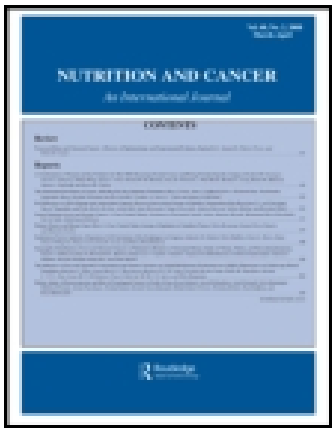

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Cultured rat hepatocytes and human hepatoma HepG2 cells were used to evaluate the hepatoprotective properties of polyphenolic extracts from the edible part of artichoke (AE). The hepatocytes were exposed to H_2O_2generated *in situ* by glucose oxidase and were treated with either AE, or pure chlorogenic acid (ChA) or with the well known antioxidant, N,N′-diphenyl-*p*-phenilenediamine (DPPD). Addition of glucose oxidase to the culture medium caused depletion of intracellular glutathione (GSH) content, accumulation of malondialdehyde (MDA) in the cultures, as a lipid peroxidation indicator, and cell death. These results demonstrated that AE protected cells from the oxidative stress caused by glucose oxidase, comparable to DPPD. Furthermore, AE, as well as ChA, prevented the loss of total GSH and the accumulation of MDA. Treatment of HepG2 cells for 24 h with AE reduced cell viability in a dose-dependent manner, however, ChA had no prominent effects on the cell death rate. Similarly, AE rather than ChA induced apoptosis, measured by flow cytometric analysis of annexin and by activation of caspase-3, in HepG2 cells. Our findings indicate that AE had a marked antioxidative potential that protects hepatocytes from an oxidative stress. Furthermore, AE reduced cell viability and had an apoptotic activity on a human liver cancer cell line.

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Abbreviations: ChA, chlorogenic acid; AE, artichoke extracts; GSH, reduced glutathione; DPPD, N,N′-diphenyl-*p*-phenylenediamine; MDA, malondialdehyde; ROS, reactive oxygen species; PI, propidium iodide; Ptx, paclitaxel.

INTRODUCTION

The presence of phytochemicals, in fruits and vegetables, has been considered of significant nutritional importance in the prevention of cancer, cardiovascular diseases, diabetes, and inflammatory diseases (1-3). Many of these phytochemicals have been found to possess antioxidant activities which are more potent than vitamins C and E and carotenoids within the same food (4,5). Synergistically or additively, these dietary antioxidants provide bioactive mechanisms to reduce free radical induced oxidative stress which results from either a decrease of natural cell antioxidant capacity or an increased production of reactive oxygen species (ROS) in organisms. Therefore for a preventive point of view, a constant supply of phytochemicals, in particular polyphenols, is essential to provide preventive and defensive mechanisms to reduce the risk of chronic diseases in human beings (6).

The current growing interest for natural antioxidants has led to a renewed attention on artichoke, because of its high polyphenolic content. Artichoke polyphenols are up to 2% of the fresh weight, mainly represented by glycoside forms of flavonoids (0.2–0.5%) such as apigenin and luteolin in the leaves and hydroxycinnamic derivatives (0.5–1.5%) in the edible part, mainly represented by mono- and di-caffeoylquinic acids (7,8). Extracts from artichoke leaves (*Cynara scolymus* L.), have been used in folk medicine against liver complaints and the total extracts or its constituents have been claimed to exert a beneficial action against hepato-biliary diseases (9–12) and to improve liver regeneration after partial hepatectomy (13,14).

It has been reported that phytochemicals have anticarcinogenic, antimutagenic and antiinflammatory activities (15–17). They can also trigger apoptosis that may be an efficient strategy for cancer prevention and treatment. Carcinogenesis is a multistage process with an accumulation of genetic alterations; thus, targets for chemoprevention could be multiple (18). Suppression of cell proliferation and induction of differentiation and apoptosis are important preventive mechanisms. Nevertheless, the modulation and induction of apoptosis by natural products are still unclear. The common dietary polyphenolic compounds can be absorbed through the gastrointestinal tract, reaching the liver, where they are mainly metabolized. Thus, this is one of the main target organs where polyphenols can exert their action.

The purpose of this study was 1) to evaluate the potential antioxidative and hepatoprotective properties of the edible part of artichoke against oxidative stress in cultured rat hepatocytes by monitoring the cellular content of glutathione, the production of MDA and the amount of cell killing and 2) to investigate the effect of polyphenolic fraction of artichoke on the viability and apoptosis induction on a human hepatoma, HepG2, a continuously growing transformed cell line.

MATERIALS AND METHODS

Cell Cultures

Male Sprague-Dawley (150–200 g) rats, obtained from Charles River Breeding Laboratories Calco, Italy, were used as hepatocytes donors. Animal handling was performed according to guidelines of the Italian Ministry of Public Health. The animals were fed *ad libitum* and fasted overnight prior to use. Isolated hepatocytes were prepared by collagenase (Roche Diagnostics, Monza Italy) perfusion according to Seglen (19). The hepatocytes were seeded and grown in Williams E medium (Invitrogen Life Technologies, Milan, Italy) containing 10 IU/ml of penicillin, 10 μg/ml streptomycin, 0.5 mg/ml gentamycin, 0.02 U/ml of insulin, and 10% heat-inactivated fetal calf serum at 37°C in humidified atmosphere of 5% CO_2-95% air.

HepG2 cells, a human hepatoma cell line, purchased from American Type Culture Collection, Rockville, MD, USA, were grown in D-MEM (Invitrogen Life Technologies, Milan, Italy) supplemented with 10% fetal calf serum, 10 IU/ml of penicillin and 10 μg/ml streptomycin at 37°C in humidified atmosphere of 5% CO_2-95% air.

