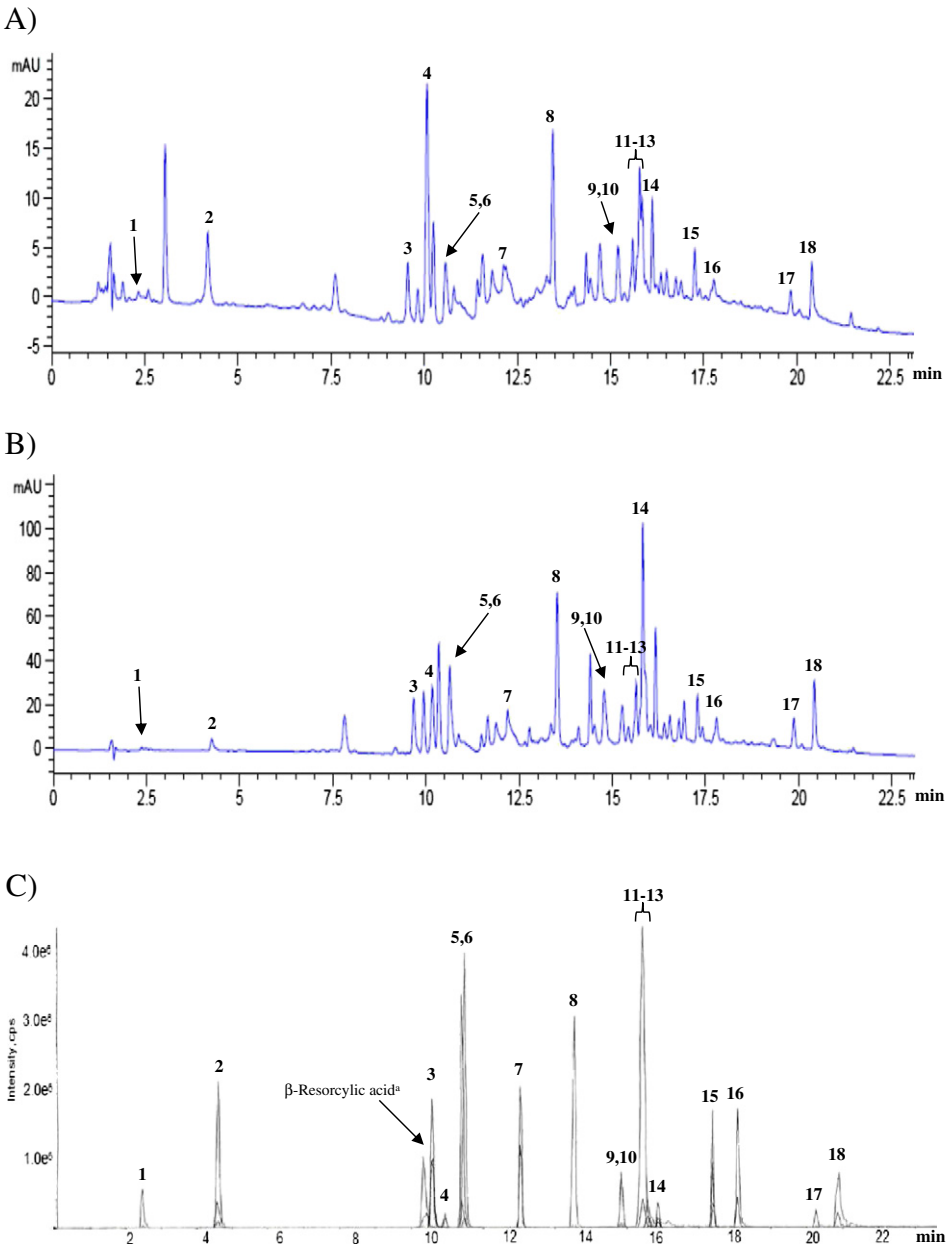

Fig 1. HPLC chromatograms of the phenolic acid, flavonol and flavanol content of CJ (A) and CE (B) powders both measured at 280 nm and MRM targeted analysis by LC/MS/MS quantification (C). Phenolic acids, flavonols and flavanols are tested, identified, and quantified by HPLC-tandem mass spectrometry (MS/MS), using multiple reaction monitoring (MRM) analysis (see number identification in Table 1). The phenolic compounds are quantified using the individual phenolic acids, flavonols and flavan-3-ols as standards. [a]β-Resorcylic acid was included as a standards in the MRM analysis, however was not detected in cranberry samples (see quantification in Table 2).

blunted the *t*-BOOH-induced raise of LDH leakage and completely prevented cell damage (Fig. 5A). Therefore, pre-treatment of HepG2 cells with the selected CJ concentrations offered a total protection against the *t*-BOOH insult. Pre-treatment of cells for 20 h with CE also significantly reduced the *t*-BOOH-induced cell damage to values that were close to those of control untreated cells (Fig. 5A), showing an attenuation of most of the cell damage induced by the pro-oxidant.

3.3.1. Redox status

Pre-treatment for 20 h of HepG2 cultures with 0.5–50 μg/mL CJ (Fig. 5B) did not evoke a significant reduction of ROS generated by *t*-BOOH. However, pre-treatment of cells for 20 h with 25–50 μg/mL CE evoked a significant protection against *t*-BOOH-induced ROS overproduction, whereas the lower doses (0.5–5 μg/mL) of CE showed no significant effect (Fig. 5B). These data suggest that high levels of ROS generated during the stress period are being more efficiently quenched in cells pre-treated with CE resulting in reduced cell damage.

As an index of the non-enzymatic antioxidant defense, the concentration of GSH was measured in HepG2 cells pre-treated with the four different concentrations of CJ and CE and exposed to *t*-BOOH. Fig. 6A shows that exposure of cells to *t*-BOOH evoked a dramatic decrease of GSH content (40%) as compared to that of the untreated control cells. Pre-treatment of HepG2 cells with the different concentrations of CJ during 20 h significantly attenuated the decrease of GSH levels induced by *t*-BOOH, but GSH values did not reach those of control untreated cells (Fig. 6A). However, treatment of cells for 20 h with CE completely prevented the GSH depletion induced by the incubation with *t*-BOOH, recovering GSH concentration to values similar to those of control untreated cells.

As an index of the enzymatic antioxidant defense, the activity of GPx and GR was tested in cells treated with CJ and CE followed by exposure

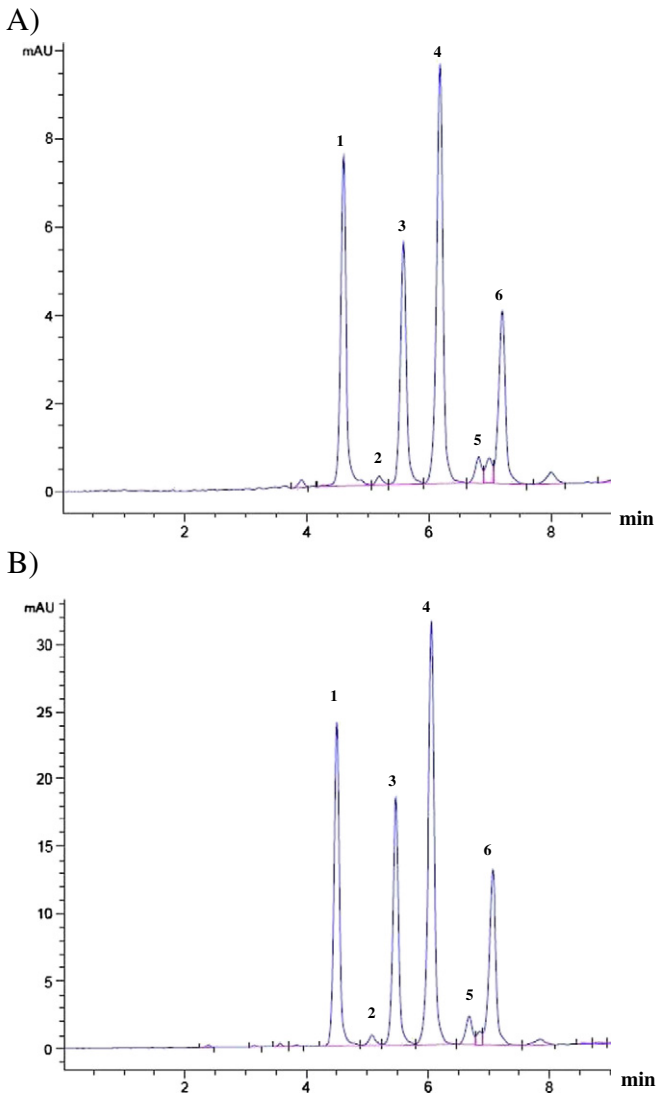

Fig 2. HPLC chromatograms of the anthocyanin content from CJ (A) and CE (B) powders at 520 nm. Anthocyanins identified and retention times in minutes: 1) cyanidin-3-galactoside (4.5); 2) cyanidin-3-glucoside (4.9); 3) cyanidin-3-arabinoside (5.5); 4) peonidin-3-galactoside (6.2); peonidin-3-glucoside (6.7); and 6) peonidin-3-arabinoside (7.1) (see quantification in Table 2).

