Comparative Effects of Food-Derived Polyphenols on the Viability and Apoptosis of a Human Hepatoma Cell Line (HepG2)

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Consumption of fruits and vegetables, which are rich in polyphenols, has been associated with a reduced risk of chronic diseases such as cancer. Dietary polyphenols have antioxidant and antiproliferative properties that might explain their beneficial effect on cancer prevention. The aim of this study was to investigate the effects of different pure polyphenols [quercetin, chlorogenic acid, and (−)-epicatechin] and natural fruit extracts (strawberry and plum) on viability or apoptosis of human hepatoma HepG2 cells. The treatment of cells for 18 h with quercetin and fruit extracts reduced cell viability in a dose-dependent manner; however, chlorogenic acid and (−)-epicatechin had no prominent effects on the cell death rate. Similarly, quercetin and strawberry and plum extracts, rather than chlorogenic acid and (−)-epicatechin, induced apoptosis in HepG2 cells. Moreover, quercetin and fruit extracts arrested the G1 phase in the cell cycle progression prior to apoptosis. Quercetin and strawberry and plum extracts may induce apoptosis and contribute to a reduced cell viability in HepG2 cells.

KEYWORDS: Polyphenols; strawberry; plum; HepG2 cells; cell viability; cell cycle; apoptosis

INTRODUCTION

There is an abundance of evidence that regular consumption of fruits and vegetables is associated with a reduced risk of chronic and degenerative diseases, such as cancer, Parkinson's disease, Alzheimer's disease, osteoporosis, and cardiovascular disease (*1−3*). Fruits and vegetables provide essential nutrients, but they also contain dietary antioxidants, such as phenols and flavonoids, which comprise a large group of compounds with low molecular weight that are present in all plants (*4*). Flavonoids exhibit a wide range of biological activities, including inhibition of lipid peroxidation, platelet aggregation, capillary permeability, and the activity of enzyme systems including lipooxigenase (*3, 5*). The flavonoids exert these effects as antioxidants and free radical scavengers and interfere with the oxidative/antioxidative potential of the cell (*6, 7*); because of those properties, they are able to prevent damage to lipids, proteins, and nucleic acids and eventual cellular damage and death (*8−10*). In addition, polyphenols can show anticarcinogenic, antimutagenic, antiinflammatory, and antiviral activities (*3, 11*), displaying antiproliferative activity. These compounds can also trigger apoptosis (*2, 7, 12*) that may be an efficient strategy for cancer chemotherapy (*2, 13*).

Carcinogenesis is a multistage process with an accumulation of genetic alterations; thus, targets for chemoprevention could be multiple (*2*). Suppression of cell proliferation and induction of differentiation and apoptosis are important preventive approaches. The apoptotic process shows characteristic morphological features, including membrane blebbing, shrinkage of the cell and nuclear volume, chromatin condensation, and a characteristic pattern of nuclear DNA fragmentation in cells due to endonuclease activation (*13*). Nevertheless, the regulation and induction of apoptosis by natural products are still not clear.

The phenolic compounds used in this study, quercetin, cholorogenic acid, and epicatechin, are common dietary polyphenols found in fruits, like strawberry or plum, vegetables, tea, wine, nuts, and seeds, and have a well-characterized in vitro antioxidant activity (*3, 4, 13*). These polyphenols can be absorbed through the gastrointestinal tract, reaching the liver, where they are partly metabolized. Thus, this is one of the main target organs where polyphenols can exert their action.

The study of the effect of those dietary polyphenols and fruits extracts on the regulation of apoptotic mechanisms at the molecular level may benefit from the use of an established cell culture line. Human hepatoma HepG2 is a well-differentiated transformed cell line that meets all biochemical requirements for the present study. This cell line has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resembles the human hepatocyte in culture (*14, 15*). In addition, this continuously growing transformed cell line permits the study of antiproliferative factors for liver cancer research.

In the present study, the effect of different pure polyphenols and natural fruit extracts on the viability and apoptosis induction

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in HepG2 cells at different incubation times (4 and 18 h) has been investigated. Cell death was detected and identified by using trypan blue staining, morphologic studies, and propidium iodide staining followed by flow cytometry and DNA electrophoresis analyses.

MATERIALS AND METHODS

Materials and Chemicals. Quercetin, chlorogenic acid, and (−)-epicatechin as well as trypan blue, propidium iodide, ethidium bromide, gentamicin, penicillin G, and streptomycin were purchased from Sigma Chemical (Madrid, Spain). Proteinase K and RNase A were obtained from Roche (Roche Molecular Biochemicals, Barcelona, Spain). Materials and chemicals for electrophoresis were from BioRad (BioRad Laboratories S. A., Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Biowhittaker Europe (Innogenetics, Madrid, Spain), respectively.

Cell Culture. Human hepatoma HepG2 cells were a gift from Dr. Paloma Martin-Sanz (Instituto de Bioquimica, CSIC, Madrid, Spain). They were grown in DMEM-F12 medium supplemented with 2.5% FBS and the following antibiotics: gentamicin, penicillin, and streptomycin (50 mg/L). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Preparation of Fruit Extracts. Lyophilized strawberry and plum powders (3 g) were treated with a solution of 0.8% HCl in methanol and water (1:1) for 1 h and centrifuged at 600g for 10 min. The supernatants were collected, and pellets were then extracted with a solution of acetone:water (7:3) and centrifuged at 600g for 10 min. The new supernatants were mixed with the previous ones (*16*). Supernatants were concentrated by rotary evaporation at 37 °C and resuspended in water. Total phenolic compounds in strawberry and plum water extracts were determined by the Folin−Ciocalteau method (*17*) and expressed as mg/g of dry extract.

Treatments of Cells with Fruit Extracts or Phenolic Components. Cells were seeded and routinely grown in DMEM-F12 medium and 2.5% FBS, but cells were changed to serum-free medium 24 h before the assay. Cells were treated with different concentrations of pure polyphenols such as quercetin (50, 75, 100, 200, and 300 μM), chlorogenic acid (250, 500, 750, and 1000 μM), (−)-epicatechin (100, 250, 500, 750, and 1000 μM), or with extracts of strawberry (0.1, 0.2, 0.4, 0.6, and 0.8 mg/mL) or plum (0.5, 0.75, 1, 1.25, and 1.5 mg/mL), respectively. In all cases, experiments were carried out using incubation times of 4 or 18 h.

