TITLE: Antioxidant and genoprotective effects of spent coffee extracts in human cells

AUTHORS: Jimena Bravo[a], Leire Arbillaga[b], M. Paz de Peña[a*], Concepcion Cid[a]

[a]Department of Nutrition, Food Science and Physiology, School of Pharmacy, University of Navarra, E-31008-Pamplona, Spain.

[b]Department of Pharmacology and Toxicology, School of Pharmacy, University of Navarra, E-31008-Pamplona, Spain.

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*Corresponding author:

M. Paz De Peña

Department of Nutrition, Food Science and Physiology

School of Pharmacy, University of Navarra

C/ Irunlarrea 1

E-31080-Pamplona, Spain

Tel: +34 948 425600 (806580)

Fax: +34 948 425740

E-mail address: mpdepena@unav.es

Abbreviations:

8-oxoguanine, 8-oxoGua; **DAPI,** 4,6-diamidino-2-phenylindole; **FPG,** formamidopyrimidine DNA glycosylase; **H_2DCF-DA,** 2′, 7′-dichlorfluorescein-diacetate; **H_2O_2,** hydrogen peroxide; **MTT,** (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide); **ROS,** reactive oxygen species; **SBs,** strand breaks.

Abstract

Spent coffee has been shown as a good source of hydrophilic antioxidant compounds. The ability of two spent coffee extracts rich in caffeoylquinic acids, mainly dicaffeoylquinic acids, and caffeine (Arabica filter and Robusta espresso) to protect against oxidation and DNA damage in human cells (HeLa) was evaluated at short (2 h) and long (24 h) exposure times. Cell viability (MTT) was not affected by spent coffee extracts (>80%) up to 1000 μg/mL after 2h. Both spent coffee extracts significantly reduced the increase of ROS level and DNA strand breaks (29-73% protection by comet assay) induced by H_2O_2. Pretreatment of cells with robusta spent coffee extract also decreased Ro photosensitizer-induced oxidative DNA damage after 24 h exposure. The higher effectiveness of Robusta spent coffee extract, with less caffeoylquinic acids and melanoidins, might be due to other antioxidant compounds, such as caffeine and other Maillard Reaction Products. This work evidences the potential antioxidant and genoprotective properties of spent coffee in human cells.

Keywords:

Coffee; Spent coffee; Antioxidant; Antigenotoxicity; DNA damage; Comet assay

1. Introduction

Global health policies promote increase consumption of plant foods, such as fruits and vegetables to contribute to the prevention of several chronic diseases related with oxidative stress, such as cancer, diabetes, cardiovascular and neurodegenerative diseases (World Health Organization, 2004). Plant foods, including beverages and extracts, are rich sources of bioactive compounds like antioxidants. A possible mechanism of antioxidants action in the prevention of chronic diseases is the decrease of oxidative stress, a condition that appears when an imbalance between the production of free radicals and antioxidant defences occurs. Free radicals, such as reactive oxygen species (ROS), damage macromolecules as proteins, lipids and DNA. DNA damage by ROS can contribute to the formation of single and double strand breaks (SBs), as well as to the oxidation of purine and pyrimidine bases, leading to genome instability and subsequent potential cancer development (Chobotova, 2009)**.** The formation of 8-oxoguanine (8-oxoGua) by ROS oxidation of guanine is one of the most common DNA lesions. This is a potential biomarker of carcinogenesis because it is relatively easily formed and is mutagenic (Valko et al., 2007). The comet assay is one of the most useful approaches for the quantification of this oxidative DNA damage (SBs and 8-oxoGua). For example, several authors have reported, employing the comet assay, that supplementation of the diet with food antioxidants decreases endogenous oxidative DNA damage in human lymphocytes (Duthie et al., 1996; Porrini and Riso, 2000). Coffee is well-known as a rich source of antioxidants in human diet that may contribute to the prevention of oxidative stress related diseases (Dorea and da Costa, 2005; Pulido et al., 2003; Svilaas et al., 2004). The preparation of instant coffee in industries and coffee beverages in restaurants, cafeterias and also at domestic levels generates tons of coffee residues. It has been proposed that spent coffee grounds could be valuable by-

products because of their antioxidant properties due to the presence of phenolic and nonphenolic bioactive compounds (Bravo et al., 2013; Bravo et al., 2012; Murthy and Madhava Naidu, 2012; Mussatto et al., 2011; Ramalakshmi et al., 2009; Yen et al., 2005). However, the chemical-based assays used for the antioxidant activity evaluation of spent coffee are suitable for the initial antioxidant screening but do not reflect the cellular physiological conditions. Therefore, there is a need for applying cell cultures models to support antioxidant research (Liu and Finley, 2005). To our best knowledge, only the cell viability in rodent cell culture systems has been evaluated to report the anti-tumor, anti-allergic and anti-inflammatory activities of spent coffee extracts obtained from instant coffee (Ramalakshmi et al., 2009). However, this is the first time that direct effect and prevention against ROS formation and DNA damage of spent coffee extracts in human cells has been reported. In a previous study, we have reported that spent coffee grounds obtained from the most common coffeemakers used at domestic and cafeterias levels (filter and espresso), and in less proportion from plunger (French press) ones, have antioxidant capacity because of the presence of relevant amounts of hydrophilic bioactive compounds, such as caffeoylquinic acids, mainly dicaffeoylquinic acids, and caffeine (Bravo et al., 2012). Because some of these coffee compounds have shown protective effects against oxidation (ROS formation) and DNA damage in human cell models when they were evaluated individually (Bakuradze et al., 2010; Cho et al., 2009; Faustmann et al., 2009; Pavlica and Gebhardt, 2005) or in coffee matrices (instant or coffee brew) in human cell models both directly or in interventional studies (Bakuradze et al., 2010; Bichler et al., 2007; Del Pino-García et al., 2012; Hoelzl et al., 2010); we hypothesize that aqueous spent coffee extracts might have antioxidant and genoprotective effects in human cells. However, up to now, the effect of these coffee by-products on oxidation and DNA damage in cells is still unknown.