Table 2
Content of phenolic compounds and other major components in cranberry juice and extract powders.

	Cranberry juice powder (mg/100 g)	Cranberry extract powder (mg/100 g)
Proanthocyanidins		
by OS-DMAC method[a]	2802.4	51,643.8
by BL-DMAC method[b]	652.7	14,987.2
Organic acids		
Citric acid	14,152.1	2854.6
Malic acid	8506.7	1663.7
Quinic acid	13,525.9	2037.5
Galacturonic acid	3305.0	78.4
Sugars		
Glucose	31,270	4700
Fructose	8120	1100
Antocyanins (520 nm)		
Cyanidin-3-arabinoside	32.8	420.0
Cyanidin-3-galactoside	40.7	507.9
Cyanidin-3-glucoside	1.3	25.4
Peonidin-3-arabinoside	17.3	238.8
Peonidin-3-galactoside	41.9	529.8
Peonidin-3-glucoside	3.0	36.6
Phenolic acids (LC/MS/MS)		
Benzoic acid	84.5	2545.1
Salicylic acid	0.6	13.4
Protocatechuic acid	15.6	120.5
Gallic acid	4.2	25.8
Vanillic acid	4.9	85.4
t-Cinnamic acid	7.7	72.8
p-Coumaric acid (4-hydroxycinnamic acid)	30.2	435.6
Caffeic acid (3,4-dihydroxycinnamic acid)	3.0	50.5
Ferulic acid (3-methoxy-4-hydroxycinnamic acid)	0.9	54.7
Chlorogenic acid	22.8	793.1
Ellagic acid	1.3	2.2
Flavan-3-ols (LC/MS/MS)		
Catechin	8.0	43.3
Epicatechin	31.7	635.9
Flavonols (LC/MS/MS)		
Quercetin	37.3	404.4
Quercitrin (quercetin-3-O-rhamnoside)	20.8	526.7
Hyperoside (quercetin-3-O-galactoside)	53.1	2720.9
Myricetin	40.5	657.3
Myricetrin (myricetin-3-O-rhamnoside	7.6	124.9
Total phenolic by Folin–Ciocalteu method (GAE)	2603.0	45,007.0

[a] Expressed in milligrams (mg) of cranberry specific PACs equivalents per 100 g of powder.
[b] Expressed in milligrams (mg) of procyanidin A-2 equivalents per 100 g of powder.

to *t*-BOOH. The presence of 400 μM *t*-BOOH in the culture medium for 2 h induced a significant increase in the enzyme activity of GPx (Fig. 6B) and GR (Fig. 6C) in response to ROS over-production. However, when cells were pre-treated for 20 h with CJ, the *t*-BOOH-induced increase in enzyme activity of GPx was significantly reduced and that of GR was completely suppressed by all concentrations. These results show a remarkable recovery of the augmented GPx and GR activity by the treatment with CJ. In the same line, cells pre-treated with any concentration of CE during 20 h averted most of the enhanced GPx activity and concentrations of 5–50 μg/mL CE also prevented the *t*-BOOH-induced increase in GR. Therefore, pre-treatment of cells with the selected concentrations of both cranberry extracts evoked a rapid recovery of the enhanced GPx and GR activities to basal values, placing cells in favorable conditions to face new oxidative insults. Since 5 μg/mL was the smallest dose of CJ and CE capable to recover all stress-induced changes, including GR, this concentration was selected for the remaining assays.

3.3.2. Biomarker of oxidative damage

The treatment of HepG2 cells with 400 μM *t*-BOOH during 2 h evoked a significant increase of over 100% in the cellular concentration of MDA, indicating oxidative damage to cell lipids (Fig. 7). Plain treatment of cells with 5 μg/mL CJ and CE did not affect MDA concentration, but pre-treatment for 20 h of HepG2 cells with 5 μg/mL of any of the powders significantly reduced the *t*-BOOH-induced increase of MDA by 20% (CJ) and 25% (CE), indicating a diminished degree of lipid peroxidation in response to *t*-BOOH in cells that had previously been in the presence of the cranberry powders (Fig. 7).

3.3.3. Regulation of signaling pathways by CJ and CE

No significant changes were observed in the expression of p-AKT, p-ERK1/2 and p-JNK when HepG2 cells were plain-treated with the two compounds CJ and CE (data not shown). Similarly, p-AKT and p-ERK1/2 were not affected by a treatment with 400 μM *t*-BOOH for 2 h, but this same treatment induced a significant increase in p-JNK (Fig. 8A). Subsequently, cells were submitted to a pre-incubation with 5 μg/mL CJ and CE for 20 h and then to a 2 h challenge with 400 μM *t*-BOOH to test for reversion of increased p-JNK. Indeed, pre-treatment with CJ completely reversed the *t*-BOOH-induced increase in p-JNK whereas no protection against enhanced p-JNK was observed in cells pre-treated with CE (Fig. 8B).

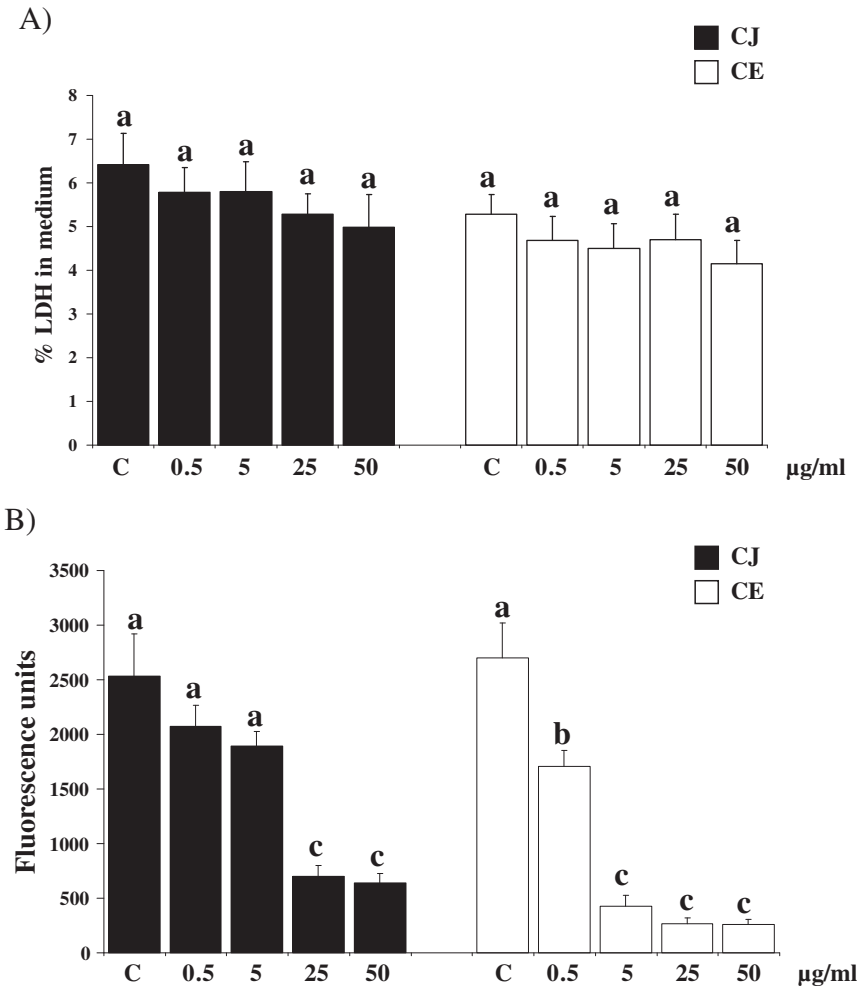

Fig 3. Direct effect of cranberry phenolic powders on cell viability and ROS generation. (A) LDH leakage is expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD ($n = 6–8$). (B) Fluorescence units corresponding to intracellular ROS production. Values are means ($n = 7–8$). Means without a common letter differ, $P < 0.05$.