Cell Viability Assay. Viability was calculated by counting the cells in a Neubauer chamber. After trypsinization, the cell suspension (1.5 × 10^5 cells) was centrifuged at 200g at 4 °C for 10 min, and the pellet was washed twice with ice-cold PBS. After resuspension in 100 μL of PBS, an aliquot of the suspension was mixed with an equal volume of trypan blue and incubated for 5 min at room temperature.

Cell Morphology. For assessing morphological changes, confluent cells were treated with the polyphenol concentrations mentioned above. After treatment, photographs were taken using an inverted microscope (Leica, Madrid, Spain) at 200× magnification.

DNA Extraction and Electrophoretic Analysis. Internucleosomal DNA fragmentation was analyzed by agarose gel electrophoresis. The cell suspension [(2−3) × 10^6 cells] was centrifuged at 200g at 4 °C for 10 min, and the pellet was washed twice with ice-cold PBS. After resuspension in 250 μL of PBS, an equal volume of 20 mM EDTA, 0.5% Triton X-100, and 5 mM Tris, pH 8, was added, and the incubation was continued for 15 min at 4 °C. Nuclei were removed by centrifugation at 500g for 10 min, and the supernatant was centrifuged at 10000g for 1 h (soluble fraction). The fragmented DNA present in the soluble fraction was incubated with 0.3 mg/mL proteinase K at 55 °C for 1 h. After two extractions with phenol/chloroform, the DNA was analyzed in a 2% agarose gel and stained with 0.5 μg/mL of ethidium bromide (*18*).

Flow Cytometry Analysis. The distribution of cells in the sub-G1 (hypodiploid peak), G1, G2-M, and S phases of the cell cycle was determined by flow cytometric analysis of DNA content after the corresponding treatment. DNA was determined after labeling the cells with propidium iodide (*19*). Briefly, cell suspensions [(0.5−1) × 10^6

cells] were prepared by trypsinization and washing twice with PBS, followed by centrifugation at 200g. Cells were fixed with 70% ethanol at 4 °C and resuspended in 0.5 mL of PBS containing 0.25 mg/mL of RNase A. The suspension was incubated for 30 min at 37 °C, and then, the cells were labeled with propidium iodide (50 μg/mL). The total DNA content was quantified by fluorescence using a Becton Dickinson (San José, CA) FACS flow cytometer. The resulting histogram was analyzed using ModFit software. Apoptosis was evaluated by determining the percentage of cells with hypodiploid DNA content, followed by cell sorting and analysis of DNA fragmentation as previously described.

Statistics. To contrast groups, one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test were used. Homogeneity of variances was evaluated by the Cochran's test, and to discriminate among means, the Fisher's least significant difference procedure was applied. The level of significance was $P < 0.05$. A Statgraphics Plus program version 4.0 (Statistical Graphics Corp., Rockville, MD) was used.

RESULTS

Viability of HepG2 Cells. The potential cytotoxic effect of pure polyphenols [quercetin, chlorogenic acid, and (−)-epicatechin] and fruit extracts was investigated, determining their effects on the viability of a human hepatoma cell line, HepG2. The cell death rate was dose-dependent after 18 h of treatment with all assayed compounds (**Figure 1**). Quercetin treatment for 18 h showed a dramatic reduction in the cell viability (up to 80% at the highest concentration tested, 200 μM). Quercetin was the most active polyphenol among the pure compounds with an estimated IC$_{50}$ (50% of cell death rate) value of 87 μM (**Figure 1A**). Treatment with the highest dose (1 mM) of chlorogenic acid or (−)-epicatechin reduced cell viability 15−20%; IC$_{50}$ values were not reached for these polyphenols at the concentrations and times assayed in this study; they displayed only a moderate effect on cell death rate even at the highest concentrations for the longest treatment (18 h) (**Figure 1B,C**). Treatment with (−)-epicatechin and chlorogenic acid for 4 h appeared to display similar and very slight inhibitory activities (8 and 4%, respectively) with the highest concentrations used; in contrast, quercetin was more effective (13% at 300 μM) after 4 h of incubation.

As to the effect of fruit extracts, different concentrations of each strawberry and plum extracts had to be used; plum extracts at concentrations lower than 0.75 mg/mL had little effect on cell vaibility, and thus, higher concentrations (up to 1.5 mg/mL) were used. Cell viability was reduced after the incubation of the human hepatoma cells with the strawberry or plum extracts (**Figure 1D,E**). That reduction showed a dose- and time-dependent fashion. After 4 h of treatment with 0.4 mg/mL strawberry extract or 1 mg/mL plum extract, a significant reduction of cell viability was observed as compared to that of lower doses (28 and 22.5%, respectively). After 18 h of treatment, the increase of cell death rate was over those ranges at lower concentrations than after 4 h of treatment (0.2 mg/mL for strawberry extract and 0.75 mg/mL for plum extract, respectively). The cell death rate of strawberry extract was higher than that of plum extract. IC$_{50}$ was estimated to be of 0.6 mg/mL for strawberry extract and of 1.5 mg/mL for plum extract at 18 h of treatment; in contrast, IC$_{50}$ was not reached, even with the highest concentrations assayed, after 4 h of treatment.

