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ORIGINAL CONTRIBUTION

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Antiproliferative effect of antioxidant polyphenols from grape in murine Hepa-1c1c7

Summary *Background* Grapes and wine contain high concentrations of polyphenolic compounds. Although their cancer protective effect has been well documented, their activity as anticarcinogens should be cautiously considered since the molecular bases of action and their applicability to human cancer prevention are still unclear. *Aim of the study* We studied the antioxidant/antiradical activity and the antiproliferative effect *in vitro* of different polyphenolic mixtures, extracted from grapes and fractionated through RP-HPLC. *Methods* The polyphenolic fractions were chemically characterized and their antioxidant/antiradical activity was determined by the DPPH assay. Mouse hepatoma Hepa-1c1c7 cells were used to study the cell growth inhibition capacity of these fractions by MTT assay. Their capacity of altering cell cycle and possible induction of apoptosis was examined using FACS analysis. *Results* The original polyphenolic fraction OW, which contained gallic acid (GA), (+)-catechin (Cat), (–)-epicatechin (Ec), glycosylated flavonols (F) and procyanidin oligomers was fractionated into fraction I, composed of monomers and small oligomers, and fraction II that included flavonols and procyanidin oligomers of higher molecular weight. The three polyphenolic fractions tested showed quite similar antiradical activity, although fraction I was the most potent antiradical agent (lowest ED_{50} value: 9 μg). Fraction II was the least potent cell growth inhibitor (highest IC_{50} value: 100 μg/ml) but showed the strongest effect on the cell cycle of Hepa-1c1c7, inducing apoptosis in those cells. The original fraction OW was demonstrated to have the most potent cell growth inhibition effect (lowest IC_{50} value: 43 μg/ml). However, it only appeared to alter cell cycle of Hepa-1c1c7 at concentrations higher than its IC_{50} and did not induce apoptosis in those cells. A similar effect on cell cycle and apoptosis was encountered for fraction I. *Conclusions* The polyphenolic fractions tested in this study were potent antiradical agents and exerted an antiproliferative effect in mouse hepatoma Hepa-1c1c7 cells; the fraction with the highest degree of polimerization and galloylation (fraction II) had the most influence on the cell cycle and induction of apoptosis on Hepa-1c1c7.

Key words polyphenols – antioxidants – RP-HPLC – Hepa-1c1c7 – cell growth inhibition – cell cycle – apoptosis

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Introduction

Cancer is one of the leading causes of death in the prosperous countries of the world, where one person in five die because of this disease [1]. Numerous experimental and epidemiological studies have shown that antioxidant-micronutrients present in food can inhibit carcinogenesis by affecting the molecular events in the initiation, promotion or progression states [2–5]. Among these micronutrients, polyphenols, constituents of plant tissues, were intensively studied in recent years [6–8].

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Grapes and wine contain high concentrations of polyphenolic compounds (12.6–22.4 mmol/l) [9], which are absorbed from the upper gastrointestinal tract [10] and distributed in the body, showing an increased affinity for the heart, liver and kidney. Chronic ingestion of products rich in polyphenols is necessary to obtain biologically effective concentrations [11, 12]. Flavonoids are the largest class of polyphenolic compounds. Thus, over 4000 structurally unique compounds have been identified in plant sources [13]. Flavanols and flavonols are important flavonoids present in grapes and wine. Flavanols such as catechins, together with oligomeric and polymeric proanthocyanidins, are constituents of both grape skins and seeds, the mean degree of polymerization being higher for skin flavanols; and flavonols as quercetin are particularly abundant in grape skins and stems [14–17]. Polyphenols have been shown to be potent antioxidants, interfering with the oxidative/antioxidative potential of the cell or acting as free radical scavengers [18–20]. The cancer protective effect of dietary polyphenols has been well documented in numerous studies [7, 21, 22]. However their activity as anticarcinogens should be cautiously considered since the molecular bases of action and their applicability to human cancer prevention are still unclear [14].

Apart from the putative anticarcinogenic effect as antioxidants, polyphenols have been described to possess antiproliferative activity [23–25]. Catechins from grape and tea influence the cell cycle by inhibiting certain kinases [26–29]. They have also been shown to trigger apoptosis [30–32].