4. Discussion

In this study we analyzed the phenolic composition of two different cranberry powders and evaluated their chemo-protective effect against a chemically-induced oxidative stress and the impact on different defense mechanisms. The results support the remarkable magnitude of the phenolic fraction in both powders, being higher in the plain cranberry extract (CE) than in the cranberry juice (CJ). The high amount of phenolic compounds in the CE powder compared to the CJ powder could be explained by the removal of simple sugars and organic acids and enrichment of phenolic compounds in the proprietary resin extraction process. In comparison, CJ was prepared by spray drying cranberry juice concentrate. This study also demonstrates that both powders may have the ability to protect human liver cells against an oxidative insult by modulating GSH concentration, ROS generation, MDA production, antioxidant enzymes activity, and, in the case of CJ, cell signaling pathways.

Cranberries are a particularly rich source of phenolic phytochemicals, including phenolic acids (benzoic, hydroxycinnamic, and ellagic acids) and flavonoids (anthocyanins, flavonols, and flavanols) (He & Liu, 2006; McKay & Blumberg, 2007; Vinson et al., 2001). The presence of these phytochemicals has been reported to be responsible for the cranberry's benefit of helping to reduce the incidence of certain diseases and infections, including cardiovascular diseases, various cancers, and infections involving the urinary tract, dental health, and *Helicobacter pylori*-induced stomach ulcers and cancers (Cote, Caillet, Doyon, Sylvain, & Lacroix, 2010; Kaspar & Khoo, 2013; McKay & Blumberg, 2007; Neto, 2007). In this study, the chemical characterization of both powders showed an expected similarity in the phenolic composition, with much higher quantities in the cranberry extract powder. Although the phenolic content of cranberries has been already reported, an exhaustive characterization of plant or fruit extract submitted to biological studies is mandatory due to purported differences in composition depending on the species, variety, localization and maturity. However, despite all these sources of disparity, values of most phenolic compounds shown in the present work are reasonably similar to those reported in previous analytical studies of cranberry (Borges et al., 2010; Brown & Shipley, 2011; He & Liu, 2006; Pappas & Schaich, 2009; Prior et al., 2010; Vinson et al., 2001; Zhang & Zuo, 2004). Additionally, in the present study the effect of a polyphenolic enrich cranberry powder was compared to that of a cranberry juice powder in order to determine whether the amount of phenolic compounds present in the latter would show similar health benefits.

Recent years have seen important breakthroughs in our understanding of the mechanisms through which these phenolic compounds exert their beneficial biological effects, yet these remain to be scientifically substantiated (Cote et al., 2010; Masella et al., 2005; Ramos, 2008). Cranberry constituents are likely to act by mechanisms that counteract oxidative stress, decrease inflammation, and modulate macromolecular interactions and expression of genes associated with disease processes (Masella et al., 2005; Neto, 2007; Ramos, 2008). Significant biological effects of cranberry extracts have been reported in cell culture, such as

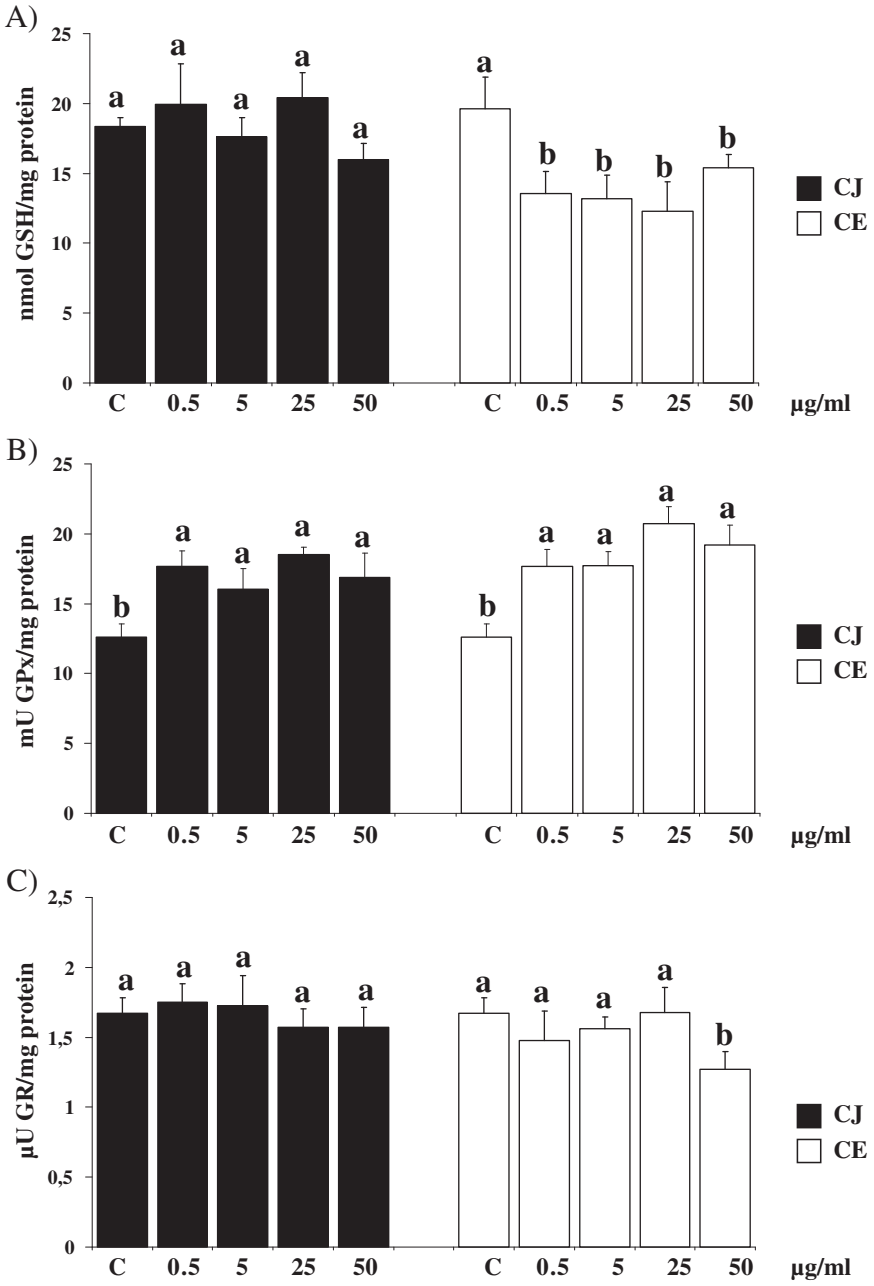

Fig 4. Direct effect of cranberry phenolic powders on antioxidant defenses. HepG2 were treated with the noted concentrations of CJ and CE for 20 h. Intracellular concentration of GSH (A) and enzyme activity of GPx (B) and GR (C) are expressed as noted units ± SD of 4–5 different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

inhibition of LDL oxidation and stimulation of LDL receptor expression in hepatocytes (Chu & Liu, 2005) as well as induction of cell cycle arrest and apoptosis in breast cancer cells (Sun & Liu, 2006). In addition, important physiological effects have been observed in animals and humans after administration of cranberries, such as decrease of cardiovascular disease risk factors and relevant cardio-protective properties (McKay & Blumberg, 2007). All these properties render cranberry polyphenolic fraction as an interesting candidate for cellular chemoprotection.

