Pro-oxidant and pro-apoptotic activity of polyphenol extract from *Annurca* apple and its underlying mechanisms in human breast cancer cells

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Abstract. Among nutraceuticals, polyphenols represent the most intriguing and studied class of compounds that can be therapeutics for a large spectrum of the most common diseases, including cancer. Although polyphenols are well known as potent antioxidants, a pro-oxidant effect has been associated with a pro-apoptotic function of these compounds in various types of tumor cells. *Annurca* apple, a southern Italian variety, is characterized by an extremely high content of polyphenols and displays a stronger antioxidant activity compared with other varieties. In the present study we explored the antiproliferative effect of *Annurca* apple polyphenol extract (APE) in human breast cancer MCF-7 cells and we investigated the underlying mechanisms. Results showed that at 500 μM catechin equivalent (EqC) APE acts as a pro-oxidant increasing thiobarbituric acid-reactive species cell content of approximately 6-fold more than the untreated cells. We found that APE strongly inhibits the proliferation of MCF-7 cells by inducing G2/M cell cycle arrest and apoptosis. Immunoblot analysis demonstrated that APE treatment increases the levels of p53 and p21, downregulates the expression of the cell cycle regulatory protein cyclin D1, and inhibits ERK1/2 phosphorylation. Moreover, APE treatment caused a marked increase of pro-apoptotic Bax/Bcl-2 ratio paralleled by caspase-9, -6, -7, and poly(ADP ribose) polymerase cleavage. Altogether our data indicate that APE, at elevated concentrations, acts as a potent pro-oxidant and antiproliferative agent able to downregulate ERK1/2 pathway leading to cell cycle inhibition and apoptosis and provides a rationale for its potential use in the development of novel therapeutics towards breast cancer.

Introduction

Considerable scientific interest in the anticancer therapy has been focused, for long time, on the identification of compounds able to efficiently commit cells to apoptosis. Apoptosis plays a crucial role in eliminating the mutated hyperproliferating cells from the system. Thus, induction of apoptosis in tumor cells may be considered as a protective mechanism against development and progression of cancer. Reactive oxygen species (ROS) play a major role in carcinogenesis (1). It has been shown that the formation of ROS contributes to the antitumor activity of several chemotherapeutic drugs. However, pro-oxidant molecules can act as selective cytotoxic agents against cancer cells by achieving toxic levels of ROS (1).

The validation and utilization of dietary components, natural products, or their synthetic analogs as chemopreventive agents in the form of foods or nutraceutical has become an important issue in health- and cancer-related research and a growing body of evidence also suggests that many natural compounds may cooperate in enhancing the therapeutic efficacy of chemotherapeutic drugs, help to by-pass cancer drug resistance or reduce side-effects of chemotherapy (2-4). Polyphenols represent one of the most interesting and investigated class of nutraceutical

compounds because of their therapeutic properties for several common diseases, including cancer (5). It is reported that flavonoids, a class of polyphenolic compounds show several anticancer effects including apoptosis induction, cell cycle arrest, antiproliferative, antioxidative, antiangiogenic and antimetastatic action against many human cancer cell lines. Interestingly, an important aspect of the chemopreventive action of polyphenols is their differential activity in targeting cancer cells but not normal cells (6,7). Most of the advantageous effects of natural polyphenols have been ascribed to their ability to scavenge free radicals endogenously generated or formed by radiation and xenobiotics. However, emerging evidence indicates that the anticancer and chemopreventive properties of plant-derived polyphenols are mainly related to their pro-oxidant activity (8). The main problem related to the use of polyphenols as anticancer agents is their poor bioavailability, which might hamper the *in vivo* effects of the single compound (9) and it has been suggested that combinations of polyphenols naturally found in fruits and vegetables could be most favorable for cancer prevention owing to synergic or additive biological effects (10).

Apples are widely consumed and represents one of the main sources of polyphenols in the Western diet (11). The antiproliferative properties of apple extracts have been described extensively by *in vitro* studies and products extracted from apple skins have been shown to be effective in preventing tumor formation in different types of cancer, including colorectal, breast, prostate, liver, cervical and lung (12-14).