In the present work, we have extracted, fractionated and characterized different polyphenolic mixtures obtained from grape. Their antioxidant/antiradical activity, together with their antiproliferative effect, capacity to alter cell cycle and induction of apoptosis were examined on mouse hepatoma Hepa-1c1c7.

Materials and methods

Materials

The starting material, provided by Bodegas Miguel Torres, S. A. (Vilafranca del Penedés, Spain) was the by-product from pressing destemmed Parellada grapes (*Vitis vinifera*) and consisted of skins, seeds and stems. The by-product was cooled immediately after pressing and then frozen (–20 °C). Deionized water and preparative grade acetonitrile (CH_3CN, Scharlau, Barcelona, Spain) were used for preparative HPLC. Analytical grade methanol was obtained from Panreac (Montcada i Reixac, Spain), HPLC grade CH_3CN was obtained from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) from Fluorochem (Derbyshire, UK). Cysteamine hydrochloride, acetic acid and hydrochloric acid 37 % (v/v) were provided by Merck. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95 %) was from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97 %) from Aldrich (Milwaukee, USA) and (–)-epicatechin from Sigma Chemical Co, (Saint Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), antibiotic (10,000 U/ml penicillin, 10,000 µg/ml streptomycin) and Dulbecco's phosphate buffer saline (PBS) were provided by Gibco-BRL (Eggenstein, Germany). Fetal calf serum (FCS) was purchased from Invitrogen (Carlsbad, CA, USA). Trypsin EDTA solution C (0.05 % trypsin –0.02 % EDTA) was obtained from Biological Industries (Kibbutz Beit Haemet, Israel). Trypan blue solution 0.2 %, 3-[4,5-dimethylthiazol-2yl]–2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI) and Igepal CA-630 were from Sigma Chemical Co (St. Louis MO, USA). α,α,α-Tris (hydroxymethyl)aminomethane was purchased from Aldrich-Chemie (Steinheim, Germany), RNase free of DNase from Roche Diagnostics (Mannheim, Germany) and rh Annexin V/FITC Kit from Bender MedSystems.

Methods

Preparative chromatography

Preparative reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters (Milford, USA) Prep LC 4000 pumping system with a Waters PrepPack 1000 module fitted with a PrepPack Waters cartridge (300 x 47 mm i. d.) filled with VYDAC (The Separations Group, Hesperia, USA) C_{18}, 300 Å pore size, 15–20 µm particle size stationary phase, at a flow rate of 100 ml/min. The column was equilibrated with filtered (0.45 µm) deionized water and the fractions were eluted with mixtures of CH_3CN/water as stated in the Results section.

Analytical chromatography

Analysis of the polyphenolic fractions was carried out by RP-HPLC on either a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC™ C_{18}, 300 Å pore size, 5 µm particle size, 250 x 4.6 mm i. d. column, or a Smart® System (Amersham-Pharmacia Biotech) equipped with a µPeak Monitor (Amersham-Pharmacia Biotech) and fitted with a µRPC C2/C18 SC 2.1/10 (100 x 2.1 mm i. d.) column (Amersham-Pharmacia Biotech). Eluents: [A] 0.10 % (v/v) aqueous TFA, [B] 0.08 % (v/v) TFA in water/CH_3CN [1:4]. Gradient elution 0 to 50 % [B] over 38 min. Flow rate 1.5 ml/min (VYDAC™ C_{18}) or 0.2 ml/min (µRPC) with detection at 214 nm (VYDAC™ C_{18}) or 214, 280, 320 and 365 nm (µRPC).

Chemical characterization of oligomeric procyanidins

The mean degree of polymerization (mDP), mean molecular weight (mMW) and galloylation (molar percentage of gallyl ester containing species) of the procyanidin oligomers within the fractions were estimated by thiolysis and HPLC essentially as described [33–34] except that cysteamine was used instead of toluene-α-thiol [35]. Briefly, the terminal flavan-3-ols units were released as such by acid cleavage, whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavonoid system. The resulting mixtures were submitted to analytical RP-HPLC (μRPC column, gradient 8–18 % [B] over 30 min) and the molar amount (nmol) of all the released moieties calculated from the peak areas and calibration curves obtained with pure samples. mDP = total nmoles/nmoles terminal units, mMW = total mass/nmoles terminal units, galloylation = [nmoles (–)-epicatechin-gallate (ECG) + nmoles cysteamine-ECG]/total nmoles.