Reagents

Glucose oxidase, grade I (Sigma-Aldrich, Milan Italy) was dissolved in PBS. Artichoke extracts, prepared as described below, and chlorogenic acid (Sigma-Aldrich, Milan Italy) were dissolved in PBS and 0.1% Me_2SO (Sigma-Aldrich, Milan Italy), DPPD (Eastman-Kodak Co. Rochester N.Y.,U.S.A.) was dissolved in 0.25% Me_2SO, Ptx (Sigma-Aldrich, Milan Italy) was dissolved in PBS.

Cell Treatments

The hepatocytes were plated in 25 cm^2 plastic flasks (Sarstedt, Numbrecht, Germany) at the density of 1.33×10^6 in 4 ml of Williams E medium. After incubation of 24 h at 37°C, the cells were washed with prewarmed Hepes buffer, pH 7.4 (0.14 M NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$ and 2.4 mM Hepes), to remove unattached dead cells. Complete William E was replaced (5 ml), and the cells were incubated for 24 h. The cultures were washed, placed in Williams E without fetal calf serum and treated with different concentrations of glucose oxidase (from 0.025 to 0.2U/ml), ChA (1 mM), AE (1 mM) and DPPD (1 μM) for 60 minutes.

HepG2 cells were seeded on sixwell dishes (Sarstedt, Numbrecht, Germany) at a density of 2×10^5/ well, after 24 h were changed to serum-free medium, treated with ChA (400, 800, 1200 μM), and either AE (400, 800, 1200 μM), or Ptx (1.5 μM) for 24 h, 48 h or 72 h .

H_2O_2Determination

The amount of $H_2O_2$was determined by direct measurement of absorbance at 240 nm by using a calibration curve from 0.1 to 30 mM ($R^2 = 0{,}9939$) according to Beers and Sizer (20).

The H_2O_2 production was determined after an incubation for 1 h at 37°C of glucose oxidase at concentrations between 0.025 U/ml and 10 U/ml in William E medium containing 11 mM glucose. Data were interpolated with a regression curve ($R^2 = 0{,}9974$).

Cell Viability, Intracellular GSH and Lipid Peroxidation Measurement

Viability of cultured cells was determined by means of trypan blue exclusion. The GSH content of the hepatocytes was determined according to Sedlak and Lindsay (21). Protein concentration was determined by a Bio-Rad protein assay kit (Bio-Rad Milan, Italy) using bovine serum albumin as standard. Malondialdehyde concentration was measured fluorometrically by the adaptation of the method of Yagi (22) as described previously by Miccadei (23). Briefly, the fluorescence of MDA was read in a Perkin-Elmer LS-5 spectrofluorometer at 515 nm excitation and 553 nm emission. Standards were prepared by diluting

malondialdehyde bis (dimethyl) acetate (Sigma-Aldrich Milan Italy) 1:1000 in methanol. The resulting solution was mixed 1:1 with 0.2 N HCl and left overnight at room temperature; 1.65 ml was added to 8.35 ml of methanol. Appropriate aliquots of this solution containing 1.25 to 5 nmol of malondialdehyde were added to 4.5 ml of Williams E medium without fetal calf serum and treated as the regular cultures. In all cases the excitation and emission spectra of the experimental samples were identical to those of the standards.

Artichoke Extracts Preparation

Ten fresh artichoke buds (cv. Violetto di Provenza) with a weight ranging between 115–125 g were used; buds were deprived of external bracts and the edible part (head) was used for extract preparation. About 25 g of fresh tissue were homogenised and refluxed with boiling methanol (1:5 w/v), twice for 1 h. After filtration through a Whatmann 1 paper filter, methanolic extracts were concentrated to dryness under vacuum to obtain a residue that was suspended and brought to the final volume of 100 ml with methanol/water (1:1 v/v). The phenolic concentration of the extracts was determined by HPLC according to Di Venere et al. (7).

Several aliquots with known concentration of these phenolic extracts, after filtration at 0.45 μm, were concentrated to dryness under vacuum and dissolved with appropriate amounts of PBS/0.1% Me_2SO to give AE used in the experiments. The extracts were stored at -40°C until use.

Detection of Apoptosis by Annexin V-FITC Staining

After overnight culture in D-MEM medium containing 10% fetal bovine serum, HepG2 cells were incubated in serum-free medium and exposed to AE or ChA (from 400 μM to 1200 μM) or Ptx (1.5 μM). The treatments lasted for 24 h. After washing with PBS the cells were harvested and resuspended (1×10^6 cells/ml) in annexin-V binding buffer containing 10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$. Aliquots of cells ($2x10^5$) were incubated with 5 μl of annexin (annexinV-FITC kit Bender Med System GmbH, Vienna, Austria.) for 15 minutes at room temperature in the dark. PI at a final concentration of 1 μg/ml was added to distinguish the necrotic cells. The apoptotic cells (annexinV^+/PI^-) were detected by fluorescence analysis performed using a flow cytometer apparatus (FACS scan, Becton Dickinson, San Josè, CA, USA). The data analysis was performed with a CELLQUEST software.

Western-Blot Analysis

To prepare the whole-cell extract, cells were washed with PBS and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1%NP 40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/ml aproteinin, 170 μg/ml leupeptin, 100 μg/ml PMSF; pH 7.5). After mixing for 30 minutes at 4°C, the mixture was centrifuged (10,000 $\times$ g) for 10 minutes and the supernatants were collected as whole-cell extracts. The protein content was determined with Bio-Rad protein assay reagent using bovine serum albumin as a standard. Western blotting analysis was performed as follows. An equal protein content of total lysates from the control, AE treated samples and Ptx sample was resolved on 10% SDS-PAGE with molecular weight markers (Bio-Rad, Milan, Italy). Proteins were then blotted onto the PVDF membrane (Millipore Billerica, MA, USA) and reacted with cleaved caspase-3 primary antibody (Cell Signaling Technology, Inc, Danvers, MA USA); as well as anti-actin (Sigma-Aldrich Milan, Italy) for protein normalization. The bands were revealed by enhanced chemiluminescence using an ECL detection kit (Amersham Bioscience Europe GmbH, Cologno Monzese Milan Italy). All the data were representative of three independent experiments.