Among the different varieties, great interest has been paid to *Annurca* apple, one of the most important cultivars of southern Italy with a 'Protected Geographical Indication' of the Campania region (15). The *Annurca* apple is characterized by an extremely high content of polyphenols, mainly catechin, epicatechin, and chlorogenic acid and displays a stronger antioxidant activity compared with other varieties (16). Many studies have recently investigated the polyphenol content of *Annurca* apple peel and have described its higher ability in comparison to other species in reducing cell cholesterol and glucose uptake (17) and its activity in chemoprevention of colorectal cancers (18,19). Moreover, the antiproliferative effect of *Annurca* apple polyphenol extract has been described in human HaCaT keratinocytes where it induced p53-independent extrinsic apoptosis (20).

Breast cancer is the most common malignancy in women with increased incidence worldwide. Poor prognosis of breast cancer is partially attributed to multiple-drug resistance and anti-apoptosis activity of cancer cells (21). Although antitumor activity of polyphenols in breast cancer has been shown, its molecular mechanism is yet to be clarified (22).

In the present study, the pro-oxidant, antiproliferative, and pro-apoptotic effects of *Annurca* APE in human breast cancer MCF-7 cells were investigated and the potential underlying molecular mechanisms were explored. We found that *Annurca* APE is able to inhibit cell proliferation by inducing cell cycle arrest at G2/M and the activation of apoptosis *via* a Bcl2- Bax-dependent mitochondrial signaling pathway. Finally, we report that the antiproliferative effects of APE involve downregulation of cyclin D1, upregulation of p53 and p21, and inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) activation.

Materials and methods

Materials. Roswell Park Memorial Institute medium RPMI-1640 medium (RPMI-1640), bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin-Ciocalteu reagent, propidium iodide (PI), thiobarbituric acid, and RIPA buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) and trypsin-EDTA were from Lonza (Milan, Italy). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Tissue culture dishes were purchased from Microtech (Naples, Italy). Annexin V-fluorescein isothiocyanate (V-FITC) Apoptosis Detection kit was purchased from eBioscience (San Diego, CA, USA). Monoclonal antibodies to caspase-9, poly(ADP ribose) polymerase (PARP), Bax, Bcl-2, cyclin D1, p21, p53, β-actin, and polyclonal antibodies to caspases 6 and 7, ERK1/2, and pERK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse secondary antibodies were obtained from ImmunoReagents, Inc., Raleigh, NC, USA. All buffers and solutions were prepared with ultra-high quality water. All reagents were of the purest commercial grade.

Apple samples. Annurca (*Malus pumila* Miller cv. *Annurca*) apple fruits (each weighing approximately 100 g) were collected in Giugliano (Napoli, Italy) in October, when fruits had just been harvested (green peel). Fruits were reddened following the typical treatment for approximately 30 days and then analyzed (15).

Polyphenol extraction. APE extraction from *Annurca* apple was carried out as previously reported by D'Angelo *et al* (20). Briefly, 40 grams of *Annurca* apple flesh were homogenized for 5 min by a Tefal Rondo 500 homogenizer using 40 ml of 80% methanol and 20% water plus 0.18 N HCl. After centrifugation (18,000 x g for 25 min), the slurry was dried under vacuum by using the Univapor Concentrator Centrifuge, model Univapo 100 H (Uni Equip). The dried extracts were dissolved in 10 ml of PBS and frozen at -80°C until use.

Polyphenolic content and HPLC analysis. The total polyphenolic content of apple extract was assessed approximately by using the Folin-Ciocalteu phenol reagent as described by Singleton *et al* (23). The extracts (100 μl) were mixed with the Folin-Ciocalteu phenol reagent (0.5 ml), deionized water (0.9 ml), and Na_2CO_3 (7.5% w/v, 4 ml). The absorbance at 765 nm was measured 1 h after incubation at room temperature using a Cary ultraviolet-visible spectrophotometer (Varian). Since APE is a mixture of different phenolic compounds, to give an arbitrary APE molar concentration the measurement was compared to a standard curve of prepared catechin solutions and expressed as milligrams of catechin equivalent (EqC) per 100 g of *Annurca* flesh fresh weight. The chemical characterization was performed in HPLC as reported by D'Angelo *et al* (24). All experiments were carried out in triplicate. HPLC separation of polyphenols was performed by reversed-phase chromatography on a 5 μm column Kromasil C_{18} column (150x4.6 mm), using a Beckman Apparatus (Gold-126) equipped with a UV detector fixed at

278 nm. The column was eluted at a flow rate of 1.0 ml/min with 0.2% acetic acid, pH 3.1 (A) and methanol (B) as the mobile phase; the gradient was changed as follows: 95% A/5% B for 1 min, 85% A/15% B in 1 min, 75% A/25% B in 20 min, 0% A/100% B in 15 min, and 95%A/5%B in 3 min. The main *o*-diphenols were identified on the basis of the retention times of authentic standard references: (+)-catechin, (-)-epicatechin, chlorogenic acid, quercetin, and quercetin glysosides.