Therefore, the aim of the present work was to evaluate the ability of two of the most antioxidant spent coffee extracts (from Arabica filter and Robusta espresso) (Bravo et al., 2012) to protect against oxidation and DNA damage in a cancer human cell model system. For this purpose, firstly, cytotoxicity was measured in order to choose the adequate extract concentration to be evaluated. Afterwards, the direct effects of the extracts on intracellular ROS level using the diclorofluorescein assay, and on DNA oxidation damage using the comet assay were determined. And last, the protection ability of the spent coffee extracts against H_2O_2-induced intracellular ROS level and DNA strand breaks and Ro-induced oxidative DNA damage (as FPG-sensitive sites) increase was assessed.

2. Materials and methods

2.1. Chemicals

Dulbecco´s Modified Eagle´s Medium (DMEM), fetal bovine serum (FBS), antibiotic solution, and trypsin solution were purchased from Gibco (Prat de Llobregat, Barcelona, Spain). Hydrogen peroxide (H_2O_2), 3-(4,5-methyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (Steinheim, Germany). Dichlorodihydrofluorescein diacetate (H_2DCF-DA) was from Invitrogen Molecular Probes (Eugene, Oregon, USA). DMSO was purchased from Panreac Quimica SAU (Barcelona, Spain). Ro (photosensitizer 19-8022) and formamidopyrimidine DNA glycosylase (FPG) were kindly supplied by Dr A. Collins (Institute for Nutrition Research, University of Oslo, Norway).

2.2. Preparation of Spent Coffee Extracts

Roasted coffee (without defective beans) from Guatemala (*Coffea arabica*, named Arabica, 3.03% water content, L* = 25.40±0.69, roasted at 219 °C for 905 s) and

Vietnam (*Coffea canephora* var. robusta, named Robusta, 1.59 % water content, L* = 24.92±0.01, roasted at 228 °C for 859 s) was provided by a local factory. Coffee beans were ground for 20 s using a grinder (model Moulinex super junior "s", París, France). The L* value was analyzed by means of a tristimulus colorimeter (Chromameter-2 CR-200, Minolta, Osaka, Japan) using the D65 illuminant and CIE 1931 standard observer. The instrument was standardized against a white tile before sample measurements. Ground roasted coffee was spread out in a 1 cm Petri plate, and the L* value was measured in triplicate on the CIELab scale. Water content was measured by weight loss after drying for 2 h at 102±3 °C in an oven JP SELECTA (Barcelona, Spain).

Arabica and Robusta spent coffee grounds were obtained as coffee by-products after the preparation of coffee brews with filter (24 g/400 mL water, model Avantis 70 Inox, Ufesa, Spain) and espresso coffeemakers (7 g/40 mL water, model Saeco Aroma, Italy), respectively. Then, spent coffee extracts were prepared according to the method described by Bravo et al. (2013). Briefly, first, spent coffee grounds were dried to a constant weight at 102 ±3 ºC in an oven JP SELECTA (Barcelona, Spain) and defatted with petroleum ether (1:11, w/v) for 3 h at 60 °C in a Soxhlet extraction system (Extraction Unit B-811 Standard Büchi, Flawil, Switzerland). Then, 24 g of spent coffee was extracted with a volume of 400 mL of water using a filter coffeemaker (model AVANTIS 70 Inox, Ufesa, Spain). Extraction took approximately 6 min at 90 ºC. Aqueous spent coffee extracts were lyophilized using a CRYODOS Telstar (Terrassa, Spain) and stored at -20 ºC until sample analysis.

2.3. Cell Culture

HeLa cells (derived from human cervical cancer) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS and 1% antibiotic

(10000 U/mL penicillin and 10000 µg/mL streptomycin, Gibco), under an atmosphere of 5% CO_2 at 37 ºC. Cells were trypsinized when nearly confluent.

2.4. Cytotoxicity

Cell viability was determined by assessing the reduction of MTT to formazan by the mitochondrial enzyme, succinate dehydrogenase, as described by Mosmann (1983). Cells were seeded in 96-well plates at 2×10^4 cells/well and maintained for 24 h until confluence. Arabica and Robusta spent coffee extracts were then added to medium at 37, 111, 333, 1000 and 3000 µg/mL. After 2 and 24 h treatments, cells were washed with phosphate-buffered saline (PBS), and 25 µL MTT (5 mg/mL) in PBS was added to 225 µL of fresh medium to each well. After an incubation of 2 h and 30 min at 37 ºC, the supernatant was removed, and the insoluble formazan crystals were dissolved with 100 µL of DMSO. The absorbance was measured at 540 nm using a spectrophotometer reader (Spectra MR, Dinex Technologies). Results were expressed as the percentage of viability (%) with respect to the control (medium treated cells) according to the following formula: [(absorbance treated cells-absorbance blank)/ (absorbance control cells-absorbance blank)] × 100.