Statistical Analysis

Statistical analysis was obtained by Anova test. The effect of pure compounds and edible artichoke extracts were analysed by repeated measure analysis of variance (Anova). P value <0.05 was considered significant. For each analysis, at least three independent experiments were carried out. Data are given as the means $\pm$ SD.

RESULTS

Artichoke Extracts Composition

The main components detected in the artichoke extracts were chlorogenic acid and two di-caffeoylquinic acids in the ratio about 1:1:1. The total phenolic concentration of the extracts was determined as chlorogenic acid equivalent, estimating also the concentration of the di-caffeoylquinic acids as chlorogenic acid (Table 1).

TABLE 1
Polyphenolic content of artichoke extracts*

Phenolic compound	Artichoke extract concentration (mg L^{-1})
mono-caffeoylquinic acid 1	17 ± 2
mono-caffeoylquinic acid 2	13 ± 1
chlorogenic acid	730 ± 78
mono-caffeoylquinic acid 3	17 ± 2
di-caffeoylquinic acid 1	4 ± 0.5
di-caffeoylquinic acid 2	8 ± 1
di-caffeoylquinic acid 3	16 ± 2
di-caffeoylquinic acid 4	745 ± 62
di-caffeoylquinic acid 5	645 ± 54
luteolin-glycoside	23 ± 5
di-caffeoylquinic acid 6	35 ± 3
apigenin-glycoside	25 ± 5

*The results represent the means $\pm$ SD of three independent extractions and are expressed as mg $L^{-1}$of chlorogenic acid (for mono- and di-caffeoylquinic acids) or luteolin-7-glucoside and apigenin-7-glucoside (for luteolin and apigenin glycosides, respectively).

Determination of Cytotoxicity in Hepatocytes

Monolayers of hepatocytes in cultures for 24 h were washed, placed in fresh medium without serum and exposed to increasing concentrations of ChA (from 0.5 to 2.0 mM), the most representative mono-caffeoylquinic acid, in terms of quantity, in polyphenolic fraction of AE. After 60 minutes the resulting cytotoxic effect was evaluated by trypan blue exclusion and occurred only at concentrations above 1 mM (Fig. 1A). Similarly the same concentration of AE, did not show any appreciable cytotoxicity in the same experimental conditions (Fig. 1C).

H_2O_2-Induced Hepatocyte Death

As previously demonstrated by others (24), cultured rat hepatocytes exposed to H_2O_2 is a suitable model to study the mechanism of cellular injury induced by oxidative stress. Cultured hepatocytes were treated with H_2O_2 generated in the cultured medium by glucose oxidase. The effects on viability are shown in Fig. 1B. The results show that the hepatocyte death is a function of the concentration of glucose oxidase added to the culture medium containing 11 mM glucose. After 60 minutes, cell killing increased progressively with concentrations of glucose oxidase in the range 0.025-0.2 U/ml. With 0.1 U/ml glucose oxidase 50% of the cells had died. Untreated hepatocytes maintained about 95% viability throughout each experiment.

Artichoke Extracts Prevented the Toxicity of H_2O_2

As reported (24), the cytotoxicity of H_2O_2 for cultured rat hepatocytes, after 1 hour of treatment, depends on an oxidative stress and is accompanied by the peroxidation of cellular lipids. The purpose of our study was to use the same model and to evaluate whether the aqueous AE are capable of preventing the toxicity of hydrogen peroxide. Cultured hepatocytes were treated with either 1 mM ChA, or 1 mM AE and immediately exposed to the H_2O_2 generated in the culture medium by 0.1 U/ml G.O. (Fig. 1C). Pretreatment with AE or ChA had the same protective effect (data not shown). Either ChA or AE were proved to have (in the same experimental conditions) no inhibitory effect on glucose oxidase activity and, consequently, on H_2O_2 production (data not shown). After 60 minutes about 50% of the cells were dead in the cultures treated with glucose oxidase alone. The presence of AE prevented by 55% the toxicity of H_2O_2. Even ChA treatment protected the cells although by a lesser extent. The antioxidant DPPD completely protected the hepatocytes from the toxicity of hydrogen peroxide generated by glucose oxidase.

GSH Content in Hepatocytes Treated with Glucose Oxidase

The tripeptide glutathione participates in many cellular functions, including the detoxification of hydrogen peroxide through the activity of the action of glutathione peroxidase. The depletion of GSH shown in Fig. 2 is due to oxidative stress caused by exogenous hydrogen peroxide. After 60 minutes of treatment,