Since the liver is the main target for dietary antioxidants once absorbed from the gastrointestinal tract and the major place for xenobiotic metabolism, studies dealing with the effect of antioxidant dietary compounds at a physiological level in the liver of live animals and at a cellular level in cultured cells from liver origin should be encouraged (Goya et al., 2009). We have previously shown that a cocoa polyphenolic extract containing mainly flavanols (Martín et al., 2010a) as well as *Corema album* (a berry consumed along the Atlantic littoral of the Iberian Peninsula)(León-González et al., 2012) and green coffee (Baeza et al., 2014) extracts containing mostly hydroxycinnamic acids protect liver-derived cells from an oxidative stress by diminishing cell damage and returning the antioxidant defense system to a steady-state activity, which enables the cell to cope with further oxidative insults. The present study extends the protection of liver cells from oxidative stress to cranberry phenolic extracts containing high quantity of hydroxycinnamic acids, flavonoids and other antioxidant polyphenols. A potential limitation of the study is the use of native phenolic compounds present in the powders instead of their microbiota/liver metabolites. Current evidences indicate that flavonoid metabolites produced by colonic microbiota, especially those of phenolic acids (di- and mono-hydroxylated phenylpropionic, phenylacetic and benzoic acids), could possess

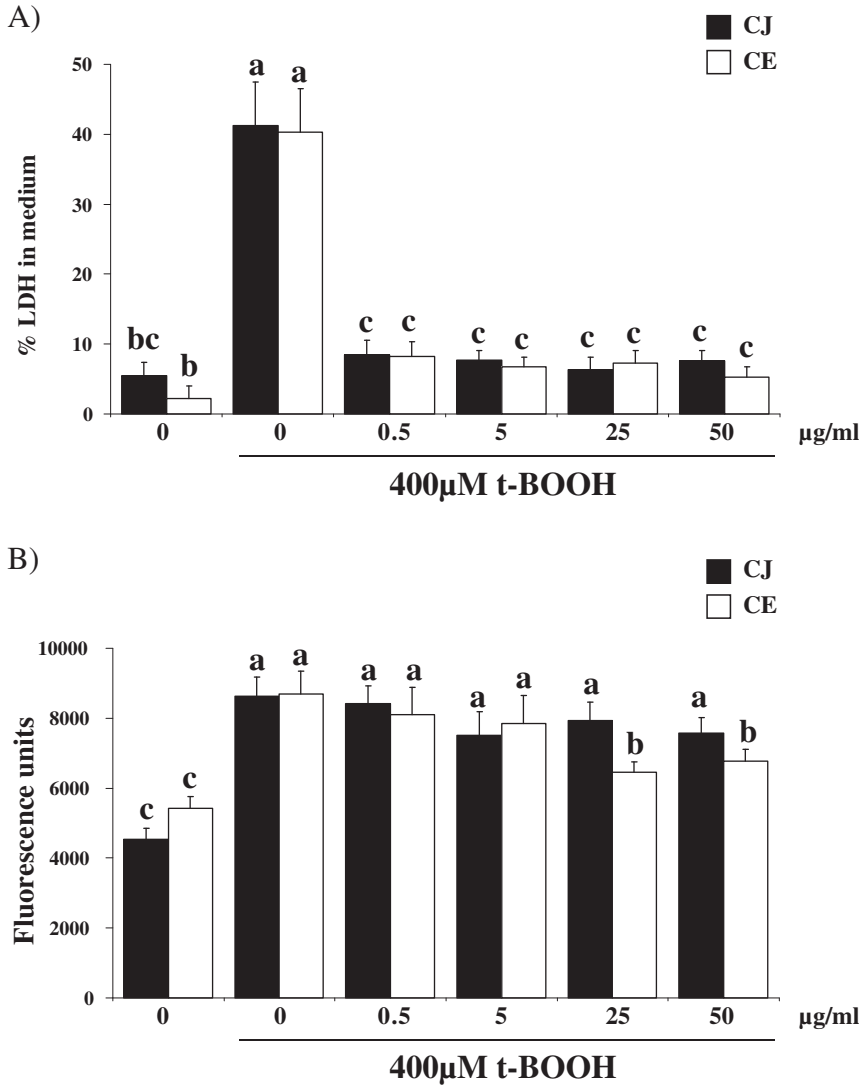

Fig 5. Protective effect of cranberry phenolic powders on cell viability and ROS generation. HepG2 were treated with the noted concentrations of extracts for 20 h, then the cultures were washed and 400 µM *t*-BOOH was added to all the cultures except controls for 2 h. Results of LDH leakage (A) are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular and intracellular. ROS production (B) was expressed as fluorescence units. Data are means ± SD ($n = 6–8$). Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

biological properties and therefore potential health beneficial effects (Fernández-Millán et al., 2014). These results support the hypothesis that not only food polyphenols but also their microbial metabolites must be taken into account when assessing the impact of polyphenols on health.

Although cranberry phenolics may have potent antioxidant effects in vitro and in vivo, elevated doses of these dietary compounds may also act as pro-oxidants in cell culture systems and provoke cellular damage (Azam, Hadi, Khan, & Hadi, 2004). Therefore, before testing the antioxidant effect, it is necessary to ensure that no direct damage is caused to the cell by the elevated concentrations; this is particularly relevant in the case of CE, with some phenolic components exceeding 20-fold the amount of those in CJ. Thus, cell integrity and redox status were first determined in cells treated with different concentrations of the two cranberry powders. The range of doses of the cranberry phenolic powders was selected according to the literature showing that concentrations in the sort of µg/mL have been found in plasma after cranberry juice intake containing 80 µg/mL polyphenols (Zhang & Zuo, 2004); besides, levels of 30–40 µM of cranberry phytochemicals have been detected in plasma after intake of cranberry juice (Pappas & Schaich, 2009; Pedersen et al., 2000). In order to evaluate the effect of cranberry phenolic extracts at the physiological level, the concentration range selected is not far from realistic, although higher concentrations in the order of mg/mL have been also used in other experimental designs (Chu & Liu, 2005). In line with this, the concentration range of CJ and CE (0.5–50 µg/mL) did not result in very significant differences among them and perhaps a higher concentration range of cranberry extracts could be better exploited in future studies.

Results testing both cranberry powders in basal conditions indicate that treatment for 20 h with the selected concentrations did not affect cell viability or integrity of HepG2. Furthermore, treatment with CJ and CE evoked a decrease in ROS production and increased GPx activity, conditions that ultimately placed the liver cells in favorable conditions to face an oxidative challenge. The decrease in GSH concentration observed in cells treated 20 h with CE may result from direct conjugation of some components to GSH, a fact previously reported for flavonoids such as catechin (Moridani, Scobie, Salehi, & O'Brien, 2001) and epigallocatechin-3-gallate (Galato, Lin, Sultan, & O'Brien, 2006, Lee, Kim, & Boo, 2008). This direct conjugation might be only relevant in the case of treatment with CE due to the larger amount of flavonoids in this powder. Then, once ascertain that treatment with the cranberry powders did not affect integrity of HepG2 cells, the response of the