Cell Morphology. Treatment of HepG2 cells with the pure polyphenols or the fruit extracts had a different effect on cell morphology (**Figure 2**). Quercetin treatment for 18 h led to apoptotic changes such as cell shrinkage and "blebbed" surfaces due to the convolutions of nuclear and plasma membranes and
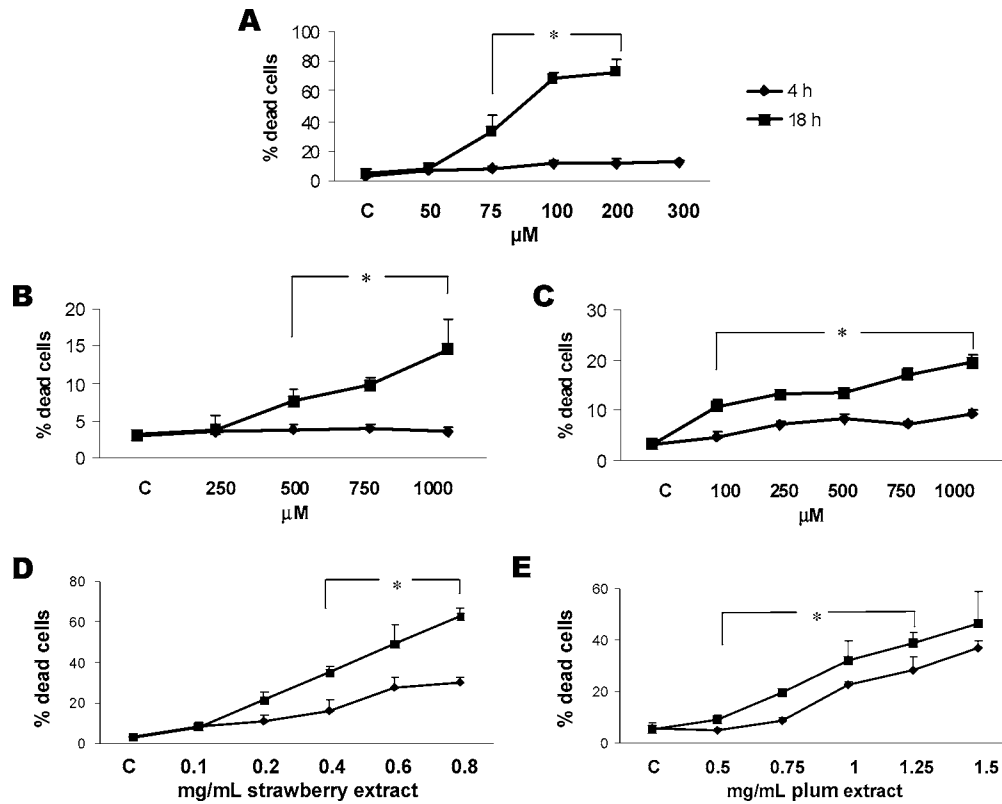
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Figure 1. Effect of quercetin (**A**), cholorogenic acid (**B**), (−)-epicatechin (**C**), and strawberry (**D**) and plum (**E**) extracts on cell viability. HepG2 cells were treated with different concentrations of each pure polyphenol for 4 (◆) and 18 h (■). Cell viability was determined by trypan blue staining as a percentage of dead cells in the total number of cells counted. Data represent means ± SD of 6–8 separate experiments. Statistical differences as compared to 4 h of treatment ($P < 0.05$) are noted as asterisks.
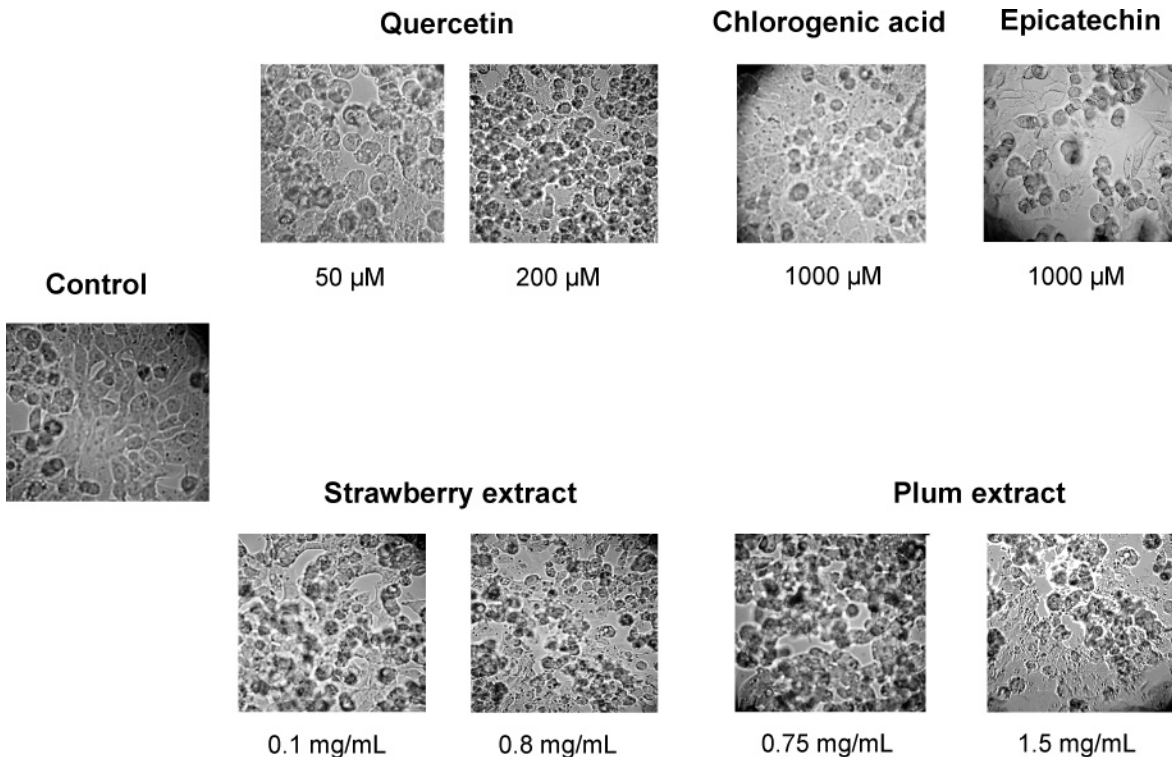

Figure 2. Morphological changes of HepG2 cells after 18 h of treatment with the pure polyphenols, quercetin, cholorogenic acid, and (−)-epicatechin, and the fruit extracts, strawberry and plum (200× magnification).

chromatin condensation. At 200 μM, those changes became more evident and the shrunken and convoluted cells with oval or rounded shape began to pinch together to form the apoptotic bodies; broken cells and debris were observed.