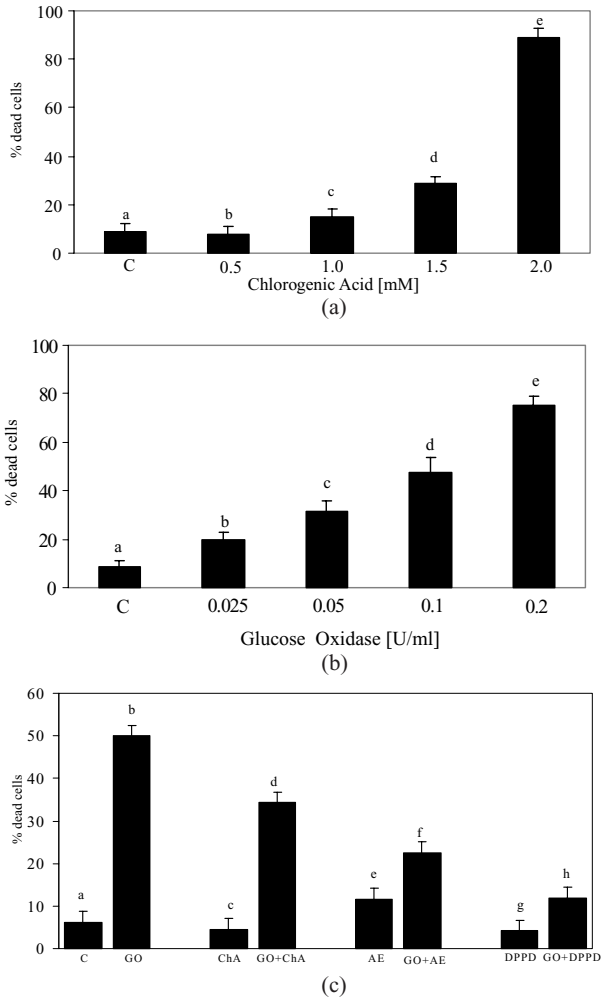

FIG. 1. Effect of Chlorogenic Acid, Glucose Oxidase and Artichoke Extracts on Hepatocytes. (a) Effect of Chlorogenic Acid on Hepatocytes. Cultured hepatocytes were treated with increasing concentrations of ChA. The viability was determined after 60 minutes. Results are the means ± SD of the determinations on three separate cultures. *P* value derived from Anova and Student's *t*-test (a = control, b = 0.5 mM concentration, c = 1.0 mM concentration, d = 1.5 mM concentration, e = 2.0 mM concentration. Significant Effect: a vs. *d*, $P < 0.05$; a vs. *e*, $P < 0.001$); (b) Dose Dependence of the Killing of Cultured Hepatocytes by Glucose Oxidase. Cultured hepatocytes were treated with increasing concentrations of Glucose Oxidase. Control cultures were maintained without any addition. The viability was determined after 60 minutes. Results are the means ± SD of the determinations on three separate cultures. *P* value derived from Anova and Student's *t*-test (a = control, b = 0.025 U/ml concentration, c = 0.05 U/ml concentration, d = 0.1 U/ml concentration, e = 0.2 U/ml concentration. Significant Effect: a vs. b, $P < 0.003$; a vs. c, $P < 0.0001$; a vs. d, $P < 0.001$; a vs. e, $P < 0.0001$); (c) Protective Effect of Artichoke Extracts on Hepatocytes Exposed to Glucose Oxidase. Cultured hepatocytes were treated with 0.1 U/ml Glucose Oxidase (GO) alone or GO plus 1 μM DPPD or GO plus 1 mM ChA or GO plus 1 mM AE, as chlorogenic equivalents. After 60 minutes the viability of the cells was measured. The results are the means ± SD of the determinations on three separate cultures. *P* value derived from Anova and Student's *t*-test (a = control, b = GO, c = ChA, d = GO+ChA, e = AE, f = GO+AE, g = DPPD, h = GO+DPPD. Significant Effect: a vs. b, $P < 0.0001$; c vs. d, $P < 0.0001$; e vs. f, $P < 0.005$; g vs. h, $P < 0.005$).

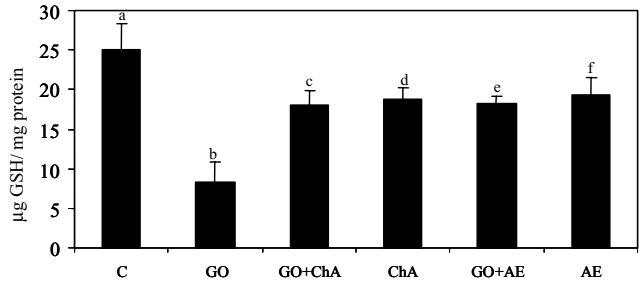

FIG. 2. Influence of Artichoke Extracts on GSH Depletion in Hepatocytes Treated with Glucose Oxidase. Cultured hepatocytes were treated with 0.1 U/ml GO alone or GO plus 1 mM ChA or GO plus 1 mM AE, as chlorogenic equivalents. After 60 minutes the cells were washed with Hepes Buffer to remove unattached dead cells and the GSH content of the hepatocytes was determined on attached cells. Results are the means ± SD) of the determinations on three separate cultures. P value derived from Anova and Student's t-test (a = control, b = GO, c = GO+ChA, d = ChA, e = GO+AE, f = AE. Significant Effect : a vs. b, $P < 0.0001$; a vs. c, $P < 0.05$; a vs. d, $P < 0.01$; a vs. e, $P < 0.05$; a vs. f, $P < 0.001$).

GSH content decreased by 70%. The presence of AE in the cultures prevented the loss of intracellular glutathione in parallel with its protective effect on cell viability. In a similar manner ChA protected the cells from depletion of GSH caused by H_2O_2.

Lipid Peroxidation in Hepatocytes Treated with Glucose Oxidase

The cell killing induced by $H_2O_2$was accompanied by detectable peroxidation of cellular lipids. It has been shown that an accumulation of malondialdehyde is a sensitive indicator of the presence of lipid peroxidation in cultured hepatocytes, in addition malondialdehyde accumulation correlates with other measurements of the peroxidation of lipids, such as the presence of conjugated dienes in cellular phospholipids (25).

In this study hepatocytes were treated with 0.1 U/ml glucose oxidase for 60 minutes. Table 2 details the resulting accumulation of MDA in the cultures. Antioxidants protect cells against a variety of oxidative stresses. Importantly, the addition of the antioxidant DPPD prevented by 60% the accumulation of MDA in parallel with the protection against the toxicity of glucose oxidase. Similarly the presence of either AE or ChA prevented the accumulation of MDA in cultured hepatocytes exposed to glucose oxidase for 60 minutes. Furthermore, MDA production was reduced ten times in the presence of AE. This influence was not due to a single direct interference of AE components with H_2O_2, since the same antioxidative capacity was found when the cultures were pretreated with AE (data not shown).