2.5. Intracellular ROS level

Intracellular ROS level was determined by using fluorescent probe dichlorodihydrofluorescein diacetate (H_2DCF-DA), according to Wang and Joseph (1999). H_2DCF-DA is enzimatically hydrolyzed by intracellular esterases to originate non-fluorescent H_2-DCF, which is then rapidly oxidized to originate highly fluorescent DCF in the presence of ROS. The DCF fluorescence intensity parallels the amount of intracellular ROS. Two different types of experiments were carried out: (1) treatment of cells with spent coffee extracts to test their direct effect on intracellular ROS level and (2) pretreatment of cells with spent coffee extracts before submitting the cells to an

oxidative stress to test their protective effect against ROS level increase induced by hydrogen peroxide. HeLa cells were seeded in 96-well plates at 2×10^4 cells/well. In experiment (1), 24 h after seeding, 200 µL of H_2DCF-DA (100 µM) in serum- and phenol red-free DMEM was added to each well for 30 min at 37 ºC. Afterwards, cells were washed once with PBS and exposed for 2 and 24 h to Arabica and Robusta spent coffee extracts in phenol red-free DMEM at 37, 111, 333 and 1000 µg/mL. For experiment (2), cells were pretreated during 2 and 24 h with Arabica and Robusta spent coffee extracts at 37, 111, 333 and 1000 µg/mL, the H_2DCF-DA (100 µM) probe was added to each well for 30 min at 37 ºC, and the cells were washed once with PBS and fresh phenol red-free DMEM containing 500 µM H_2O_2 was added to all cultures except controls for 10 min at 37 ºC. In both experiments, intracellular ROS were measured using a microplate fluorometer Fluoroskan Ascent (Thermo Labsystems) at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. ROS level was expressed as the fluorescence of the treated samples (spent coffee treated cells) compared to the fluorescence of the control samples (medium treated cells) = [(fluorescence treated cells/fluorescence control cells) × 100].

2.6. DNA damage

DNA damage was determined by comet assay able to detect SBs and oxidative DNA damage in culture cells. The inclusion of FPG digestion allowed the detection of the main purine oxidation product 8-oxoguanine as well as other altered purines. Two different types of experiments were carried out: (1) treatment of cells with spent coffee extracts to rule out their ability to cause SBs and oxidative DNA damage and (2) pretreatment of cells with spent coffee extracts before submitting the cells to an oxidative damage to evaluate their genoprotective effect against induced DNA damage. HeLa cells were seeded in 24-well plates at 8×10^4 cells/well. A negative control with

cells treated with medium and two positive controls were also included: cells treated with a solution of 500 μM H_2O_2 for 10 min (on ice) to induce SBs and cells treated with 1 μM Ro plus visible light from a 500 W tungsten-halogen source at 33 cm (10 min on ice) to induce 8-oxoGua. In experiment (1), 24 h after seeding, cells were exposed for 2 and 24 h to spent coffee extracts in medium at 111 and 333 μg/mL. For experiment (2), cells were preincubated for 2 and 24 h at 37 ºC with spent coffee extracts in medium and then were washed with PBS and treated on ice with H_2O_2 (500 μM) for 10 min or with 1 μM Ro plus visible light (10 min) depending on the DNA damage to evaluate, as described previously. DNA damage (SBs and 8-oxoGua) was evaluated by the comet assay without and with FPG, respectively.

The comet assay technique was applied according to Collins and Dusinska (2002), Singh et al. (1988), and Tice et al. (2000) with some modifications. Thirty microliters of each cell suspension (1×10^6 cells/mL in PBS) were mixed with 140 μL of 1% low melting point agarose, and two drops of 70 μL of this mixture were placed on a microscope slide precoated with 1% of normal melting point agarose. Three slides were prepared for each condition. Slide 1 for observing DNA SBs, and slides 2 and 3 for obtaining information regarding the presence of oxidized DNA bases using FPG enzyme. A cover slip was put on top of each drop and the gels were allowed to set for 5 min at 4 ºC. Then the cover slip was removed and the slides were immersed in lysis solution (2.5 M NaCl, 0.1 M Na_2-EDTA, 0.01 M Trizma-BASE, pH 10, with 1% Triton X-100 added prior to use) at 4 ºC for 1 h. After that, slides 2 and 3 were washing three times (5 min each time) with enzyme buffer (0.1 M KCl, 0.04 M HEPES-KOH, 0.0005 M Na_2-EDTA, 0.2 mg/mL BSA, pH 8.0) prior to incubation for 30 min at 37 ºC with 50 μL of buffer (slide 2) or FPG in the enzyme buffer (slide 3) in a humid chamber. The slides then were placed on a horizontal gel electrophoresis tank filled with

freshly prepared alkaline buffer (0.3 M NaOH and 0.001 M Na_2-EDTA, pH > 13), at 4 ºC for 20 min, to allow DNA unwinding. Electrophoresis was carried out for 30 min at 25 V (~0.8 V/cm across the gels and ~300 mA). Finally, slides were neutralized in PBS during 10 min, washed two times (5 min each) with deionized water and fixed with ethanol 96% (5 min).

Gels were stained with 35 µL of 1 µg/mL DAPI and comets were analyzed in a fluorescence microscopy (Eclipse 50 i NIKON). A total of 100 comets on each slide were scored through a computer-assisted image analysis (Comet assay IV, Perceptive Instruments), by measuring the percentage of DNA in the tail.

2.7. Statistical analysis

Data are presented by descriptive analysis [mean ± standard deviation (SD) for three independent experiments]. Comparisons were performed by the non-parametric Mann-Whitney *U*-test. The $p \leq 0.05$ probability was accepted as the level of significance. All statistical analyses were performed using the SPSS v.15.0 software package.