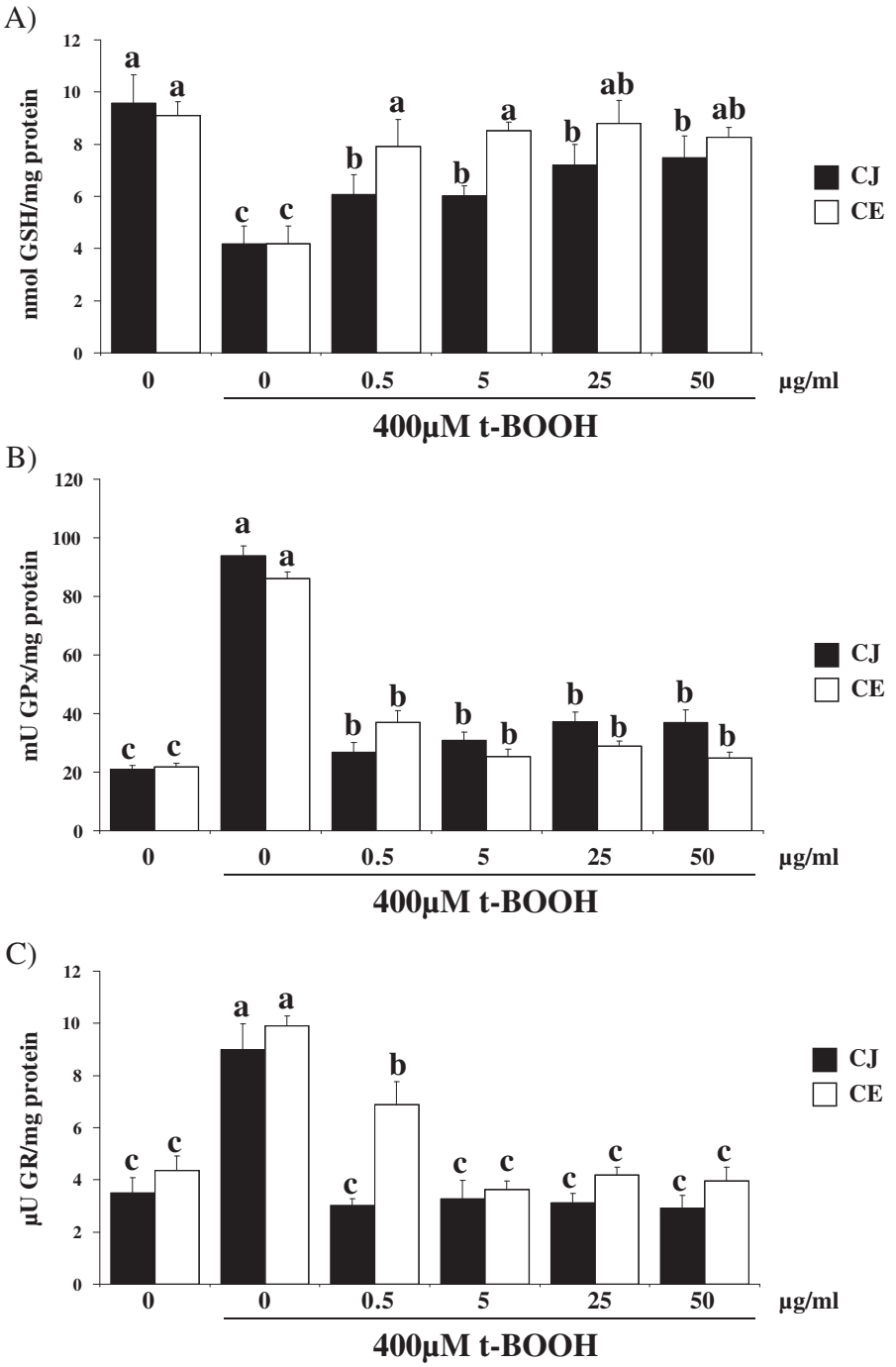

Fig 6. Protective effect of cranberry phenolic powders on the antioxidant defenses. GSH content (A) and activity of GPx (B) and GR (C) in HepG2 cells treated with the noted concentrations of the extracts for 20 h before the exposure to 400 µM *t*-BOOH for 2 h. Values are means ± SD ($n = 4–5$). Means without a common letter differ, $P < 0.05$.

cranberry extract-conditioned cells to the challenge induced by a potent pro-oxidant, was subsequently tested.

Our previous results demonstrated that treatment of HepG2 cells with *t*-BOOH was an excellent model of oxidative stress in cell culture (Alía et al., 2006; Goya et al., 2009). As other organic peroxides, *t*-BOOH can decompose to other alkoxyl and peroxyl radicals in a reaction aided by metal ions that can generate ROS, including H_2O_2 (Alía et al., 2006). In these stressful conditions, the complete inhibition of cyto-toxicity induced by *t*-BOOH when HepG2 cells was pre-treated with both cranberry phenolic powders for 20 h indicated that the integrity of the challenged cells was remarkably protected against the potent oxidative insult. The amount of the bioactive phenolics in CJ was effective enough for a complete cell protection and no further effects were observed with CE. Similar to what was reported with other phenolic extracts such as a cocoa (Martín et al., 2010a), *C. album* (León-González et al., 2012) and green coffee (Baeza et al., 2014), the *t*-BOOH-induced increase in ROS generation was to some extent restrained when cells were pre-treated with CJ and CE. CE prevented ROS overproduction more efficiently than CJ. In any case, this partial recovery of enhanced ROS suggested that the immense levels of ROS generated during the stress period were being moderately but significantly quenched by the antioxidant compounds in cells pre-treated with cranberry powders

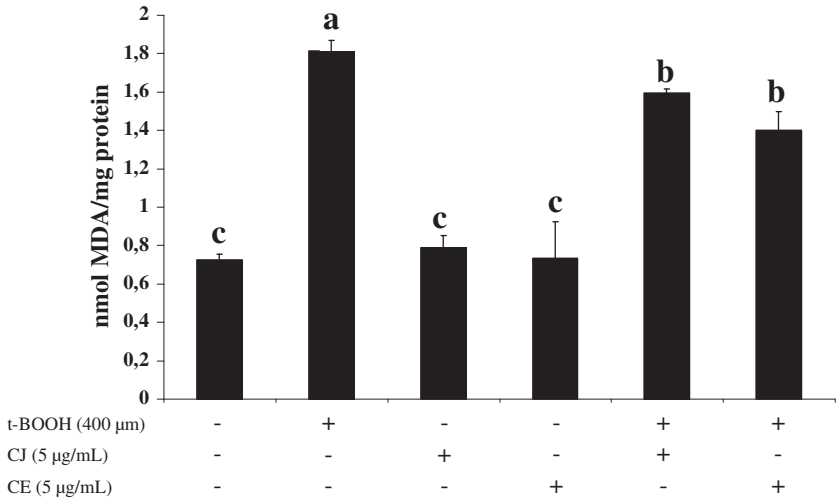

Fig 7. Protective effect of cranberry phenolic powders on oxidative stress biomarker. HepG2 were treated with 5 µg/mL of the cranberry extracts for 20 h, then the cultures were washed and 400 µM *t*-BOOH was added to all the cultures except controls for 2 h. Values of MDA are expressed as means ± SD of 4 different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

for 20 h. This ROS-quenching effect by the cranberry antioxidant phenolics could be a first explanation for the reduced oxidative stress and subsequent cell protection.

As the main non-enzymatic antioxidant defense within the cell, GSH depletion reflects intracellular oxidation (Goya et al., 2009). The critical decrease in the concentration of GSH induced by *t*-BOOH was partly (CJ) or completely (CE) prevented by pre-treatment for 20 h with the cranberry powders. A similar response had been reported for other polyphenol-rich extracts (Baeza et al., 2014; Lee et al., 2008; Martín et al., 2010a). The GSH recovery was more notable in the case of CE, since plain treatment with this extract provoked a decrease in steady-state levels of GSH. The higher amount of phenolics in CE may represent a redox challenge to the cell in basal conditions but may also strengthen the antioxidant defense in a stressful situation. Overall, the results of this parameter indicate that, despite the increased consumption of GSH in the enzymatic and non-enzymatic quenching of ROS generated by *t*-BOOH, pre-treatment with these two cranberry extracts prevented the lethal depletion of the intracellular GSH stock. Maintaining GSH concentration above a critical threshold while facing a stressful situation represents a crucial advantage for cell survival.