TABLE 2
Influence of artichoke extracts on MDA production in hepatocytes treated with glucose oxidase*

Treatment	Lipid peroxidation (nmolMDA/mg protein)
No addition	Not detectable (5)
Glucose Oxidase alone	1.26 ± 0.01 (51)
Glucose Oxidase plus ChA	Not detectable. (36)
Glucose Oxidase plus AE	0.10 ± 0.01 (25)
Glucose Oxidase plus DPPD	0.48 ± 0.00 (16)
ChA	Not detectable (10)
AE	Not detectable (11)
DPPD	Not detectable (6)

*Cultured hepatocytes were treated with 0.1 U/ml glucose oxidase alone or glucose oxidase plus 1 μM DPPD or glucose oxidase plus 1mM ChA or glucose oxidase plus 1 mM AE. After 60 minutes the content of MDA in the cultures was measured and the percentage of the dead cells was determined (numbers in parentheses). The results are the means ± SD of the determinations on three separate cultures.

Cytotoxicity Induced by AE in HepG2 Cells

The potential cytotoxic activity of AE on cancer cells was investigated by determining the viability of a human hepatoma cell line, HepG2. Figure 3 shows the time course of the resulting cell killing assessed by trypan blue exclusion. The cell death rate was dose and time-dependent for 48 h. Treatment with AE for 24 h showed a dramatic reduction in the cell viability up to 80% at the highest concentration tested, 1200 μM. Once induced with the highest concentration of AE, the level of cell death remained almost constant during incubation time of 72 h .

Apoptosis Induced by Artichoke Extracts Treatment

In view of the above mentioned effects on cell viability, we examined whether AE treatment induced apoptosis in HepG2 cells. After 24 h treatment with increasing concentrations of AE in the absence of serum, cell death was assessed with flow

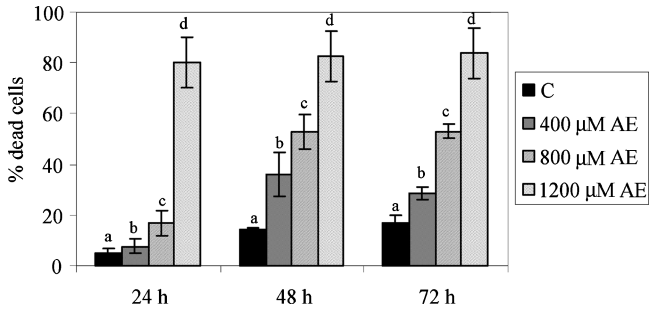

FIG. 3. Cytotoxicity of AE in HepG2 Cells. The HepG2 cells were placed in serum-free medium and treated with increasing concentrations of AE (from 400 to 1200 μM) for 24 h, 48 h or 72 h. Cells were then harvested by trypsinization and cytotoxicity was determined by a trypan blue exclusion method. The results are the means ± SD of at least three independent experiments. P value derived from Anova and Student's t-test a = control, b = 400 μM AE concentration, c = 800 μM AE concentration, d = 1200 μM AE concentration. Significant Effect : 24 h) a vs. c, $P < 0.01$; a vs. d, $P < 0.0001$; 48 h) a vs. b, $P < 0.05$; a vs. c, $P < 0.01$; a vs. d, $P < 0.001$; 72 h) a vs. b, $P < 0.01$; a vs. c, $P < 0.0001$; a vs. d, $P < 0.0001$).