3. Results

3.1. Cytotoxicity

The cytotoxicity of Arabica and Robusta spent coffee extracts was measured in HeLa cells at short (2 h) and long (24 h) exposure times using the MTT reduction assay (Fig. 1). After 2 h incubation, the viability of HeLa cells was not affected (>80%) by both spent coffee extracts up to 1000 µg/mL, but a remarkable decrease (~60%) was observed for Arabica filter spent coffee extract at 3000 µg/mL (Fig. 1A). After 24 h of treatment, HeLa cells viability decreased in a concentration-dependent manner, and a clear toxic effect at 3000 µg/mL was observed for both spent coffee extracts (Fig. 1B). Therefore, in order to evaluate the possible protective effect of Arabica filter and

Robusta espresso spent coffee extracts on intracellular ROS level and DNA damage, concentrations between 37-1000 μg/mL were chosen, under the same exposure times.

3.2. Effect of spent coffee extracts on intracellular ROS level

After 2 and 24 h of treatment with spent coffee extracts, the level of intracellular ROS was evaluated in HeLa cells (Fig. 2). Robusta espresso spent coffee extract did not induce any relevant ROS level increase at none of the concentrations studied after 2 and 24 h exposure. However, at the highest concentrations (1000 μg/mL after 2 h and also at 333 μg/mL after 24 h) a slight but not relevant ROS level increase was observed for Arabica filter spent coffee extract. A significant basal ROS level decrease was observed after treatment with 37 and 111 μg/mL of both spent coffee extracts at short and long exposure times in comparison to control cells.

3.3. Protection of spent coffee extracts against intracellular ROS level increase

The protective effect of spent coffee extracts against ROS level increase induced by 500 μM H_2O_2 (10 min, 37 ºC) was evaluated (Fig. 3). After 2 h of exposure, spent coffee extracts pretreatment showed a significant decrease in ROS level at 1000 μg/mL (Fig. 3A). At the long pretreatment of 24 h, the ROS level significantly decreases at the highest concentration (1000 μg/mL) for both spent coffee extracts, but also at 333 μg/mL for Robusta espresso spent coffee extract (Fig. 3B).

3.4. Genotoxicity of spent coffee extracts

Firstly, the effect of Arabica and Robusta spent coffee extracts on induction of SBs and oxidized bases was assessed by the comet assay without and with FPG treatment respectively. HeLa cells were treated with 111 and 333 μg/mL of Arabica and Robusta spent coffee extracts for 2 and 24 h. The percentage of DNA in the tail was less than 3%, similar to control cells (data not shown). Therefore, none of the studied

concentrations induce relevant SBs or oxidized purines (FPG-sensitive sites) in the cells.

3.5. Protective effect of spent coffee extracts against DNA SBs and oxidative DNA damage

Then, to evaluate the ability of Arabica filter and Robusta espresso spent coffee extracts to protect against DNA damage, HeLa cells were incubated for 2 and 24 h with 111 and 333 μg/mL spent coffee extracts before treatment with H_2O_2 or Ro plus visible light. In previous experiments, two concentrations of H_2O_2 were initially evaluated: 25 and 500 μM. However, the highest one (500 μM) was chosen to exacerbate DNA damage (36.7% of damage for 500 μM vs 8.8% for 25 μM) and highlight cells able to protect themselves from the oxidative stress. At tested concentrations, DNA strand breaks were significantly reduced by the pretreatment with both spent coffee extracts (except for Arabica 111 μg/mL at 2 h exposure) showing a strong protective effect against H_2O_2-induced DNA damage with a significant dose-dependent decrease ($p \leq 0.05$) after a long period of incubation (24 h) (Fig. 4).

To evaluate oxidative DNA damage in pilot studies, we exposed HeLa cells for 5 and 10 min to Ro plus visible light to induce 8-oxoGua. However, we chose 10 min because the percentage of DNA damage obtained in our experimental conditions was more suitable (59.1% of damage for 10 min vs 33.8% of damage for 5 min) to determine the ability of spent coffee extracts to protect against this oxidative damage. In this study, after 2 h of incubation, there was not any significant decrease of oxidized DNA bases at none of the concentrations of Arabica and Robusta spent coffee extracts assessed (Fig. 5A). However, after 24 h of incubation, Robusta espresso spent coffee extract at 333 μg/mL significantly reduced the DNA damage detected by the FPG enzyme (Fig. 5B).

4. Discussion

Spent coffee that is produced in tons by restaurants and cafeterias, and by consumers at domestic levels, could be a good opportunity to have an important source of natural antioxidants. Spent coffee grounds obtained from the preparation of coffee brews (Arabica from Guatemala and Robusta from Vietnam) with the most common coffeemakers (filter, espresso, plunger and mocha) have antioxidant capacity (Folin-Ciocalteau, ABTS and DPPH) because the presence of relevant amounts of caffeoylquinic acids (CQAs), mainly dicaffeoylquinic acids (diCQAs), caffeine and melanoidins, with the exception of those obtained from mocha coffeemaker (Bravo et al., 2012). Moreover, in this previous work, it is shown that aqueous extracts from spent coffee grounds obtained by filter coffeemaker for Arabica coffee and by espresso coffeemaker for Robusta coffee were those with the highest antioxidant capacity. For these reasons and because these antioxidant assays in test tubes do not necessarily reflect the cellular conditions, in the present study we have evaluated the possible antioxidant and antigenotoxic effects of these two spent coffee extracts in a biological system, and the HeLa cell line was selected for that. Two treatment times were assessed, one short of 2 h in order to avoid cell reparation of the possible DNA damage, and the other, a longer treatment time of 24 h in order to take advantage of the spent coffee extracts uptake into the HeLa cells.