Incubation of the human hepatoma cells for 18 h with the highest concentrations of fruit extracts (0.8 mg/mL for strawberry or 1.5 mg/mL for plum) resulted in an apoptotic morphology; that is, rounded shape cells and debris were
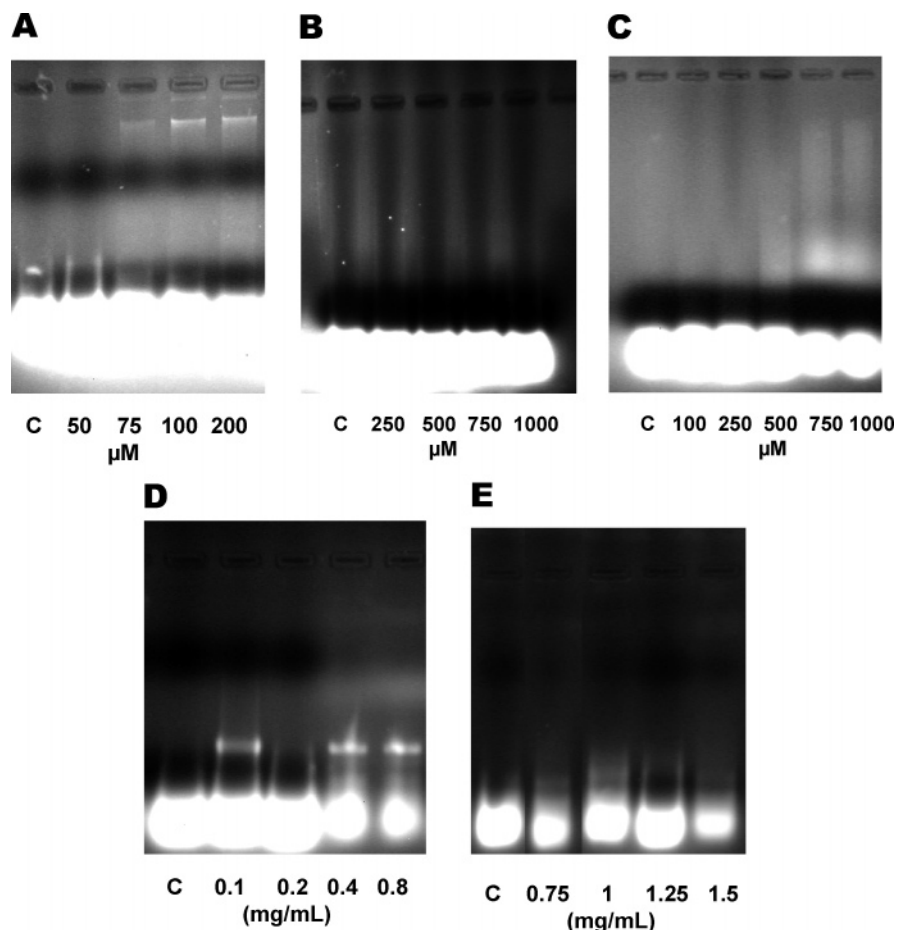
Figure 3. Effect of quercetin (**A**), cholorogenic acid (**B**), (−)-epicatechin (**C**), and strawberry (**D**) and plum (**E**) extracts on DNA fragmentation detected by agarose gel electrophoresis. HepG2 cells were treated with the different concentrations of each pure polyphenol during 18 h. Three independent experiments were carried out with similar results. A representative experiment is shown.

observed, contrary to what was observed when lower concentrations were used. The morphological changes in HepG2 cells after the treatment with (−)-epicatechin or cholorogenic acid for 18 h appeared to be very slight, and only a small percentage of cells acquired a rounded shape.

Apoptosis Induction. In view of the above-mentioned effects on cell viability and morphological features, it was examined whether those pure polyphenols or fruit extracts induced apoptosis in the human hepatoma cell line. To simultaneously demonstrate decreased cell viability, apoptosis, and cell cycle arrest, the same concentrations were used in all assays.

Pure polyphenols had different effects on the induction of apoptosis. Parallel to the effect on cell viability, treatment of HepG2 cells with quercetin and (−)-epicatechin for 18 h resulted in internucleosomal DNA fragmentation, a characteristic of apoptosis (**Figure 3A**,**C**). In fact, the appearance of DNA ladder was observed after 18 h of treatment with 50 μM quercetin and 500 μM (−)-epicatechin and became more obvious at higher concentrations. DNA fragmentation was not detected after treatment with cholorogenic acid even at concentrations as high as 1 mM (**Figure 3B**), which agreed with the little effect of this phenol on cell death rate. Because (−)-epicatechin and cholorogenic acid did not show a prominent effect on cell viability and apoptosis was only induced at very high concentrations (corresponding to pharmacological doses in vivo), only quercetin was assayed in a 4 h treatment. Treatment of HepG2 cells with 100 μM quercetin for 4 h resulted in internucleosomal DNA fragmentation (see **Figure 4A**).

Concerning the effect of fruit extracts, induction of nuclear fragmentation was observed after 18 and 4 h of treatment with both fruit extracts. The appearance of DNA ladder was observed after 18 h of treatment with 0.1 mg/mL strawberry or 0.75 mg/mL plum extracts (**Figure 3D**,**E**), and this fragmentation became more obvious at higher concentrations. DNA laddering appeared also after 4 h of treatment with 0.2 or 1.25 mg/mL for the strawberry or plum extracts, respectively (**Figure 4B**,**C**). As happened with the cell viability assay, DNA fragmentation was not so obvious after 4 h of treatment as it was after 18 h of treatment and, again, it seemed that strawberry extract was more effective than plum extract for the apoptosis induction at both 4 and 18 h of treatment.