In addition to their antioxidant capacity by directly scavenging intracellular ROS, polyphenols have been recently shown to provide a parallel protection by modulating the activity of a number of protective enzymes (Martín et al., 2010a; Masella et al., 2005). GPx aids in eliminating peroxides (Lei, Cheng, & McClung, 2007) while GR regenerates oxidized glutathione (Argyrou & Blanchard, 2004). Thus, the enhancement of these glutathione-related enzymes plays a major role in helping the cells to overcome ROS production in the presence of *t*-BOOH (Alía et al., 2006). A rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will position the cell in a favorable condition to deal with a new insult (Goya et al., 2009). Accordingly, we have previously reported that realistic concentrations of a cocoa (Martín et al., 2010a), *C. album* (León-González et al., 2012) and green coffee (Baeza et al., 2014) extracts averted cell damage by preventing the permanently increased activity of GPx and GR induced by *t*-BOOH. In line with those results, in the present study we show for the first time that a treatment of human liver cells with realistic concentrations of cranberry extracts prevents the long-lasting increase in the activity of GPx and GR induced by oxidative stress. These results indicate that, at the end of an induced stress period the antioxidant defense system of the cells which had been pre-treated with cranberry phenolic powders had efficiently returned to a steady-state activity, diminishing cell damage and enabling the cells to cope in better conditions with further oxidative challenges.

MDA, a three-carbon compound formed by scission of peroxidized PUFAs, mainly arachidonic acid, is one of the main products of lipid peroxidation (Mateos et al., 2004). Since MDA has been found elevated in various diseases thought to be related to free radical damage, it has been widely used as proof of lipoperoxidation in biological and medical sciences (Mateos & Bravo, 2007). We have found that the *t*-BOOH-induced increase of MDA was partly reduced when cells were pre-treated for 20 h with 5 µg/mL of either cranberry powder. Therefore, the rapid recovery of the redox homeostasis evoked by the pre-treatment with cranberry powders would ensure a diminished lipid peroxidation and negligible cell damage. This protection by cranberry phenolic powders against an induced lipid peroxidation in cultured HepG2 cells is in line with previous studies that showed a similar effect by individual flavonols (Alía et al., 2006), flavanols (Granado-Serrano et al., 2007; Martín et al., 2010b) and hydroxycinnamic acids (Baeza et al., 2014; Granado-Serrano et al., 2007), all of them major components of both cranberry powders.

Oxidative stress-induced injury results not only from direct chemical interactions by altering cellular macromolecules including DNA, proteins and lipids, but also from profound alterations in signal transduction pathways (Ramos, 2008; Singh & Czaja, 2007). Signaling cascades involving the MAPK-JNKs pathways are key mediators of stress signals and seem to be mainly responsible for protective responses and stress-dependent apoptosis reactions (Ramos, 2008; Singh & Czaja, 2007). In this regard, ROS generation has been described as a critical upstream activator of JNKs, and the persisted activation of JNKs has been directly involved in the development of apoptosis in hepatocytes and nonhepatic cells (Singh & Czaja, 2007). In a previous study we have shown that *t*-BOOH was able to induce an increase in the phosphorylated levels of JNKs proteins in HepG2 cells and that these events occurred after increased ROS generation and prior to apoptotic activation and cell death (Martín et al., 2010a). Therefore, p-JNK activation seems to be involved in ROS mediated apoptosis in HepG2 cells. In that same study, pre-treatment of cells with a cocoa phenolic extract rich in flavonoids attenuated the generation of intracellular ROS induced by *t*-BOOH and prevented the increase of p-JNKs levels that preceded cell apoptosis and death. In line with those results, pre-treatment with CJ was able to prevent lethal p-JNKs activation induced by *t*-BOOH and to protect cells from its cytotoxic effect. However, CE treatment could not avert p-JNK activation induced by *t*-BOOH, indicating that other mechanism,

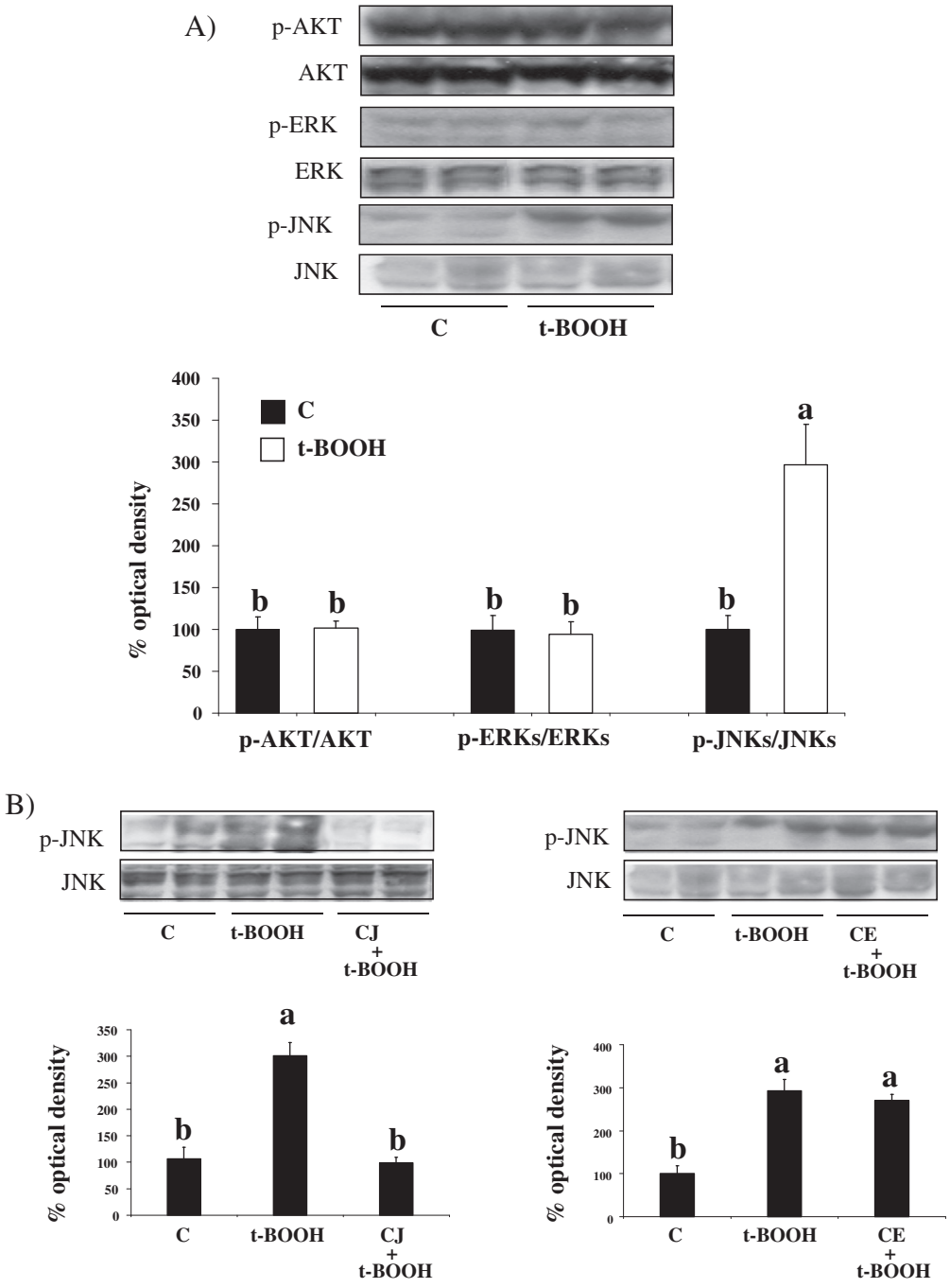

Fig 8. Effect of *t*-BOOH on AKT, ERKs and JNKs and protective effect of cranberry phenolic powders on p-JNK protein levels. (A) HepG2 cells were treated with 400 μM *t*-BOOH for 2 h and cell homogenates were assayed for total and phosphorylated AKT, ERKs and JNK by western blot. (B) HepG2 cells were treated with 5 μg/mL CJ and CE for 20 h, then the cultures were washed and 400 μM *t*-BOOH was added to all plates except controls for 2 h and JNK and p-JNK were assayed by western blot. Representative blots are depicted in the upper side of both panels and densitometric results from 3 different assays are shown in the lower side. Different letters upon symbols indicate statistically different data ($P < 0.05$).

such as redox regulation, might be primary in the protection from oxidative stress by CE in HepG2 cells.