Antioxidant/antiradical activity

The free radical scavenging activity was evaluated by the DPPH method [36, 37]. The samples (0.1 ml) were added to aliquots (3.9 ml) of a solution made up with 4.8 mg DPPH in 200 ml of MeOH (initial concentration of DPPH ca. 60 μM) and the mixture incubated for 1 h at room temperature. The results were plotted as the degree of absorbance disappearance at 517 nm ($(1-A/A_0)x100$) against μg of sample. Each point was acquired in triplicate. A dose-response curve was obtained for every product. The results were expressed as the efficient dose ED_{50} given as the amount (μg) of product able to consume half the amount of the initial free radical.

Cell culture

Mouse hepatoma Hepa-1c1c7 cells (ECACC 95090613) were cultured in DMEM supplemented with 10 % heat-inactivated fetal calf serum and 0.1 % antibiotic. Cells were maintained as monolayer cultures at 37 °C in a humidified atmosphere with 5 % CO_2.

Cell growth inhibition

The assay was performed by a variation of the method described by Mosmann [38]. Samples containing 200 μl cell suspension (2 x 10^4 cells/ml) were plated in 96-well-flat-bottomed microtiter plates. After adherence of the cells within 24 h of incubation at 37 °C, different polyphenolic fraction dilutions on a scale of 10 μg/ml to 100 μg/ml were added separately. After incubation for 72 h at 37 °C in a humidified incubator with 5 % CO_2, MTT dissolved in PBS at 5 mg/ml and sterile filtered was added to all the wells at a final concentration of 0.5 mg/ml. Following 1 hour of incubation, the generated formazan was dissolved with 100 μl DMSO per well. The optical density was measured on a ELISA plate reader (Merck ELISA System MIOS version 3.2.) at 550 nm. Absorbance was proportional to the number of cells. The concentrations that caused 50 % of inhibition of cell growth (IC_{50}) were calculated.

Cell cycle analysis

Cell cycle was assessed through flow cytometry by using a fluorescence-activated cell sorter (FACS). Cells were cultured in 6-well flat-bottomed microtiter plates containing 2 ml of cell suspension. The number of cells [116400] was determined by the relationship number of cells/area wells, considering the 4000 cells that were cultured in 96-well plates. After 24 h of incubation at 37 °C with 5 % CO_2, polyphenolic fractions were added at their respective IC_{50} values and at a higher dose. Following 72 h of incubation, cells were harvested by mild trypsinization, collected by centrifugation and stained in Tris-buffered saline (TBS) containing 50 μg/ml PI, 10 μg/ml RNAse free of DNAse and 0.1 % Igepal CA-630 for 1 h at 4 °C. FACS analysis was carried out at 488 nm in an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL, USA). Data from 12,000 cells were collected and analyzed using Multicycle program (Phoenix Flow Systems, San Diego, CA, USA). All experiments were performed in triplicate.

Assessment of apoptosis

Detection of apoptosis was performed using an Annexin V-FITC kit binding assay and analyzed by FACS. Cell culture, treatment with polyphenolic fractions and cell collection were carried out as was described in the preceding section. Thereafter cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC (Bender System kit) was added according to the product insert and incubated for 30 minutes at room temperature in the dark. One minute before FACS analysis PI was added at 20 μg/ml. Approximately 3 X 10^4 cells were measured for each histogram and experiments were performed in triplicate.

Results

Extraction and fractionation of polyphenolic compounds from a winery by-product was performed. The lyophilized fraction OW soluble in both ethylacetate and water (2 g) was further fractionated by RP-HPLC using the system described in the Methods. After loading and washing with water (1.7 l), fractions were eluted with

mixtures of CH_3CN in water: fraction I with 12 % CH_3CN (2 l) and fraction II with 24 % CH_3CN (1.6 l). The eluants were concentrated under vacuum and lyophilized to give slightly colored fluffy solids.