Effects on Cell Cycle Progression. The effects of the pure polyphenols and fruit extracts on cell cycle progression were examined. In agreement with the previous analysis (cell viability and DNA fragmentation), the pure polyphenols had different effects on the HepG2 cell cycle. The treatment of these cells for 18 h with quercetin resulted in a marked accumulation of cells in sub-G1 phase (hypodiploid peak) and a slight reduction in G1 and G2/M and S phases (**Figure 5** and **Table 1**). When the cells were treated with the different concentrations of quercetin, the percentage of cells undergoing apoptosis increased from <1% in controls to 9% at 50 μM and 18% at 200 μM, whereas cells in G1 decreased from 66 (controls) to 57% at 200 μM and those in G2/M and S phases decreased from 34 (controls) to 26% at 200 μM. This result suggests that quercetin
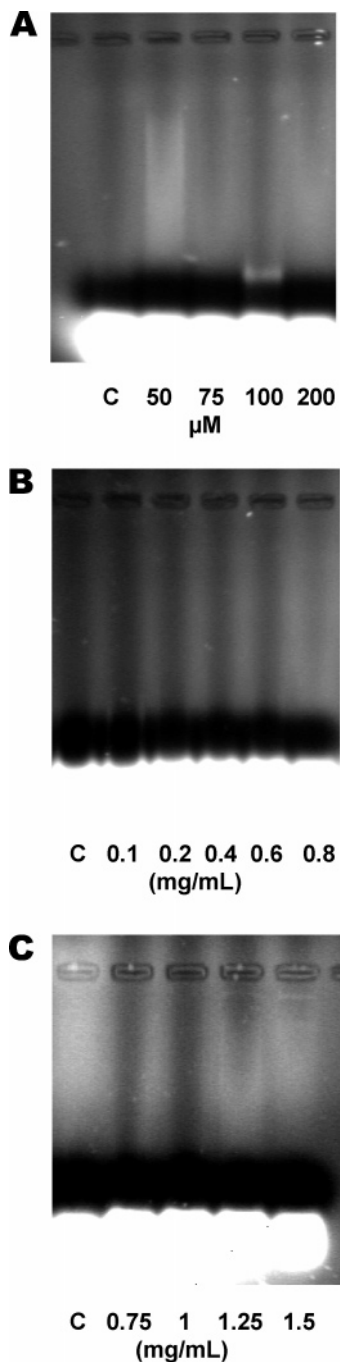
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Figure 4. Effect of quercetin (**A**) and strawberry (**B**) and plum (**C**) extracts on DNA fragmentation detected by agarose gel electrophoresis. HepG2 cells were treated with the different concentrations during 4 h. Three independent experiments were carried out with similar results. A representative experiment is shown.

induces apoptosis shown as an accumulation of HepG2 cells at the sub-G1 phase in a dose-dependent fashion.

(−)-Epicatechin and cholorogenic acid, which showed not so prominent effects on viability and apoptosis in HepG2 cells after a 18 h treatment, did not alter the hypodiploid peaks nor G1, G2/M, and S phases on the histogram of flow cytometry even at the highest concentrations used (**Figure 5** and **Table 1**).

Both fruit extracts influenced the cell cycle progression (**Figure 6** and **Table 2**). The treatment of HepG2 cells with 0.2 mg/mL of strawberry extract for 18 h resulted in a decreased proportion of cells in the G1 phase, from 66% in controls to

51% at 0.2 mg/mL, and a concomitant increase in the hypo-diploid peak, from 1% in controls to 17% at 0.2 mg/mL. Similarly, quantitative analysis of flow cytometry of cells treated with plum extract showed a relevant increase of cells in the sub-G1 phase (1.35% in controls and 7.7% at 0.75 mg/mL) and a concomitant reduction in G2/M-S phases (35% in controls and 24% at 0.75 mg/mL) suggesting induction of apoptosis (**Figure 6** and **Table 2**). It is worth noting that the highest concentrations of the strawberry (0.6 and 0.8 mg/mL) and plum (1.25 and 1.5 mg/mL) extracts showed a lower increase of cells in the hypodiploid peak than smaller concentrations of strawberry and plum extracts (6.2% at 0.8 mg/mL and 3.28% at 1.5 mg/mL, respectively), which can be considered as an apparent aberrant cell cycle profile. The strawberry extract was more efficient than the plum extract since the percentage of cells undergoing apoptosis was higher with lower concentrations for the berry extract.

The effect of quercetin and fruit extracts on the HepG2 cell cycle was also analyzed after 4 h of incubation. Although treatment of HepG2 cells with quercetin for 4 h resulted in internucleosomal DNA fragmentation at 100 μM, as shown above, when the cell cycle was studied, there was an accumulation of cells in the G1 phase, from 57% in controls to 69% at 200 μM, and a reduction in G2/M and S phases, from 43% in controls to 31% at 200 μM (**Figure 7** and **Table 3**). Treatment of HepG2 cells with the fruit extracts for 4 h resulted in a slight DNA fragmentation, and interesting changes were also found in the cytometric analysis. A very slight increase in the percentage of cells undergoing apoptosis (hypodiploid peak) was observed, increasing from 1.19% in controls to 1.63% with 0.8 mg/mL of strawberry extract and to 3.0% with 1.5 mg/mL of plum extract. Also, a concomitant increase in the G1 phase (57% in controls and 63% with strawberry extract at 0.8 mg/mL and 64% with plum extract at 1.5 mg/mL) and a decrease in G2/M and S phases (43% in controls and 37% with strawberry extract at 0.8 mg/mL and 33% with plum extract at 1.5 mg/mL) were observed (**Figure 7** and **Table 3**).

DISCUSSION

Many studies in different cell lines, animal models, and human epidemiological trials have shown the potential of dietary polyphenols as antiproliferative agents (*7*, *12*). Uncontrolled cell growth is a multistage genetic change affecting protooncogenes or tumor suppressor genes, and that sequence of events has many steps for intervention with the aim of preventing, slowing down, or reversing the process. One of the important targets as a preventive approach is the induction of apoptosis. This form of programmed cell death is defined by a set of characteristic morphological and biochemical features. The present study was carried out to investigate the role of different pure polyphenols and natural fruit extracts in the viability and the apoptotic process in HepG2 cells.

As quoted above, the phenolic compounds used in this study, quercetin, cholorogenic acid, and epicatechin, are common dietary polyphenols. Quercetin is present in varying amounts in most fruits and vegetables, chlorogenic acid is found in cherry, kiwi, and plum, and (−)-epicathechin is abundant in green tea and cocoa (*20*). The antiproliferative effect of green tea and cocoa extracts has been previously studied (*21*−*23*). Strawberry and plum, two of the most consumed fruits, are characterized for having high contents in pelargonidin and chlorogenic acid, respectively, quercetin being the second most abundant polyphenol in both fruits (*24*, *25*).