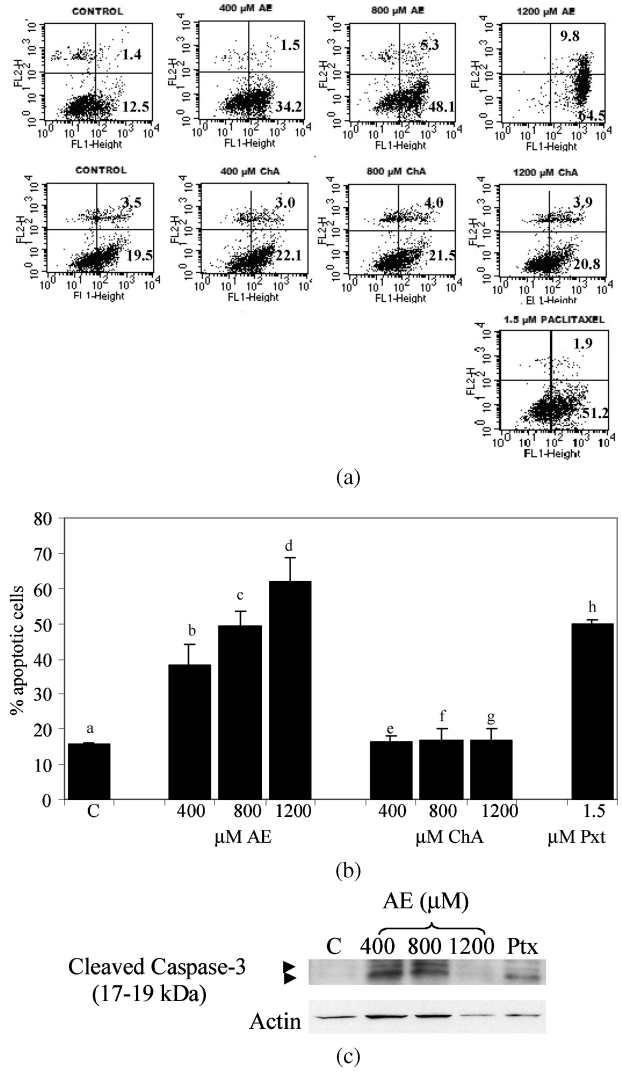

FIG. 4. Assessment of apoptosis. (a) Analysis of cell apoptosis by flow cytometer. Cells (2×10^5) were treated with AE or ChA or Ptx at indicated concentrations. After 24 h, the cells were stained with Annexin V-FITC and PI for flow cytometry analysis as described in Materials and Methods. Annexin V-FITC-generated signals were detected with the FL1 detector and PI signals were detected with the FL2 detector. Early apoptosis cells, which are the Annexin V-FITC-positive/PI negative population of cells, are reported in the lower right-hand quadrant. Necrosis or late apoptotic cells, which are the Annexin V-FITC-positive/PI-positive population of cells, are reported in the upper right-hand quadrant. The figures show typical dot plots for two representative experiments. (b) Statistical analysis of cell apoptosis test. The results are the means ± SD of at least three independent experiments. P value derived from Anova and Student's t-test (a = control, b = 400 μM AE concentration, c = 800 μM AE concentration, d = 1200 μM AE concentration, e = 400 μM ChA concentration, f = 800 μM ChA concentration, g = 1200 μM ChA concentration, e = 1.5 μM Pxt concentration . Significant Effect : a vs. b, $P < 0.01$; a vs. c, $P < 0.001$; a vs. d, $P < 0.001$; a vs. h, $P < 0.0001$). (c) Effect of AE on apoptotic protein Caspase-3. After being cultured in medium containing no treatment (c), or 400, 800, 1200 μM AE or Ptx 1.5 μM HepG2 cells were harvested at 24 h. Then cell lysates were subjected to Western blot analysis of cleaved products (17–19 kDa) of Caspase-3 protein in whole cell lysates. Actin was used as a loading control in each experiment.

cytometry after double staining with annexin V and PI. The annexin V-FITC-positive/PI negative population of cells (early apoptotic cells) was increased in a dose dependent manner as shown in Fig. 4A. While there was 15% early apoptosis in the control cells, the apoptotic cell population was more than 50% in the cells exposed to 800 μM AE. The highest concentration tested, 1200 μM, caused 65% of apoptosis. In comparison, the positive control Ptx 1.5 μM induced 50% of apoptotic cell death after 24 h. Caspase-3 plays a pivotal role during the process of apoptosis and is synthesized as a proenzyme cleaved into active heterodimers (17–19 kDa) when cells undergo apoptosis. As shown in Fig. 4C, cellular caspase-3 was activated in response to 400 and 800 μM AE, the highest concentration tested, 1200 μM, caused a less activation of caspase-3 probably due to the presence of late apoptotic and necrotic cells, according to the annexin V dot plots (see Fig. 4B). Since activation of caspase-3 is an irreversible commitment to cell death, apoptosis of HepG2 cells treated with AE is thus confirmed and is caspase-3 mediated. According to literature data (26), treatment with ChA for 24 h did not show a significant apoptotic activity in our experiments. To follow the time course of toxicity, HepG2 cells were incubated with either AE or ChA for 48 h. No significant increased number of annexin positive cells was detected with AE treatment, whereas the ChA exposure led to a pronounced apoptotic cell death reaching 50 % at the highest concentration tested, 1200 μM (data not shown). This observation suggests that the combination of polyphenolic compounds present in AE is most likely responsible for a faster and more pronounced apoptotic activity.

DISCUSSION

In the present study, we have investigated the antioxidative and the hepatoprotective potential of extracts from artichoke heads. Cultured rat hepatocytes were exposed to H_2O_2 generated in the medium by glucose oxidase. This resulted in a depletion of GSH store, accompanied by the peroxidation of cellular lipids and loss of cell viability. In presence of AE loss of GSH and MDA production were significantly reduced with prevention of cell death.

In situ generation of H_2O_2 by glucose oxidase has previously been shown (24,27) to induce irreversible injury of hepatocytes by at least two mechanisms. The first one involves the GSH-GSSG cycle in the defense of rat hepatocytes against toxic concentration of H_2O_2 generated by 1 hour of exposure to glucose oxidase, is inhibited by the presence of the antioxidant DPPD and is related to the peroxidation of membrane phospholipids. The second one is independent of lipid peroxidation and appears within 3 hour of treatment. The results of the current study can be explained by a sequence of events wherein the depletion of GSH and an unbalance of GSSG-GSH cycle most likely leads to an accumulation of endogenous peroxide followed by the formation of a potent oxidizing agent. This latter species initiates the peroxidative decomposition of the lipids of the hepatocyte

membrane, a process leading to cell death. Such a hypothesis attributes the cell death to oxidative damage and implies that the protective effect of DPPD is a consequence of its antioxidant activity. It is important to emphasize that our data show that DPPD as well as ChA or AE reduced MDA production, reflecting lipid peroxidation, and interfere with the pathways resulting into irreversible cell injury. This influence is not due only to the interference of the extracts components on peroxides in the medium since the same antioxidative capacity was found when the cultures were pretreated with the extracts. Therefore the protective activity of AE against the toxicity of H_2O_2 is likely to be related to the antioxidative role of the artichoke.

The cells were exposed to glucose oxidase that depletes cellular stores of GSH accompanied by the peroxidation of cellular lipids and by the death of the hepatocytes. It is quite interesting that AE as well as ChA prevent the loss of GSH. These findings are in line with other studies carried out on protective activity of artichoke leaves against an oxidative stress (10), which underlined the relevance of high levels of intracellular GSH and a proper functioning of the protective machinery against an oxidative stress. It might be suggested that depletion of GSH induced by glucose oxidase is counteracted by either AE or ChA, the pathways activated under these conditions are presently under investigation in our laboratory.