RP-HPLC analysis showed that the original fraction (OW) contained gallic acid (GA), (+)-catechin (Cat), (–)-epicatechin (Ec), glycosylated flavonols (F) and other peaks corresponding mainly to procyanidin oligomers. Fraction I was composed of monomers and small oligomers, whereas fraction II included flavonols and procyanidin oligomers of higher molecular weight. Table 1 summarizes the estimated values of mean size and composition of the catechin components (monomers and procyanidin oligomers) of fractions OW, I and II, obtained by thiolysis with cysteamine and RP-HPLC analysis. The fraction retained longer on RP-HPLC (II) presented the highest degree of polimerization and galloylation. Since the monomers were all included in fraction I, its mDP was the lowest.

The free radical scavenging efficiency of the fractions was evaluated by the DPPH radical method as described in the Methods section and compared with that of (–)-epicatechin and trolox (soluble analogue of vitamin E). The ED_{50} values obtained were the following: fraction OW, 12 µg; fraction I, 9 µg; fraction II, 11 µg; (–)-epicatechin, 13 µg; trolox, 15 µg. The three fractions, (–)-epicatechin and trolox presented similar ED_{50} values, with fraction I being the most potent of the products tested.

The antiproliferative effect of the three polyphenolic fractions (OW, I and II) was investigated. The concentration of fractions that caused 50 % inhibition of cell growth was obtained by determination of formazan dye uptake as explained in the Methods section. The fraction concentration was plotted against the percentage of relative cell proliferation after 72 h of treatment, which was calculated assuming that the cells cultured in the absence of the tested fraction had 100 % cell proliferation (Fig. 1). The IC_{50} values obtained were as follows: fraction OW, 43 µg/ml; fraction I, 61 µg/ml; fraction II, 81 µg/ml. The fraction with the highest degree of polimerization and galloylation (fraction II) caused 50 % inhibition of cell growth at a concentration approximately double the concentration required for fraction OW to produce the same effect.

Cell cycle analysis of Hepa-1c1c7 cells was performed 72 hours after treatment with fractions OW, I or II at concentrations equal to their respective IC_{50} values and

Table 1 Chemical characterisation of the catechin components

Fraction	mDP	mMW	galloylation
OW	1.7	539	15%
I	1.4	422	7%
II	3.0	1005	31%

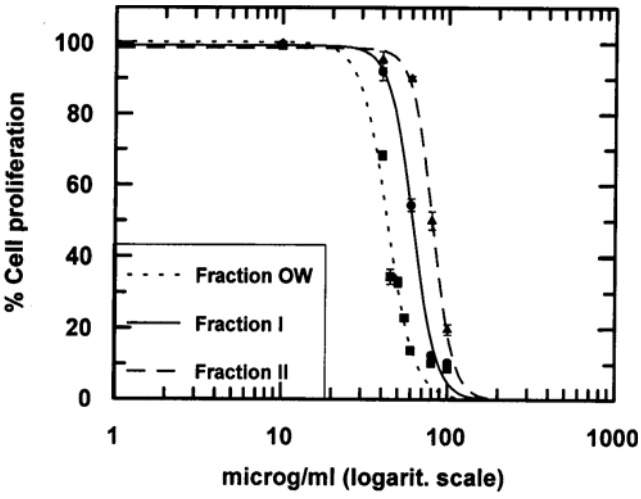

Fig. 1 Effect of polyphenolic fractions from grape (fractions OW, I and II) on cell proliferation of mouse hepatoma Hepa-1c1c7 after 72 hours of treatment. The relative percentage 72 hours, and relative percentage of cell proliferation was calculated considering untreated control cells after 72 hours as 100 % cell proliferation. Each point represented the mean of triplicates of experiments. Vertical bars indicate standard error of the means

at a higher dose which results in 80 % inhibition of cell proliferation, with respect to the untreated control cells (fraction OW, 60 µg/ml; fraction I, 80 µg/ml; fraction II, 100 µg/ml). Both analysis at the IC_{50} and at a higher dose showed that the fraction with the highest degree of polimerization and galloylation (fraction II) exerted the strongest effect on the cell cycle, producing an apparent aberrant cell cycle profile (Fig. 2. A). Compared to the untreated control cells, this fraction produced a decrease of cells in G0/G1 as well as an increase of cells in S and G2 phases. Neither fraction OW nor fraction I affected the cell cycle of Hepa-1c1c7 cells at concentrations equal to their respective IC_{50} values. However, when the concentrations were higher than their IC_{50} values, fraction OW increased the number of cells in S and G2/M phases by decreasing number of them in G1 and fraction I appeared to interfere with the Hepa-1c1c7 cell cycle by slightly arresting cells in G2/M phase (Fig. 2. B).