Firstly, cytotoxicity was tested in order to select non cytotoxic concentrations for further protection assays. For both spent coffee extracts, 3000 μg/mL was the highest concentration that we could evaluate, because evident signs of precipitation were observed under the microscope at higher concentrations. No cytotoxic effects were observed after 2 and 24 h treatment with both spent coffee extracts in the dose range of 37-1000 μg/mL, with the latter being slightly cytotoxic (cell viability < 60%) after 24 h.

It was also checked that cell viability was not less than 70% after the treatment with both spent coffee extracts at the tested concentrations, prior to H_2O_2 or Ro treatments in the protection assays. On the other hand, non cytotoxic selected concentrations might be considered within physiological ranges when is taken into account that a cup of coffee (125 mL for filter and 45 mL for espresso) provides 12-70 mg of CQAs, 13-50 mg of diCQAs and 84-113 mg of caffeine whereas the tested spent coffee extracts have up to 52.35 µg of CQAs, 45.80 µg of diCQAs and 94.06 µg of caffeine.

It is well-known that some of the compounds with antioxidant properties present in coffee, such as phenolic compounds, caffeine and molecules of the early phases of Maillard reaction may have prooxidant activity under certain conditions such as high doses or in the presence of metal ions (Azam, et al. 2003; Caemmerer et al., 2012; Zheng et al., 2008). Therefore, due to the fact that oxidative stress can be induced by prooxidant agents, either through the formation of ROS or the inhibition of antioxidant systems (Yordi et al., 2012), the possible induction of ROS by the spent coffee extracts was evaluated. A significant ROS increase was detected only for Arabica spent coffee extract at high concentrations. At both exposure times (2 and 24 h) and in the absence of an induced oxidative stress, both spent coffee extracts at the concentrations of 37 and 111 µg/mL significantly reduced the basal ROS production. This suggests that spent coffee extracts could alter the oxidative environment of cells.

When the H_2O_2-induced oxidative stress was applied, both spent coffee extracts were able to significantly reduce ROS production at the highest concentrations (333-1000 µg/mL). Bakuradze et al. (2010) reported that ROS production was reduced by pre-incubating HT-29 cells with Arabica and Robusta filter coffee brews, and explained this capacity by the presence of 5-caffeoylquinic acid (5-CQA) and thermal degradation products. In line with this, many studies have shown the potential of 5-CQA for

decreasing ROS induced by hydrogen peroxide (Cho et al., 2009; Pavlica and Gebhardt, 2005). In a previous study, we have reported that 5-CQA is the major phenolic compound quantified in the tested spent coffee extracts (Bravo et al., 2012), but this chlorogenic acid was in lesser amounts in Robusta espresso spent coffee extract (18.20 μg/mg) which showed slightly higher protective effects against ROS than in Arabica filter one (24.28 μg/mg). However, the amount of dicaffeoylquinic acids that exhibit more potential antioxidative effect than CQAs due to the esterification of an additional caffeoyl group to the quinic core (Iwai et al., 2004; Ohnishi et al., 1994) was 1.2-fold higher in Robusta espresso spent coffee. Moreover, caffeine was 2.7-fold higher in Robusta espresso spent coffee whereas Maillard reaction products (MRPs) measured as browning index were higher in Arabica filter spent coffee extract than in Robusta (0.165 vs 0.133, Abs 420 nm). Taking into account the results of the present study and in accordance with previous literatures, the similar efficiency of both spent coffee extracts might be due to a balance between CQAs and MRPs, more abundant in Arabica filter spent coffee, and diCQAs and caffeine, higher in Robusta espresso spent coffee extract.

No genotoxic effects (SBs and FPG-sensitive sites) were detected using the comet assay in HeLa cells after 2 and 24 h treatment with spent coffee extracts at the highest non cytotoxic concentration (cell viability > 80%). Therefore, the concentrations selected for studying spent coffee extracts protection against DNA damage by the comet assay were 111 and 333 μg/mL. DNA damage is a useful biomarker of the oxidative status. Thus, in order to evaluate the effectiveness of Arabica filter and Robusta espresso spent coffee extracts to protect against induced DNA strand breaks, an oxidative insult with H_2O_2 was applied. H_2O_2 produces DNA strand breaks mimicking the effect of radiation on DNA (Dahm-Daphi et al., 2000). After a short treatment (2 h) with 333 μg/mL extracts,

Arabica and Robusta spent coffee showed a strong protective effect against H_2O_2–induced DNA damage in HeLa cells (73 and 64% protection, respectively). However, at 111 µg/mL, only Robusta espresso spent coffee extract protected DNA against this damage (47% protection). After a long treatment (24 h), all the tested concentrations of spent coffee extracts showed a protective effect against H_2O_2–induced DNA damage, ranging from 29% to 65% protection. These results are in concordance with the protective effect observed in mouse lymphoma cells after N-methyl-N-nitro-N-nitrosoguanidine exposure by caffeinated instant coffee (Abraham and Stopper, 2004). In a recent study, the intermediate and final MRPs have been found to contribute to the capacity of instant coffee to diminish DNA damage in HT-29 cells (Del Pino-García et al., 2012).