Studies conducted in vitro and epidemiological evidences have clearly shown the potential antiproliferative activity of dietary polyphenols (28,29). There has been a growing interest in the multiple biological properties of hydroxycinnamic acids (30); several investigations were conducted in order to clarify the positive role of dietary plant phenolic acids such as caffeic (CA), chlorogenic (ChA), and ferulic (FA) and their conjugates on human health (31). Furthermore, it has been reported that dietary phenolic acids with antioxidant activity have anticarcinogenic activity (32); in several experimental studies conducted in animal models nutraceuticals were reported to prevent several malignancies (33,34).

The aim of our study is to search for novel agents with potential chemopreventive properties and low (or no) toxicity on liver cells. However, to consider these polyphenolic extracts from artichoke as chemopreventive agents, the present study needs to be verified by further extensive *in vivo* testings. Our results, as well as other studies (10) suggest that ChA contributes to the protective potential but do not account for the full properties of the artichoke extracts. We propose that the combination of several ubiquitous as well as artichoke specific compounds are responsible for the potent antioxidant activity of AE. These findings are consistent with epidemiological studies which have shown that the action of antioxidant nutrients *per se* do not explain completely the observed health benefit of diet rich in fruit and vegetables.

Uncontrolled cell growth of transformed cells deriving from impaired activity of oncogenes and suppressor genes as well evasion from apoptosis are two major highly linked hallmarks of cancer. Limitless replicative potential can in fact be balanced by the apoptotic process. Many agents have exhibited selective anti-tumor activity for certain type of tumor cells. Since Ptx is a widely used cancer chemotherapeutic drug that exhibits clinical activity in a range of human malignancies and has been reported to induce apoptosis on HepG2 cells (35), we compared the activity of such a drug to the activity of either ChA or AE, only quality-wise and not concentration-wise, in the same experimental conditions. The artichoke extracts showed an apoptotic induction, caspase-3 dependent, on HepG2 in a dose dependent manner within 24 h. ChA, as reported in the literature (26), does not induce apoptosis on HepG2 cells during the same incubation time.

We performed cytotoxicity assays working within a concentration range (400μM-1200μM) for both chlorogenic acid and artichoke extract; a faster and more pronounced apoptotic activity of the artichoke extract than chlorogenic acid activity could be ascribed to the combined or synergistic activities of a number of bioactive compounds present in the edible part of the plant. Other studies showed proapoptotic properties of chlorogenic acid at physiological concentrations (36–39). In conclusion, since polyphenols are significant components of the diet, their potential health benefits observed *in vitro* and in animal models require to be correlated with studies in humans; furthermore, their positive role in human diet depends, after ingestion, on their metabolism, absorption and excretion. Thus, future research in order to assess their *in vivo* biological significance were required (40).

In summary, our results demonstrate that extracts from the edible part of artichoke show a pronounced antioxidative and apoptotic activity which may be of interest with respect to a possible chemopreventive activity associated to artichoke consumption.

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REFERENCES

1. Singh AV, Franke AA, Blackburn GL, and Zhou JR: Soy phytochemicals prevent orthotopic growth and metastasis of bladder cancer in mice by alternations of cancer cell proliferation and apoptosis and tumor angiogenesis. *Cancer Res* **66,** 1851–1858, 2006.
2. Seeram NP, Henning SM, Niu Y, Lee R, Scheuller HS, et al.: Catechin and caffeine content of green tea dietary supplements and correlation with antioxidant capacity. *J Agric Food Chem* **54,** 1599–1603, 2006.
3. Canene-Adams K, Campbell JK, Zaripheh S, Jeffery EH, and Erdman JW Jr: The tomato as a functional food. *J. Nutr* **135,** 1226–1230, 2005.
4. Cao GH, Sofic E, and Prior RL: Antioxidant capacity of tea and common vegetables. *J Agric Food Chem* **44,** 3426–3431, 1996.
5. Eberhardt MV, Lee CY, and Liu RH: Nutrition-Antioxidant activity of fresh apples. *Nature* **405,** 903–904, 2000.