Assessment of apoptosis in Hepa-1c1c7 cells was performed 72 hours after treatment with fractions OW, I or II at the same concentrations mentioned above for the analysis of cell cycle. FACS analysis using Annexin V-FITC staining and PI accumulation was used to differentiate early apoptotic cells (Annexin V^+ and PI^-) from late apoptotic/necrotic cells (Annexin V^+ and PI^+). Results showed that fractions OW and I hardly induced apoptosis in Hepa-1c1c7 cells at either concentrations equal to their respective IC_{50} (Fig. 3. A) or higher than them (Fig. 3. B), whereas the treatment with fraction II generated 38 % apoptotic cells with respect to the untreated control (15 % early apoptosis plus 23 % late apoptosis/necrosis) when cells were treated with a concentration equal to its IC_{50} (Fig. 3. A) and 35 % apoptosis

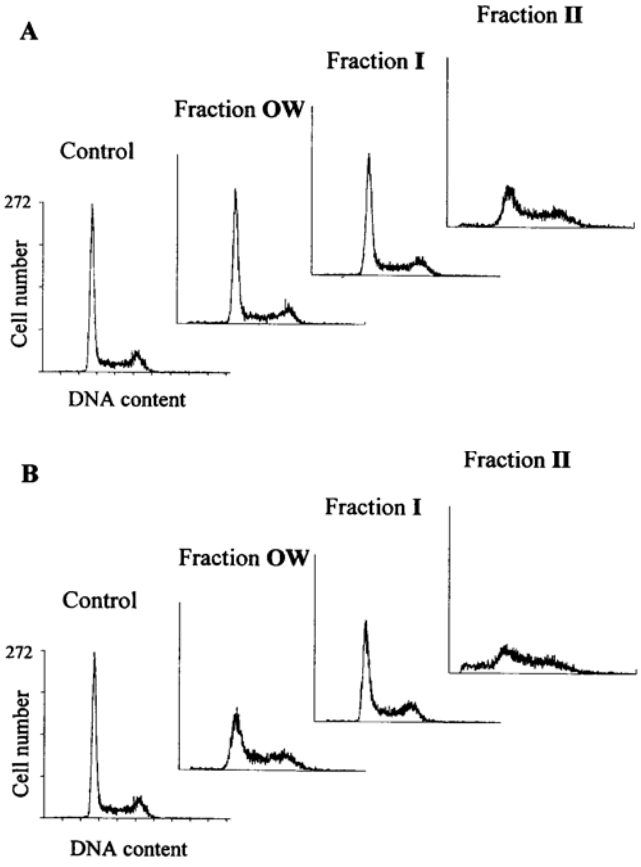

Fig. 2 Cell cycle analysis of Hepa-1c1c7 cells untreated or treated 72 hours with polyphenolic fractions from grape, at concentrations equal to their respective IC_{50} values (fraction OW, 43 µg/ml; fraction I, 61 µg/ml; fraction II, 80 µg/ml) (**A**) and at a higher concentration (fraction OW, 60 µg/ml; fraction I, 80 µg/ml; fraction II, 100 µg/ml) (**B**) Experiments were carried out in triplicate