Quercetin concentrations that significantly decreased HepG2 cell viability have been previously reported to have similar

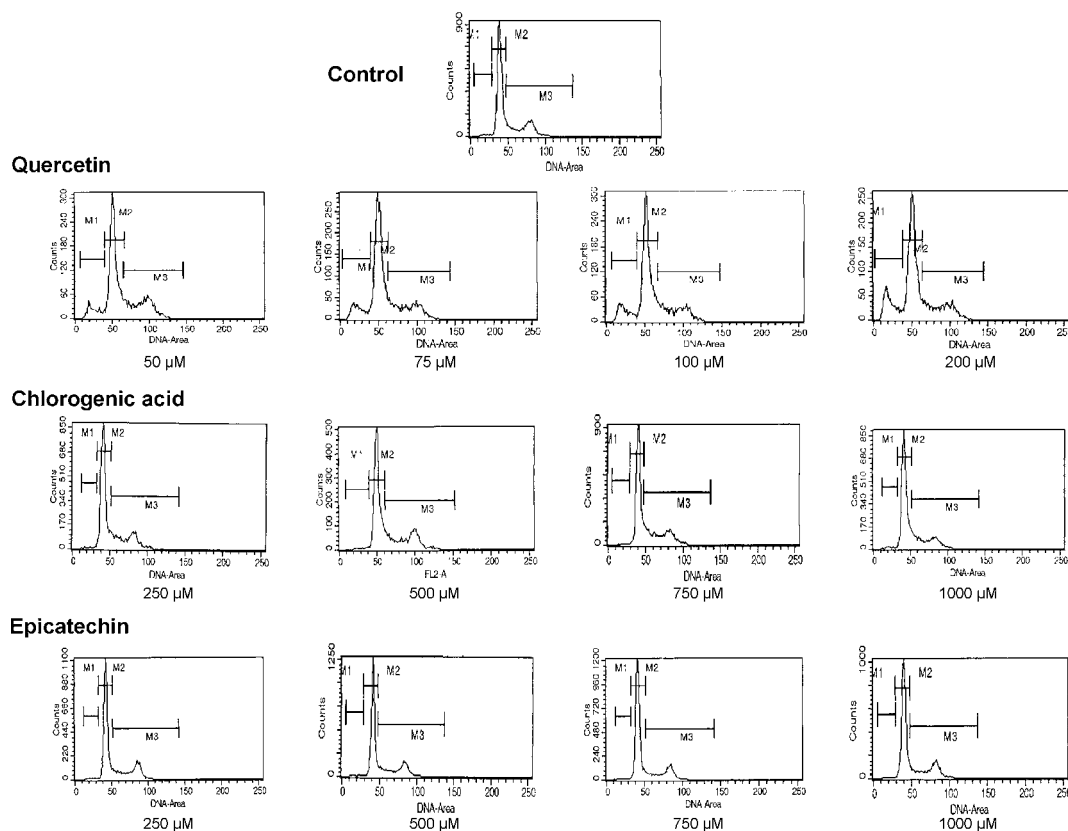**1276** *J. Agric. Food Chem.,* Vol. 53, No. 4, 2005

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Figure 5. DNA content frequency histograms of HepG2 cells after treatment with different concentrations of pure polyphenols [quercetin, cholorogenic acid, and (−)-epicatechin] during 18 h. Cells were stained with propidium iodide, harvested, and submitted to flow cytometry analysis. Cell cycle sub-G1 (or hypodiploid peak), G1, G2/M-S phases were named M1, M2, and M3, respectively. A representative histogram from two separate experiments is shown.

Table 1. Effect of the Pure Polyphenols [Quercetin, Cholorogenic Acid, and (−)-Epicatechin] on DNA Cell Cycle Analysis in HepG2 Cells after 18 h of Treatment with Different Concentrations[a]

	concn (μM)	% sub-G1 phase	% G1 phase	% G2/M-S phases
control		0.83	66.08	34.11
quercetin	50	8.93	60.04	32.75
	75	11.72	61.16	28.52
	100	12.05	61.30	27.70
	200	17.45	57.28	26.31
chlorogenic acid	250	1.12	65.47	34.71
	500	1.41	62.20	36.53
	750	0.77	61.08	41.92
	1000	1.60	67.13	32.68
(−)-epicatechin	250	0.91	64.92	35.27
	500	0.62	63.68	38.93
	750	0.91	70.98	28.93
	1000	1.00	66.27	35.47

[a] Cells were stained with propidium iodide, harvested, and submitted to flow cytometry analysis.

effects in other cancer cell cultures, such as human colon cancer (*26*), ovarian cancer (*27*), breast cancer (*28*), leukemia (*29*), human lung cancer (*30*), and murine hepatoma (*31*), and in vivo, ingestion of a quercetin enriched diet (2%) decreased the frequency of tumors in azoxymethane treated mice (*32*). Thus, our results with quercetin support an observed viability effect on tumor cells of this flavonoid where a small percentage of secondary necrosis after an initial apoptotic cell death cannot be ruled out.

(−)-Epicatechin and chlorogenic acid seemed not to have a very obvious effect on HepG2 cell death. Numerous studies have

demonstrated the cancer protective effect of tea or cocoa polyphenols in cancer cell lines from lung, pancreas, or colon (*21*, *33*), as well as in mammary and pancreatic tumorigenesis in rats (*23*). Nevertheless, just a few studies have evaluated the effect of (−)-epicatechin alone; in agreement with our results, (−)-epicatechin showed no growth inhibitory effect neither on human colon carcinoma LoVo cells, on the contrary, it seemed to slightly promote the cell proliferation (*34*), nor in human breast, central nervous system, and lung cancer cells (*35*). Concerning chlorogenic acid, just a few references have been found, but recently, its preventive role has been demonstrated in human colon cancer (*5*), leukemia (*36*), and oral tumor cell lines (*37*).

The cell viability effect of strawberry and plum extracts, as well as quercetin, on the HepG2 cells decreased in a dose-dependent manner. Such an effect has been previously reported in this human hepatoma cell line and other cancer cell cultures, i.e., breast, cervical, leukemia, and prostate cancer cells (*8*, *38*, *39*). Moreover, Sun et al. (*10*) have evaluated the potential antiproliferative activities of 11 different fruit extracts in HepG2 cells, and it can be highlighted that strawberry showed one of the highest inhibitory effects on cell proliferation, which supports the higher efficiency of the strawberry extract observed in our study when compared with the plum extract.