Strand breaks are not the only kind of DNA damage in cells, also oxidized bases are present and 8-oxoguanine is one of the most common DNA lesions. In this study, we exposed HeLa cells to Ro plus visible light to induce 8-oxoGua. Only Robusta espresso spent coffee extract, at the highest concentration tested (333 µg/mL) and after a long period of incubation (24 h), showed a significant protection against Ro plus visible light-induced purines oxidation (11.5% protection). To our knowledge, this is the first time that the ability of spent coffee to protect against DNA SBs and oxidative DNA damage has been shown. However, the capacity of coffee to protect human lymphocytes against oxidative DNA-damage (Bichler et al., 2007; Hoelzl et al., 2010) and the effect of standards of coffee compounds on DNA damage have been studied. Thus, 5-CQA that is one compound quantified in Arabica and Robusta spent coffee extracts (Bravo et al., 2012) has been found to decrease radical induced DNA damage in bacterial and mammalian cells (Faustmann et al., 2009). However in the present study, Robusta espresso spent coffee extract which has less amount of 5-CQA (18.20 µg/mg) than

Arabica filter (24.28 μg/mg) (Bravo et al., 2012) was more effective in protection against induced oxidative DNA damage. Indeed, similar results were obtained by Schaefer et al. (2006), who reported that the most active polyphenolic apple juice extracts protecting against menadione-induced DNA damage in Caco-2 and HT-29 cells were those which contained low concentrations of this chlorogenic acid. Moreover, Robusta espresso spent coffee extract contains higher concentrations of caffeine (94.06 μg/mg vs 35.19 μg/mg) than Arabica filter. This xanthine has been found to protect against oxidative damage of calf thymus DNA and radical induced migration in human lymphocytes (Faustmann et al., 2009). These results suggest that other phenolic compounds, such as diCQAs with higher antioxidative effect as discussed above, but also other non-phenolic bioactive compounds such as caffeine might contribute to the antigenotoxic activity of spent coffee extracts. The higher effectiveness of Robusta espresso spent coffee extract on protection against DNA purines oxidation might be explained by the enhance intracellular antioxidant capacity, due to the fact that under the same conditions where this genoprotective effect was detected, a significant decrease level of ROS was also observed. Also other mechanisms such as endogenous defence systems could contribute because this genoprotective effect was only observed after the long incubation (24 h).

In conclusion, in the experimental conditions of this work, spent coffee extracts show antioxidant and antigenotoxic properties, revealing that the use of these coffee by-products which contain bioactive compounds may provide new strategies to protect against oxidative stress related diseases, such as cancer. Besides, the results of the present work highlight the relevance of studying the bioactivity of these compounds in the spent coffee matrices, or in general real food matrices, instead of the pure compounds because it allows to explore the balance of compounds with different

efficiency, but also to prevent the loss of synergistic and/or antagonistic effects among spent coffee compounds. Additionally, further experimental and clinical studies about the compounds responsible, the underlying mechanisms, and to clarify dose and time dependence would be necessary to enlarge the significance of these results.

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FIGURE CAPTIONS

Fig. 1. Viability curves of HeLa cells after 2 and 24 h of incubation with spent coffee extracts, obtained with the MTT assay. Results are expressed as mean ± SD of three independent experiments.

Fig. 2. Intracellular ROS level of HeLa cells treated during 2 and 24 h with different concentrations of spent coffee extracts. Results are expressed as mean ± SD of three independent experiments.* Significantly different from medium-treated cells (C-) ($p \leq 0.05$).

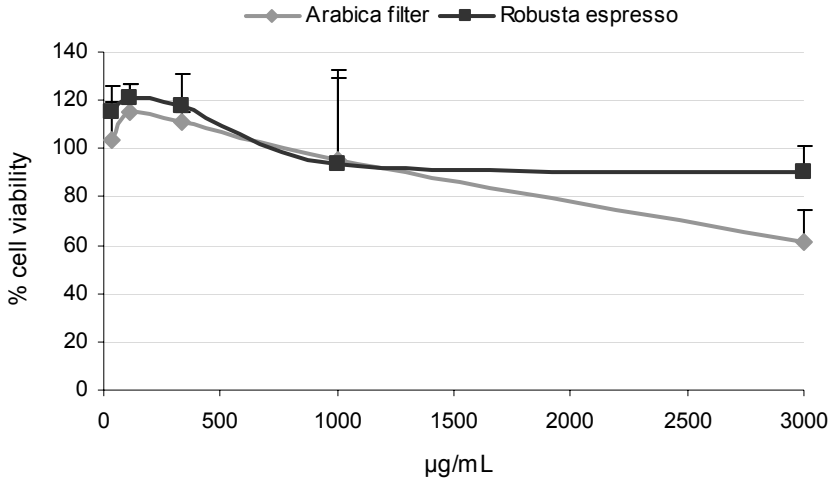
Fig. 3. Protective effect of spent coffee extracts against H_2O_2–induced ROS production, evaluated by the dichlorofluorescein assay. HeLa cells were incubated for 10 min (37 ºC) in the presence of 500 μM H_2O_2. Spent coffee extracts were added to the cells 2 and 24 h prior to the addition of the oxidative stimulus. Results are expressed as mean ± SD of three independent experiments.* Significantly different from H_2O_2 treated cells ($p \leq 0.05$).

Fig. 4. Protective effect of spent coffee extracts against H_2O_2–induced formation of DNA strand breaks, evaluated by the comet assay. HeLa cells were incubated for 10 min (on ice) in the presence of 500 μM H_2O_2. Spent coffee extracts were added to the cells 2 and 24 h prior to the addition of the oxidative stimulus. Results are expressed as mean ± SD of three independent experiments.* Significantly different from H_2O_2 treated cells ($p \leq 0.05$).