5. Conclusions

The overall results seem to indicate that CJ has a lower antioxidant capacity than CE but it is capable to regulate protein signaling pathways at molecular level in order to reduce cell damage, whereas CE shows a higher capacity than CJ to reduce ROS generation and recover GSH concentration in order to protect cell viability in conditions of oxidative stress. In summary, our results demonstrate that the phenolic-rich powders obtained from cranberry have the ability to protect human HepG2 cells against an oxidative challenge by different mechanisms such as reducing free radical activity, enhancing antioxidant defenses and regulating protein signaling pathways. Therefore, phenolic-rich cranberry powders could be used as bioactive ingredients in the production and development of functional foods having the added value of contributing to the protection against oxidative stress related diseases.

Disclosure information

J.M. and C.K. are employees of Ocean Spray Cranberries Inc.

Acknowledgments

This study was funded by Ocean Spray Cranberries. J.M. and C.K. are employees of Ocean Spray Cranberries Inc.

References

Ahn, W. S., Yoo, J., Huh, S. W., Kim, C. K., Lee, J. M., Namkoong, S. E., et al. (2003). Protective effects of green tea extracts (polyphenon E and EGCG) on human cervical lesions. *European Journal of Cancer Prevention*, *12*, 383–390.

Alía, M., Ramos, S., Mateos, R., Bravo, L., & Goya, L. (2006). Quercetin protects human hepatoma cell line (HepG2) against oxidative stress induced by tertbutyl hydroperoxide. *Toxicology and Applied Pharmacology*, *212*, 110–118.

Anhe, F. F., Roy, D., Pilon, G., Dudonne, S., Matamoros, S., Varin, T. V., et al. (2014). A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. Population in the gut microbiota of mice. *Gut* (DOI: gutjnl-2014-307142) [Epub ahead of print].

Apostolidis, E., Kwon, Y. -I., & Shetty, K. (2006). Potential of cranberry-based herbal synergies for diabetes and hypertension management. *Asia Pacific Journal of Clinical Nutrition*, *15*, 433–441.

Argyrou, A., & Blanchard, J. S. (2004). Flavoprotein disulfide reductases: Advances in chemistry and function. *Progress in Nucleic Acid Research and Molecular Biology*, *78*, 89–142.

Azam, S., Hadi, N., Khan, N. U., & Hadi, S. M. (2004). Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: Implications for anticancer properties. *Toxicology in Vitro*, *18*, 555–561.

Baeza, G., Amigo-Benavent, M., Sarriá, B., Goya, L., Mateos, R., & Bravo, L. (2014). Green coffee hydroxycinnamic acids but not caffeine protect human HepG2 cells against oxidative stress. *Food Research International*, *62*, 1038–1046.

Bettuzzi, S., Rizzi, F., & Belloni, L. (2007). Clinical relevance of the inhibitory effect of green tea catechins (GtCs) on prostate cancer progression in combination with molecular profiling of catechin-resistant tumors: An integrated view. *Polish Journal of Veterinary Sciences*, *10*, 57–60.

Blumberg, J. B., Camesano, T. A., Cassidy, A., Kris-Etherton, P., Howell, A., Manach, C., et al. (2013). Cranberries and their bioactive constituents in human health. *Advances in Nutrition*, *4*, 618–632.

Borges, G., Degeneve, A., Mullen, W., & Crozier, A. (2010). Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. *Journal of Agricultural and Food Chemistry*, *58*, 3901–3909.

Brandon, E. F., Bosch, T. M., Deenen, M. J., Levink, R., van der Wal, E., van Meerveld, J. B., et al. (2006). Validation of in vitro cell models used in drug metabolism and transport studies: Genotyping of cytochrome P450, phase II enzymes and drug transporters polymorphisms in the human hepatoma (HepG2), ovarian carcinoma (IGROV-1) and colon carcinoma (CaCo-2, LS180) cell lines. *Toxicology and Applied Pharmacology*, *211*, 1–10.

Brown, P. N., & Shipley, P. R. (2011). Determination of anthocyanins in cranberry fruit and cranberry fruit products by high-performance liquid chromatography with ultraviolet detection: Single-laboratory validation. *Journal of AOAC International*, *94*, 459–466.

Caillet, S., Côté, J., Doyon, G., Sylvain, J. -F., & Lacroix, M. (2011). Antioxidant and antiradical properties of cranberry juice and extracts. *Food Research International*, *44*, 1408–1413.

Cassidy, A., Huang, T., Rice, M. S., Rimm, E. B., & Tworoger, S. S. (2014). Intake of dietary flavonoids and risk of epithelial ovarian cancer. *American Journal of Clinical Nutrition*, *100*, 1344–1351.

Chen, C., & Kong, A. N. (2004). Dietary cancer-chemopreventive compounds: From signaling and gene expression to pharmacological effects. *Free Radical Biology and Medicine*, *36*, 1505–1516.

Chu, Y. -F., & Liu, R. H. (2005). Cranberries inhibit LDL oxidation and induce LDL receptor expression in hepatocytes. *Life Sciences*, *77*, 1892–1901.

Cote, J., Caillet, S., Doyon, G., Sylvain, J. F., & Lacroix, M. (2010). Bioactive compounds in cranberries and their biological properties. *Critical Reviews in Food Science and Nutrition*, *50*, 666–679.

Cunningham, D. G., Vannozzi, S., O'Shea, E., & Turk, R. (2002). In C. -T. Ho, & Q. Y. Zheng (Eds.), *Quality Management of Nutraceuticals ACS Symposium series 803, Washington DC*.

Da Silva Pinto, M., Ghaedian, R., Rahul Shinde, R., & Shetty, K. (2010). Potential of cranberry powder for management of hyperglycemia using *in vitro* models. *Journal of Medicinal Food*, *13*, 1036–1044.

Fernández-Millán, E., Ramos, S., Alvarez, C., Bravo, L., Goya, L., & Martín, M. A. (2014). Microbial phenolic metabolites improve glucose-stimulated insulin secretion and protect pancreatic beta cells against oxidative stress via ERKs and PKC pathways. *Food and Chemical Toxicology*, *66*, 245–253.

Galato, G., Lin, A., Sultan, A. M., & O'Brien, P. J. (2006). Cellular and in vivo hepatotoxicity caused by green tea phenolic acids and catechins. *Free Radical Biology and Medicine*, *40*, 570–580.

Goya, L., Martín, M. A., Ramos, S., Mateos, R., & Bravo, L. (2009). A cell culture model for the assessment of the chemopreventive potential of antioxidant compounds. *Current Nutrition and Food Science*, *5*, 56–64.

Granado-Serrano, A. B., Martín, M. A., Bravo, L., Goya, L., & Ramos, S. (2012). Quercetin modulates Nrf2 and glutathione-related defences in HepG2 cells. Involvement of p38. *Chemical-Biological Interactions*, *195*, 154–164.

Granado-Serrano, A. B., Martín, M. A., Haegeman, G., Goya, L., Bravo, L., & Ramos, S. (2010). Epicatechin induces NF-κB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related (Nrf2) via phosphatidylinositol-3-kiinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signaling in HepG2 cells. *British Journal of Nutrition*, *103*, 168–179.

Granado-Serrano, A. B., Martín, M. A., Izquierdo-Pulido, M., Goya, L., Bravo, L., & Ramos, S. (2007). Molecular mechanisms of (−)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line (HepG2). *Journal of Agricultural and Food Chemistry*, *55*, 2020–2027.

He, X., & Liu, R. H. (2006). Cranberry phytochemicals: Isolation, structure elucidation, and their antiproliferative and antioxidant activities. *Journal of Agricultural and Food Chemistry*, *54*, 7069–7074.

Heinonen, M. (2007). Antioxidant activity and antimicrobial effect of berry phenolics—A Finnish perspective. *Molecular Nutrition and Food Research*, *51*, 684–691.