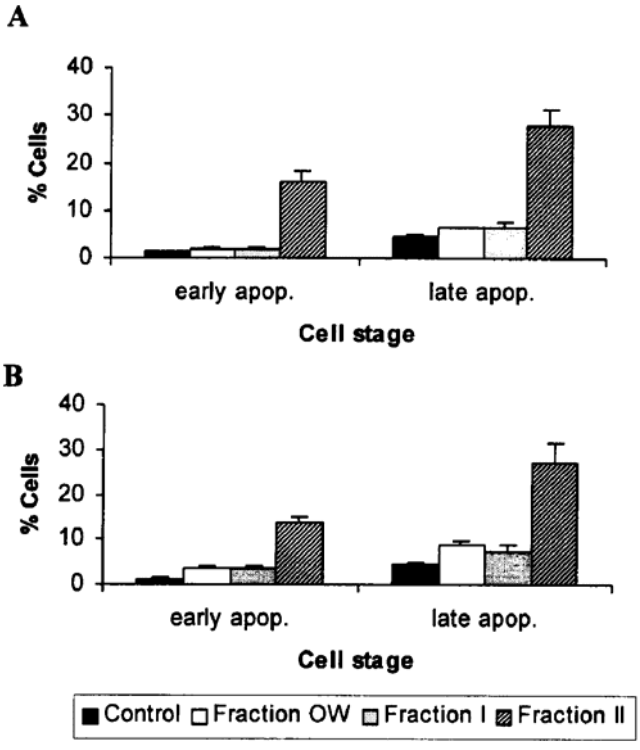

Fig. 3 Cytometric analysis of Annexin V-FITC staining and PI accumulation after exposure of Hepa-1c1c7 to polyphenolic fractions from grape. Each fraction was applied at a concentration equal to its IC_{50} value (fraction OW, 43 µg/ml; fraction I, 61 µg/ml; fraction II, 80 µg/ml) (**A**) and at a higher value (fraction OW, 60 µg/ml; fraction I, 80 µg/ml; fraction II, 100 µg/ml) (**B**) Early apoptotic cells were Annexin V^+ PI^-, whereas late apoptotic/necrotic cells were Annexin V^+ PI^+ . Values are expressed as means ± sem of triplicates of experiments

(13 % early apoptosis plus 22 % late apoptosis) after treatment with a concentration higher than its IC_{50} (Fig. 3. B).

Discussion

Numerous studies have demonstrated the cancer protective effect of dietary polyphenols [7, 21, 22]. Nevertheless, antitumoral properties of these compounds have been a central point of discussions in the last few years [14].

A focus of our study points to a particular efficiency of grape polyphenolic compounds as antioxidants. The results of the DPPH assay clearly indicate a high antioxidant/antiradical activity of polyphenols from grapes, which is in line with previous studies [39, 40]. It has been described that the antioxidant activity depends on polymerization and increases with galloylation [41, 42]. However, although fraction II is the most galloylated it does not show the highest antioxidant/antiradical capacity. This is probably due to the fact that fraction II also contains glycosilated flavonols which are probably less potent antioxidants than gallate compounds. Moreover, our results show that the antioxidant capacity of the polyphenolic fractions does not correlate with the antiproliferative potency. Thus, even though the three fractions present similar antioxidant potency, the capacity to inhibit cell proliferation is higher for the original fraction OW (IC_{50}: 43 µg/ml) than for the two derived fractions I (IC_{50}: 61 µg/ml) and II (IC_{50}: 81 µg/ml), which is indicative of a synergistic effect on the polyphenolic compounds of the two fractions I and II. Interestingly, while fraction II showed the lowest capacity to inhibit cell proliferation, its effect on the cell cycle of Hepa-1c1c7 and its capacity to induce apoptosis in this cell line were stronger than those obtained for fractions OW or I. Thus, fraction II was the only one that affected the cell cycle and induced apoptosis in Hepa-1c1c7 at a concentration equal to its IC_{50} (81 µg/ml). However, fraction I at the same concentration induced neither apoptosis nor resulted in the apparent aberrant cell cycle profile described for fraction II, but produced a slight arrest of Hepa-1c1c7 cells in the G2/M phase.

Whereas there is general agreement on the beneficial effect of natural antioxidants in carcinogenesis prevention, some controversy exists on their effect on tumor proliferation. Thus, on the one hand, it has been described that antioxidants can prevent apoptosis in cancer cells and, on the other hand, that they can induce apoptosis in some systems [43]. The results obtained in the present study are in agreement with the induction of apoptosis in vivo by polyphenols described in colon tumors [22] and show that the degree of galloylation of polyphenolic fractions correlates with the capacity to alter cell cycle and induce apoptosis. These results, together with the findings obtained from recent studies which demonstrate that galloyl esters of catechins such as (–)-epigallocatechin-3-gallate inhibit the activities of several key cell cycle regulatory proteins [28, 30] and trigger apoptosis in diverse cell lines [30–32], suggest that galloylation is important to consider in the study of the antitumoral properties of polyphenolic compounds. In conclusion the high antioxidant potency and capacity to induce apoptosis in cancer cells of fraction II makes it potentially interesting as a food supplement and deserves further characterization.

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