6. Scalbert A, Johnson T, and Saltmarsh M: Polyphenols antioxidants and beyond. *Am J Clin Nutr* **81,** 215S–217S, 2005.
7. Di Venere D, Linsalata V, Calabrese N, Cardinali A, Sergio L, et al.: Biochemical characterization of new seed propagated artichoke cultivars. *Acta Hortic* **681,** 517–522, 2005.
8. Di Venere D, Linsalata V, Pace B, Bianco VV, and Perrino P: Polyphenol and inulin content in a collection of artichoke. *Acta Hortic* **681,** 453–459, 2005.
9. Gebhardt R: Antioxidative and protective properties of extracts from leaves of the Artichoke (*Cynara Scolymus* L.) against hydroperoxide–induced oxidative stress in cultured rat hepatocytes. *Toxicol Appl Pharmacol* **144,** 279–286, 1997.
10. Chinou I and Harvala C: Polyphenolic constituent from the leaves of two *Cynara* species growing in Greece. *Planta Med* **63,** 469–470, 1997.
11. Adzet T, Camarasa J, and Laguna JC: Hepatoprotective activity of polyphenolic compounds from *Cynara Scolymus* L. against CCl_4toxicity in isolated rat hepatocytes. *J Nat Prod* **50,** 612–617, 1987.
12. Wojcicki J: Effect of 1.5-dicaffeylquinic acid (Cynarine) on cholesterol levels in serum and liver acute ethanol-treated rats. *Drug Alcohol Depend* **3,** 143–145, 1978.
13. Maros T, Racz G, Katonai B, and Kovacs VV: Wirkung der Cynara Scolymus- Extrakte auf die Regeneration der Rattenleber. *1 Mitt Arzneimittelforschung* **16,** 127–129, 1978.
14. Maros T, Seres-Sturm L, Racz G, Rettegi C, Kovacs VV, and Hints M.: Wirkung der Cynara Scolymus- Extrakte auf die Regeneration der Rattenleber. *2. Mitt Arzneimittelforschung* **18,** 884–886, 1968.
15. Middleton E Jr., Kandaswami C, and Theoharides TC: The effect of plant flavonoids on mammalian cells: Implications for inflammation, heart disease and cancer. *Pharmacol Rev* **52,** 673–751, 2000.
16. Singh RP and Agarwal R: Natural flavonoids targeting deregulated cell cycle progression in cancer cells. *Curr Drug Targets* **7,** 345–354, 2006.
17. Jiang C, Lee HJ, LiG X, Guo J, Malewicz B, Zhao Y, Lee EO, Lee HJ, Lee JH, Kim MS, Kim SH, and Lu J.: Potent antiandrogen and androgen receptor activities of an Angelica Gigas-containing herbal formulation: identification of decursin as a novel and active compound with implications for prevention and treatment of prostrate cancer. *Cancer Res* **66**, 453–463, 2006.
18. Manson MM: Cancer prevention-The potential for diet to modulate molecular signalling. *Trends Mol Med* **9,** 11–18, 2003.
19. Seglen PO: Preparation of isolated liver cells. *Methods Cell Biol* **13,** 29–83, 1976.
20. Beers RF and Sizer IW: A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* **195**, 133–140, 1952.
21. Sedlak J and Lindsay RH: Estimation of total, protein-bound, and noprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* **25,** 192–205, 1968.
22. Yagi K: A simple fluorimetric assay for lipoperoxide in blood plasma. *Biochem Med* **15,** 212–216, 1976.
23. Miccadei S, Kyle EM, Gilfor D, and Farber JL: Toxic consequence of an abrupt depletion of glutathione in cultured rat hepatocytes. *Arch Biochem Biophys* **265,** 311–320, 1988.
24. Rubin R and Farber JL: Mechanisms of the killing of cultured hepatocytes by hydrogen peroxide. *Arch Biochem Biophys* **228,** 450–459, 1984.
25. Casini A, Giorli M, Hyland RJ, Serroni A, and Farber JL: Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. *J Biol Chem* **257,** 6721– 6728, 1982.
26. Ramos S, Alia M, Bravo L and Goya L: Comparative effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2). *J Agric Food Chem* **53,** 1271–1280, 2005.
27. Starke PE and Farber JL: Endogenous defenses against the cytotoxicity of hydrogen peroxide in cultured rat hepatocytes. *J Biol Chem* **260,** 86–92, 1985.
28. Matito C, Mastorakou F, Centelles JJ, Torres JL, and Cascante M: Antiproliferative effect of antioxidant polyphenols from grape in murine Hepalclc7. *Eur J Nutr* **42,** 43–49, 2003.
29. Watson WH, Cai J, and Jones DP: Diet and apoptosis. *Annu Rev Nutr* **20,** 485–505, 2000.
30. Laranjinha JA, Almeida LM, Madeira VM: Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. *Biochem Pharmacol* **48,** 487–494, 1994.
31. Clifford MN: Chlorogenic acids and other cinnamates: nature, occurrence and dietary burden. *J Sci Food Agric* **79,** 362–372, 1999.
32. Nagano J, Kono S, Preston DL, Mabuchi K: A prospective study of green tea consumption and cancer incidence, Hiroshima and Nagasaki (Japan). *Cancer Causes Control* **112,** 501–508, 2001.
33. Kanadaswami C, Lee LT, Lee PP, Hwang JJ, Ke FC, Huang YT, Lee MT: The antitumor activities of flavonoids. *In Vivo* **19,** 895–909, 2005.
34. Roudebush P, Davenport DJ, Novotny BJ: The use of nutraceuticals in cancer therapy. *Vet Clin North Am Small Anim Pract* **34,** 249–269, 2004.
35. Kuo HC, Lee HJ, Hu CC, Shun HI, and Tseng TH: Enhancement of esculetin on taxol-induced apoptosis in human hepatoma HepG2 cells. *Toxicol Appl Pharmacol* **210,** 55–62, 2006.
36. Yagasaki K, Miura Y, Okauchi R, Furuse T: Inhibitory effects of chlorogenic acid and its related compounds on the invasion of hepatoma cells in culture. *Cytotechnology* **33,** 229–235, 2000.
37. Feng R, Lu Y, Bowman L, Qian Y, Castranova V, and Ding M: Inhibition of Activator Protein-1, NF-kB, and MAPKs and Induction of Phase 2 Detoxifying Enzyme Activity by Chlorogenic Acid. *J Biol Chem* **280,** 27888–27895, 2005.
38. Bandyopadhyay G, Biswas T, Roy KC, Mandal S, Mandal C, Pal BC, Bhattacharya S, Rakshit S, Bhattacharya DK, Chaudhuri U, Konar A and Bandyopadhyay S: Chlorogenic acid inhibits Bcr-Abl tyrosine kinase and triggers p38 mitogen-activated protein kinase–dependent apoptosis in chronic myelogenous leukemic cells. *Blood* **104,** 2514–2522, 2004.
39. Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP and Gesher AG: Dietary polyphenolic phytochemicals—promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int J Cancer* **120,** 451–458, 2007.
40. Azzini E, Bugianesi R, Romano F, Di Venere D, Miccadei S, Durazzo A, Foddai MS, Catasta G, Linsalata V and Maiani G: Absorption and metabolism of bioactive molecules after oral consumption of cooked edible heads of Cynara scolymus L. (cv Violetto di Provenza) in humans. *Br J Nutr* **5**, 963–969, 2007.