Fig. 5. Protective effect of spent coffee extracts against Ro-induced purines oxidation (FPG-sensitive sites). HeLa cells were incubated for 2 and 24 h with spent coffee extracts, prior to the treatment with 1 μM Ro (10 min, on ice) plus light. Results are expressed as mean ± SD of three independent experiments.* Significantly different from Ro treated cells ($p \leq 0.05$).

Fig. 1

(A) 2 HOURS



(B) 24 HOURS

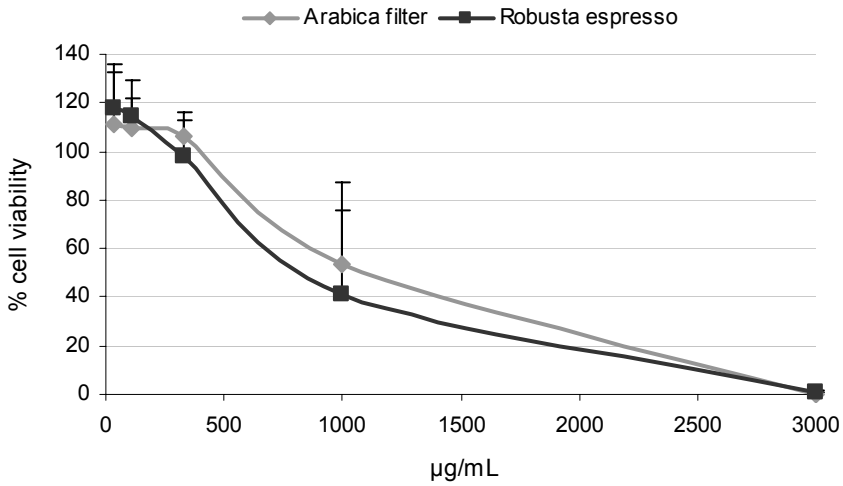
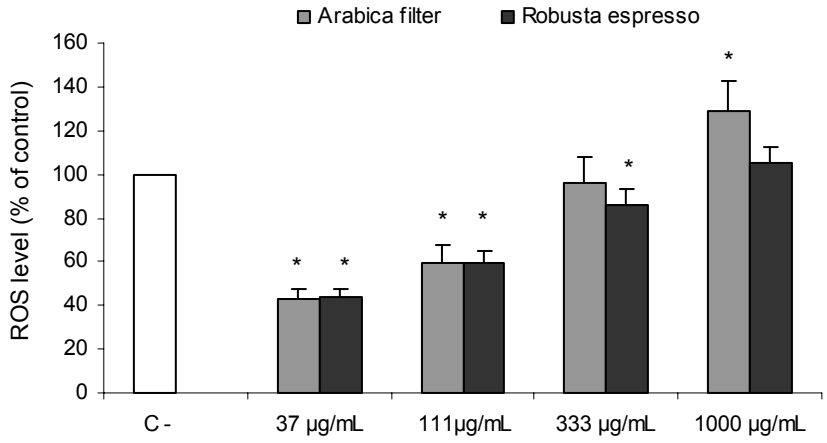


Fig. 2

(A) 2 HOURS



(B) 24 HOURS

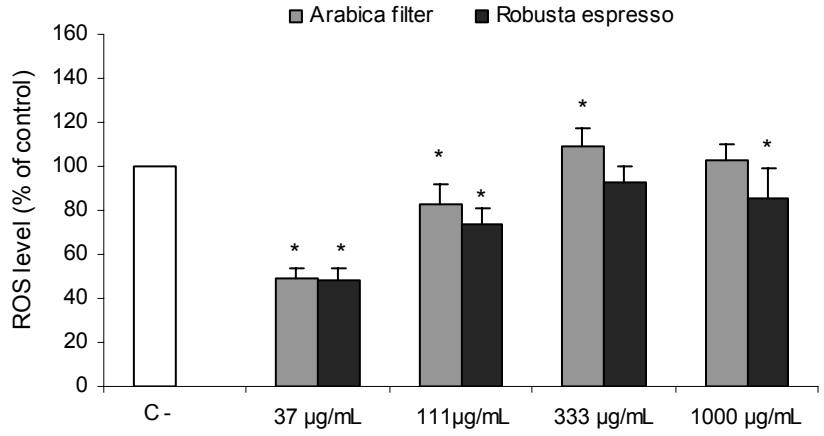


Fig. 3

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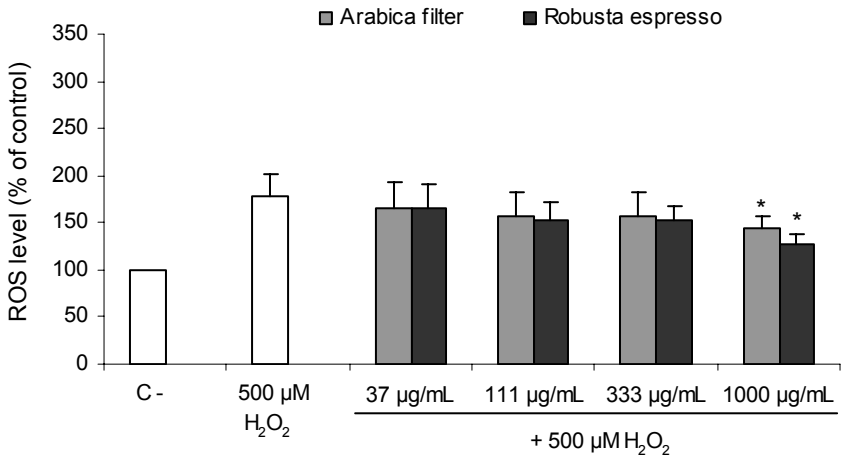