Cell culture. Human breast carcinoma cell line (MCF-7) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37˚C.

Cell viability. MCF-7 cells were seeded in 96-well plates at the density of 3×10^3 cells/well in RPMI complete medium. After 24 h incubation, cells were treated with 100, 250, and 500 μM APE EqC for 24 and 48 h. Cell viability was assessed by adding MTT solution in PBS to a final concentration of 0.5 mg/ml. Cells were then incubated at 37˚C for 4 h and the MTT-formazan crystals were solubilized in a solution of 4% 1 N isopropanol/hydrochloric acid on a shaking table for 20 min. The absorbance values of the solution in each well were measured at 595 nm using a Bio-Rad iMark microplate reader (Bio-Rad Laboratories, Milan, Italy). Cell viability was expressed as a percentage of absorbance values in treated samples respect to that of control (100%). All MTT experiments were performed in quadruplicate.

Thiobarbituric acid-reactive species. Thiobarbituric acid-reactive species (TBARS) were determined on aliquots (250 μl) of cell culture medium of not treated cells (control) or after 48 h treatment with 100, 250 and 500 μM EqC APE. TBARS were measured as previously described (25). Briefly, samples were incubated with a solution consisting of 0.5 ml of 20% acetic acid, and 0.5 ml of 0.78% aqueous solution of thiobarbituric acid (pH 3.5), heated at 95˚C for 45 min, and then centrifuged at 4000 rpm for 5 min. TBARS content was quantified at 532 nm with reference to malondialdehyde (extinction coefficient at 532 nm, 1.56×10^5 M^{-1} cm^{-1}). Result is the average of triplicate measurements of each individual experiment performed in duplicate.

Evaluation of morphological changes by phase-contrast microscopy. MCF-7 cells were cultured in 6-well tissue culture plates (Becton-Dickinson, Franklin Lanes, NJ, USA) at a seeding density of 9.0×10^4 cells. After overnight attachment, the cells were treated with different concentrations (100, 250, and 500 μM EqC) of APE and the untreated cells were used as control. The cells were cultured for up to 48 h under standard culture conditions and their morphological changes were imaged using a phase-contrast microscope (Axiovert-10 Zeiss microscope).

Western blot analysis. MFC-7 cells were cultured in 10-cm culture dishes at a seeding density of 5.5×10^5 cells. After overnight attachment, the cells were treated for 48 h with 100, 250, and 500 μM EqC APE and the untreated cells used as control.

After the treatment, the cells were collected by centrifugation, washed twice with ice-cold PBS, and the pellets were lysed using 100 μl of RIPA buffer. After incubation on ice for 30 min, the samples were centrifuged at 18,000 x g in an Eppendorf microcentrifuge for 10 min a 4˚C, and the supernatants were quantified for protein content. Aliquots containing approximately 30 μg of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes by Trans blot turbo (Bio-Rad Laboratories). The blots were blocked with 5% non-fat dry milk in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl plus 0.5% Tween-20 (TBS-T). The membranes were subsequently incubated at 4˚C overnight in the 5% non-fat dry milk-TBS-T buffer, containing, for each experiment, one of the specific primary antibodies. After three times washing with TBS-T, the blots were incubated for 1 h at room temperature with the HRP-conjugated secondary antibodies. After four times washing, the blots were developed using enhanced chemiluminescence detection reagents ECL (Millipore Corp., Billerica, MA, USA) and exposed to X-ray film. The film was scanned by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Flow cytometry analysis of the cell cycle. Cell cycle distribution was studied by PI staining and flow cytometry analysis using a FACScalibur (Becton-Dickinson, San Jose, CA, USA) interfaced with a Hewlett-Packard computer (model 310) for data analysis. Briefly, MCF-7 cells were plated in 6-multiwell plates at the density of 9.0×10^4 cells/well. After 24 h, the medium was changed and APE was added at concentrations of 100, 250, or 500 μM EqC; conversely fresh medium was added to the control well. After 48 h treatment, the cells were recovered by incubation with trypsin-EDTA, washed in PBS, collected by centrifugation, fixed by resuspension in 70% ice-cold methanol/PBS and incubated overnight at 4˚C. After fixing, samples were centrifuged at 400 x g for 5 min, washed twice with ice-cold PBS, resuspended in 0.5 ml DNA staining solution (50 μg/ml PI and 25 μg/ml RNaseA in PBS), and incubated at room temperature for 1 h in the dark. Samples were then transferred to 5-ml Falcon tubes and stored until assayed. PI fluorescence was collected as FL2-A (linear scale) by the ModiFIT Cell Cycle Analysis software (Becton-Dickinson). For each sample, at least 20,000 events for each point were analyzed in at least three different experiments giving an SD of <5%.