Kanazawa, K., Uehara, M., Yanahitani, H., & Hashimoto, T. (2006). Bioavailable flavonoids to suppress the formation of 8-OHdG in HepG2 cells. *Archives of Biochemistry and Biophysics*, *455*, 197–203.

Kaspar, K. L., & Khoo, C. (2013). Cranberry polyphenols in the promotion of urinary tract, cardiovascular and emerging health areas. In M. Skinner, & D. Hunter (Eds.), *Bioactives in fruit: Health benefits and functional foods* (pp. 273–292). New York: Wiley Blackwell.

Kim, M. J., Kim, J. H., & Kwak, H. K. (2014). Antioxidant effects of cranberry powder in lipopolysaccharide treated hypercholesterolemic rats. *Preventive Nutrition and Food Science*, *19*, 75–81.

Lee, S. I., Kim, H. J., & Boo, Y. C. (2008). Effect of green tea and (−)-epicatechin gallate on ethanol-induced toxicity on HepG2 cells. *Phytotherapy Research*, *22*, 669–674.

Lee, Y. Y., Kim, H. G., Jung, H. I., Shin, Y. H., Hong, S. M., Park, E. H., et al. (2002). Activities of antioxidant and redox enzymes in human normal hepatic and hepatoma cell lines. *Molecular Cells*, *14*, 305–311.

Lei, X. G., Cheng, W. -H., & McClung, J. P. (2007). Metabolic regulation and function of glutathione peroxidase-1. *Annual Review of Nutrition*, *27*, 41–61.

León-González, A., Mateos, R., Ramos, S., Martín, M. A., Sarriá, B., Martín-Cordero, C., et al. (2012). Chemo-protective activity and characterization of phenolic extracts from *Corema album*. *Food Research International*, *49*, 728–738.

Lima, C. F., Fernandes-Ferreira, M., & Pereira-Wilson, C. (2006). Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life Sciences*, *79*, 2056–2068.

Martín, M. A., Granado-Serrano, A. B., Ramos, S., Izquierdo-Pulido, M., Bravo, L., & Goya, L. (2010a). Cocoa flavonoids up-regulate antioxidant enzymes activity via ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *Journal of Nutritional Biochemistry*, *21*, 196–205.

Martín, M. A., Ramos, S., Mateos, R., Izquierdo-Pulido, M., Bravo, L., & Goya, L. (2010b). Protection of human HepG2 cells against oxidative stress induced by the flavonoid epicatechin. *Phytotherapy Research*, *24*, 503–509.

Masella, R., Di Benedetto, R., Vari, R., Filesi, C., & Giovannini, C. (2005). Novel mechanisms of natural antioxidant compounds in biological systems: Involvement of glutathione and glutathione-related enzymes. *Journal of Nutritional Biochemistry*, *16*, 577–586.

Mateos, R., & Bravo, L. (2007). Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). *Journal of Separation Science*, *30*, 175–191.

Mateos, R., Goya, L., & Bravo, L. (2004). Determination of malondialdehyde (MDA) by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. A marker for oxidative stress in cell cultures of human hepatoma HepG2. *Journal of Chromatography B*, *805*, 33–39.

Mateos, R., Goya, L., & Bravo, L. (2006). Uptake and metabolism of hydroxycinnamic acids (chlorogenic, caffeic, and ferulic acids) by HepG2 cells as a model of the human liver. *Journal of Agricultural and Food Chemistry*, *54*, 8724–8732.

McKay, D. L., & Blumberg, J. B. (2007). Cranberries (*Vaccinium macrocarpum*) and cardiovascular disease risk factors. *Nutrition Reviews*, *65*, 490–502.

McKay, D. L., Chen, C. Y., Zampariello, C. A., & Blumberg, J. B. (2015). Flavonoids and phenolic acids from cranberry juice are bioavailable and bioactive in healthy older adults. *Food Chemistry*, *168*, 233–240.

Mersch-Sundermann, V., Knasmuller, S., Wu, X. J., Darroudi, F., & Kassie, F. (2004). Use of a human-derived liver cell line for the detection of cytoprotective, antigenotoxic and cogenotoxic agents. *Toxicology*, *198*, 329–340.

Moridani, M. Y., Scobie, H., Salehi, P., & O'Brien, P. J. (2001). Catechin metabolism: Glutathione conjugate formation catalyzed by tyrosinase, peroxidase, and cytochrome p450. *Chemical Research in Toxicology*, *14*, 841–848.

Mukhatar, H., & Ahmad, N. (2000). Tea polyphenols: Prevention of cancer and optimizing health. *American Journal of Clinical Nutrition*, *71*, 1698S–1702S.

Neto, C. C. (2007). Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition and Food Research*, *51*, 652–664.

Pappas, E., & Schaich, K. M. (2009). Phytochemicals of cranberries and cranberry products: characterization, potential health effects and processing stability. *Critical Reviews in Food Science and Nutrition*, *49*, 741–781.

Pedersen, C. B., Kyle, J., Jenkinson, A. M., Gardner, P. T., PcPhail, D. B., & Duthie, G. G. (2000). Effects of cranberry and blueberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. *European Journal of Clinical Nutrition*, *54*, 405–408.

Pérez-López, F. R., Haya, J., & Chedraui, P. (2009). Vaccinium macrocarpon: An interesting option for women with recurrent urinary tract infections and other health benefits. *Journal of Obstetrics and Gynaecology Research*, *35*, 630–639.

Prior, R. L., Fan, E., Ji, H., Howell, A., Nio, C., Payne, M. J., et al. (2010). Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture*, *90*, 1473–1478.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and signaling pathways. *Molecular Nutrition and Food Research*, *52*, 507–526.

Sánchez-Rangel, J. C., Benavides, J., Heredia, J. B., Cisneros-Zevallos, L., & Jacobo-Velázquez, D. A. (2013). The Folin–Ciocalteu assay revisited: Improvement of its specificity for total phenolic content determination. *Analytical Methods*, *5*, 5990–5999.

Seeram, N. P., Adams, L. S., Hardy, M. L., & Heber, D. (2004). Total cranberry extract versus its phytochemical constituents: Antiproliferative and synergistic effects against human tumor cell lines. *Journal of Agricultural and Food Chemistry*, *52*, 2512–2517.

Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., et al. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *Journal of Agricultural and Food Chemistry*, *54*, 9329–9339.

Singh, R., & Czaja, M. J. (2007). Regulation of hepatocyte apoptosis by oxidative stress. *Gastroenterology and Hepatology*, *1*, S45–S48.

Sohal, R. S., Mockett, R. J., & Orr, W. C. (2002). Mechanisms of aging. An appraisal of the oxidative stress hypothesis. *Free Radical Biology and Medicine*, *33*, 575–586.

Sun, J., & Liu, R. H. (2006). Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Letters*, *241*, 124–134.

Tan, S. -A., Goya, L., Ramanathan, S., Sulaiman, S. F., Alam, M., Navaratnam, V., et al. (2014). Chemopreventive effects of standardized papaya leaf fraction on oxidatively stressed human liver cells. *Food Research International*, *64*, 387–395.

Vinson, J. A., Su, X., Zubik, L., & Bose, P. (2001). Phenol antioxidant quantity and quality in foods: Fruits. *Journal of Agricultural and Food Chemistry*, *49*, 5315–5321.

Wang, C. H., Fang, C. C., Chen, N. C., Liu, S. S., Yu, P. H., Wu, T. Y., et al. (2012). Cranberry-containing products for prevention of urinary tract infections in susceptible populations: A systematic review and meta-analysis of randomized controlled trials. *Archives of Internal Medicine*, *172*, 988–996.

Wedick, N. M., Pan, A., Cassidy, A., Rimm, E. B., Sampson, L., Rosner, B., et al. (2012). Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. *American Journal of Clinical Nutrition*, *95*, 925–933.

Zhang, K., & Zuo, Y. (2004). GC-MS determination of flavonoids and phenolic and benzoic acids in human plasma after consumption of cranberry juice. *Journal of Agricultural and Food Chemistry*, *52*, 222–227.