(B) 24 HOURS

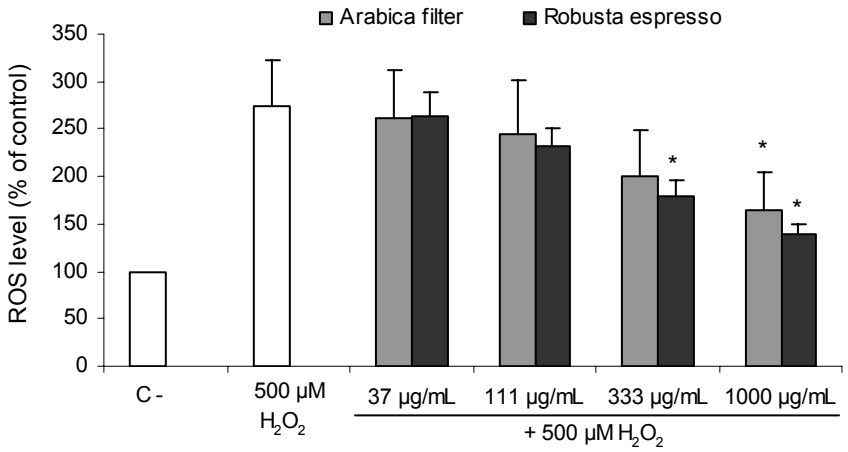
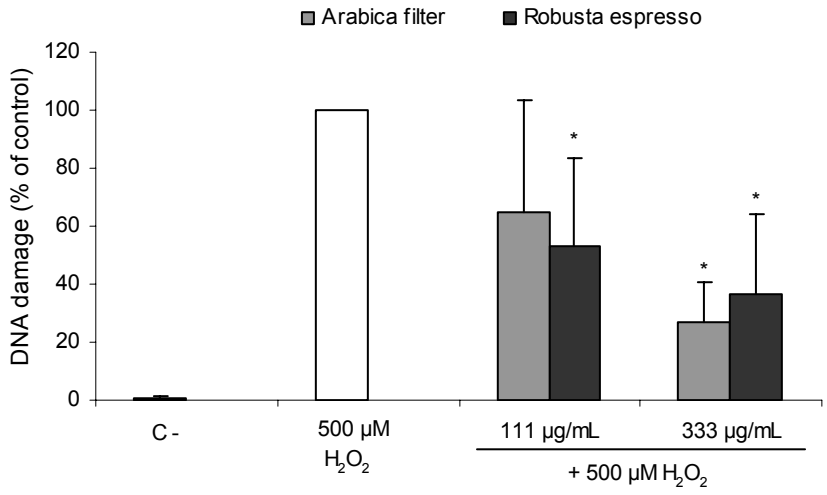


Fig. 4

(A) 2 HOURS



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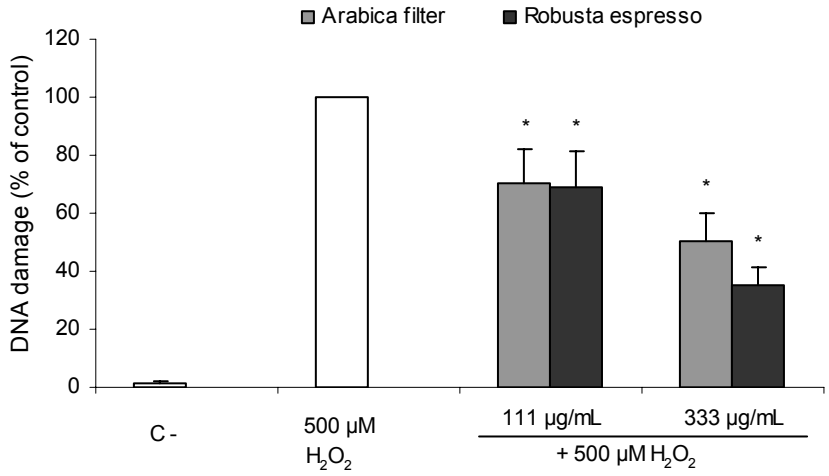
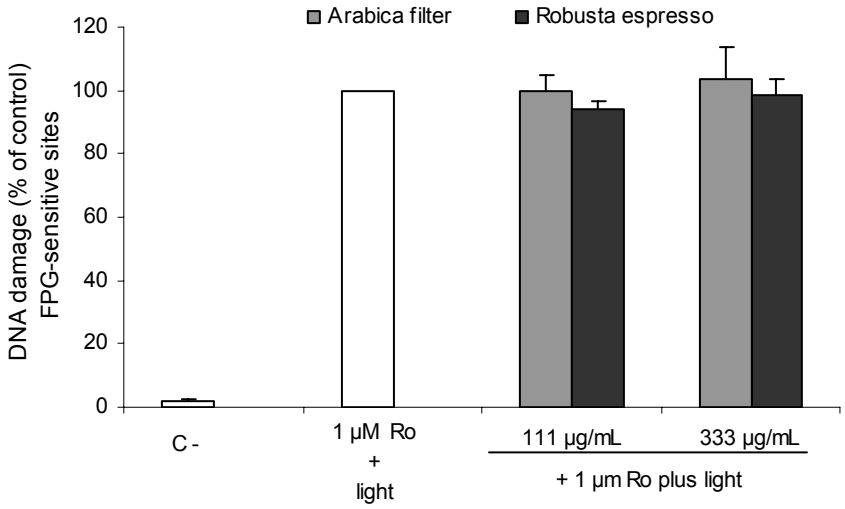


Fig. 5

(A) 2 HOURS



(B) 24 HOURS