Cell proliferation could be balanced by the apoptotic process and represents an efficient way to eliminate damaged cells. Programmed cell death is characterized by morphological and biochemical changes in the cell, i.e., DNA fragmentation (*13*). Quercetin induced morphological alterations in HepG2 and the appearance of DNA ladder according to other studies carried out in different human hepatocarcinoma, leukemia (*40*, *41*) cell

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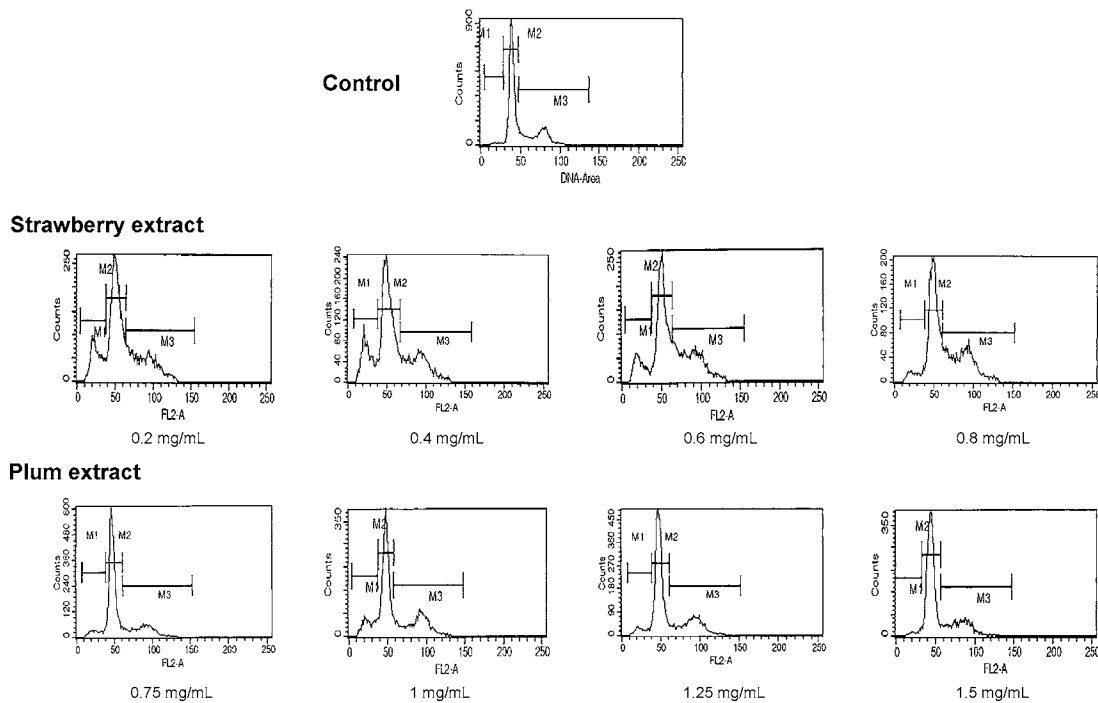*J. Agric. Food Chem.,* Vol. 53, No. 4, 2005   **1277**

Figure 6. DNA content frequency histograms of HepG2 cells after treatment with different concentrations of fruit extracts (strawberry and plum) during 18 h. Cells were stained with propidium iodide, harvested, and submitted to flow cytometry analysis. Cell cycle sub-G1 (or hypodiploid peak), G1, G2/M-S phases were named M1, M2, and M3, respectively. A representative histogram from two separate experiments is shown.

Table 2. Effect of Fruits Extracts (Strawberry or Plum) on DNA Cell Cycle Analysis in HepG2 Cells after 18 h of Treatment with Different Concentrations[a]

	concn (mg/mL)	% sub-G1 phase	% G1 phase	% G2/M-S phases
control		1.35	66.15	35.45
strawberry extract	0.2	17.15	51.35	31.91
	0.4	18.36	53.56	29.46
	0.6	12.56	49.99	37.83
	0.8	6.20	51.52	42.55
plum extract	0.75	7.68	68.34	24.43
	1.00	14.55	53.95	33.14
	1.25	6.82	64.29	29.32
	1.50	3.28	69.54	27.52

[a] Cells were stained with propidium iodide, harvested, and submitted to flow cytometry analysis.

lines, and mouse thymocytes (*42*). (−)-Epicatechin and chlorogenic acid seemed to neither induce the internucleosomal DNA fragmentation nor the apoptotic changes in the cell morphology as previously shown in human colon carcinoma and oral tumor cells (*34, 36, 37*). Studies about induction of DNA laddering with pharmacological doses with (−)-epicatechin have not been found.

The apoptotic morphological and biochemical changes assayed for the strawberry and plum extracts were more evident after 18 h of treatment, as well as for quercetin. Such effects have not been reported before, although the apoptotic potential of those fruits is known (*8, 10, 38, 39*).

It is well-known that apoptosis is generally caused by cell cycle arrest in the G1/S phase or G2/S phase (*2*). In this study, quercetin and fruit extracts, rather than chlorogenic acid and (−)-epicatechin, induced apoptosis after 18 h, shown as an increased hypodiploid peak. The results at 4 h seemed to indicate that this apoptotic effect was preceded by an accumulation of cells in the G1 phase. Accordingly, some studies have shown that quercetin can interfere with tumor development by arresting

proliferation in G1 of cells from human gastric, endometrial, and nasopharyngeal cancer in culture (*43−45*), whereas no references were found for strawberry and plum related to the cell cycle. In addition, this blockage seems to be quite specific of cells with high proliferative capability (*2*). Interestingly, the highest concentrations of strawberry and plum extracts induced an apparent aberrant cell cycle profile, consisting of a lower increase of cells in the hypodiploid peak than with smaller concentrations. When trying to correlate the proliferative effect to the antioxidant capacity of polyphenolic extracts, other authors have also found this aberrant response (*7, 10, 46*). Nevertheless, these studies highlighted that the antioxidant capacity could not be associated with the antiproliferative effect; thus, this activity could not be correlated with the capability for inducing apoptosis (*7, 10, 46*); those effects seem to be associated to the degree of polymerization and galloylation (*7, 35, 46*).

Chlorogenic acid and (−)-epicatechin had little modifying effects on cell viability, cell cycle progression, and apoptosis assays in the present study. (−)-Epicatechin has been previously reported to have minor effects in other cancer cell cultures, such as different human colon cancer cell lines (*34, 46*), although tea and cocoa flavanols and procyanidins induced apoptosis in different colon and lung human cancer cell lines (*21, 22*) and arrested the G2/M phase in the cell cycle progression in colon cancer cells (*22*) or the G0/G1 phase in human prostate carcinoma cells (*47*). Altogether, the results suggest that chlorogenic acid and (−)-epicatechin may exert their tumor inhibitory activity through a different mechanism rather than the direct induction of apoptosis in tumor cells. As previously suggested, these dietary polyphenols may elevate the activity of enzymes having the capacity to enhance carcinogen detoxification (*5, 14, 35*) and scavenge free radicals (*6, 13, 15*) or inhibit tumor angiogenesis (*3, 47, 48*).

Although plum extract was able to induce a reduction of cell viability and apoptosis in HepG2 cells and its main polyphenol
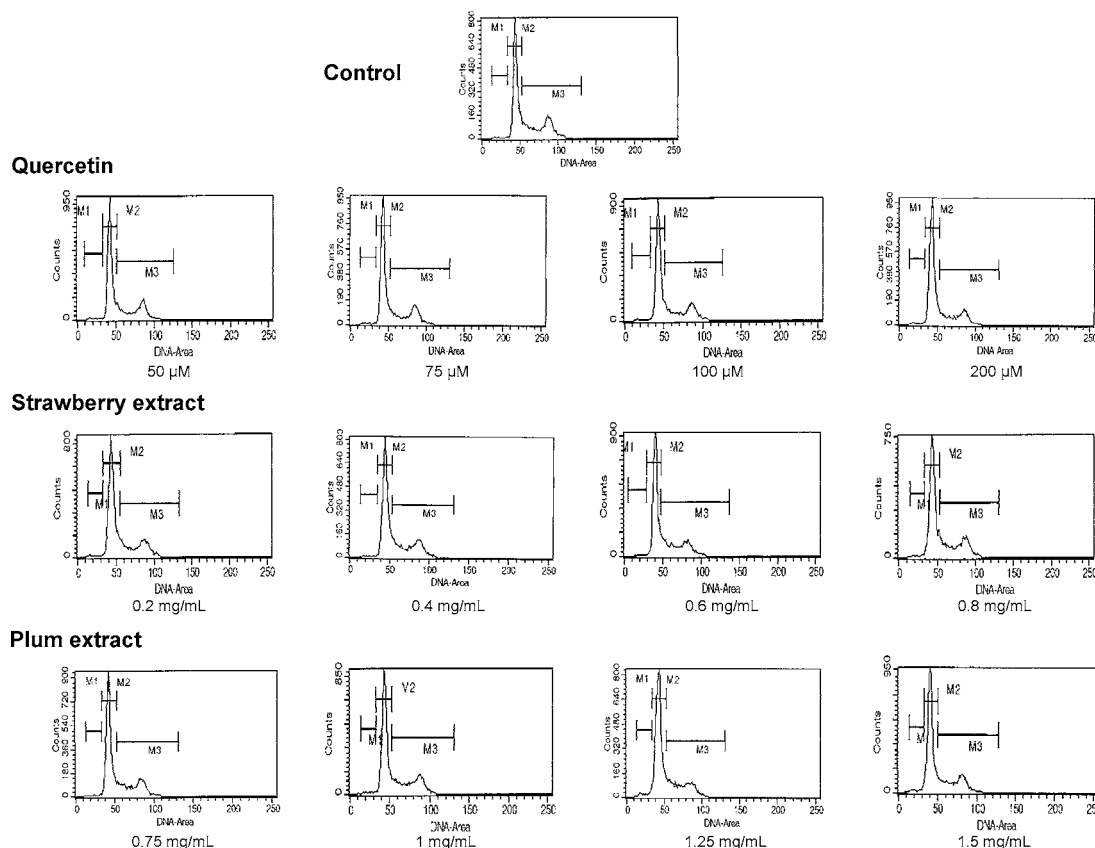
Figure 7. DNA content frequency histograms of HepG2 cells after treatment with different concentrations of quercetin and fruit extracts (strawberry and plum) during 4 h. Cells were stained with propidium iodide, harvested, and submitted to flow cytometry analysis. Cell cycle sub-G1 (or hypodiploid peak), G1, G2/M-S phases were named M1, M2, and M3, respectively. A representative histogram from two separate experiments is shown.

Table 3. Effect of Quercetin or the Fruits Extracts (Strawberry or Plum) on DNA Cell Cycle Analysis in HepG2 Cells after 4 h of Treatment with Different Concentrations[a]

	concn	% sub-G1 phase	% G1 phase	% G2/M-S phases
control		1.19	57.09	43.25
quercetin (μM)	50	1.14	63.44	36.36
	75	1.26	64.66	35.38
	100	1.16	63.64	36.76
	200	1.64	68.79	30.77
strawberry extract (mg/mL)	0.2	1.20	66.18	32.69
	0.4	1.13	61.62	38.96
	0.6	1.42	63.74	34.84
	0.8	1.63	62.80	37.03
plum extract (mg/mL)	0.75	1.12	63.55	36.60
	1.00	1.51	61.59	37.95
	1.25	3.12	65.50	32.99
	1.50	3.02	64.08	33.29

[a] Cells were stained with propidium iodide, harvested, and submitted to flow cytometry analysis.

is chlorogenic acid, the pure flavonoid did not show the same effects. However, it has been suggested that the combination of bioactive compounds and synergistic mechanisms in the fruit matrix might be responsible for the potent activities (*10*, *20*).

Quercetin and fruit extracts (strawberry and plum) induced a specific G1 arrest in the cell cycle progression of human hepatoma HepG2 as a previous step for apoptosis, but the biochemical mechanism of this blockage remains unknown, and further studies to clarify the regulation of their cytotoxic action are necessary. Finally, the reason for the differences among the pure polyphenols and fruit extracts in the regulation of the

induction of apoptosis and cell cycle in HepG2 cells is not yet clear and remains a current topic of investigation in our laboratory.

In summary, the effects of naturally occurring plant polyphenols on cell viability and apoptosis in a human hepatoma cell line (HepG2) have been evaluated. Quercetin and natural fruit extracts (strawberry and plum), rather than chlorogenic acid and ($-$)-epicatechin, showed apoptosis-inducing effects, and these results suggest that those compounds might exert a tumor preventive action through apoptosis- and/or cell proliferation-dependent mechanisms. This work provides an initial tool to study the molecular mechanisms and signals involved in the programmed cell death induced by those polyphenols.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; h, hour; IC$_{50}$, 50% of cell death rate; PBS, phosphate-buffered saline; RNase, ribonuclease.

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