Flow cytometry analysis of apoptosis. Annexin V-FITC binds phosphatidylserine residues which are translocated from the inner to the outer leaflet of the plasma membrane during the early stages of apoptosis. A flow-based Annexin V-FITC/PI double staining was used to distinguish apoptotic (Annexin V-FITC-positive, PI-positive) from necrotic (Annexin V-FITC-negative, PI-positive) cells (26). MCF-7 cells were plated in 6-multiwell plates at the density of 9.0×10^4 cells/well. After 24 h, the medium was changed and APE was added at concentrations of 100, 250, or 500 μM EqC while fresh medium was added to the control well. After 48 h of treatment, the cells were detached by incubation with trypsin-EDTA, washed in PBS, collected by centrifugation, resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and
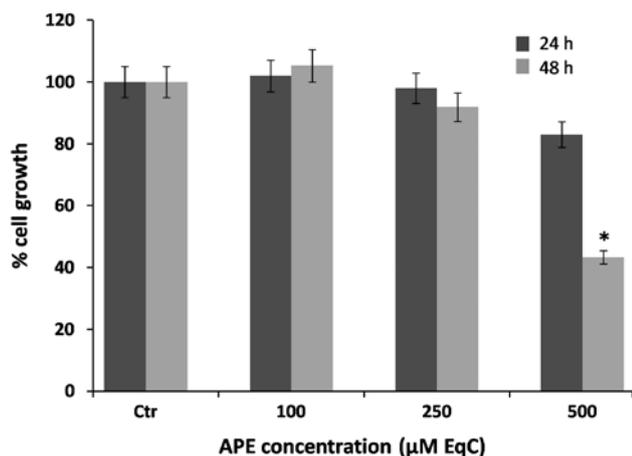
Figure 1. Effects of APE on MCF-7 cell proliferation. MCF-7 cells were cultured for 24 and 48 h in medium supplemented or not (Ctr) with APE 100, 250 and 500 μM EqC. Cell viability was then assessed by MTT assay and expressed as a percentage of untreated cells. Values represent the mean ± SD of three independent experiments (*P<0.05).

Figure 2. Effects of APE on TBARS content in MCF-7 cells. MCF-7 cells were incubated for 48 h in RPMI medium containing increasing amounts of APE (100, 250 and 500 μM EqC). TBARS content was measured at 532 nm as described in Materials and methods by using as reference malondialdehyde (extinction coefficient at 532 nm, 1.56×10^5 M^{-1} cm^{-1}). Values represent the mean ± SD of three independent experiments (*P<0.05).

then stained with 5 μl of Annexin V-FITC and 10 μl PI (20 μg/ml) for 30 min in the dark, according to the manufacturer's instructions. Analyses were performed with flow cytometry apparatus (Becton-Dickinson). Apoptosis was detected in red and green fluorescence channels with excitation wavelength of 488 nm. For each sample, 2×10^4 events were acquired. Analysis was carried out by triplicate determination on at least three separate experiments.

Statistical analysis. All experiments were performed three times with replicate sample. Data are expressed as mean ± standard deviation (SD). Comparisons between treated samples and control were performed using analysis of variance (ANOVA) plus Bonferroni's t-test. A P-value <0.05 was considered to indicate a statistically significant result.

Results

Total polyphenolic content of APE. The total polyphenolic content of the *Annurca* apple extract measured by Folin-Ciocalteu phenol reagent resulted in 125.2±7.1 mg of catechin per 100 g of sample and is similar to that evaluated in other studies (24,25). The HPLC analysis of APE identified (+)-catechin, (-)-epicathechin, chlorogenic acid, quercetin, and quercetin glycosides as the main *Annurca* apple *o*-diphenols (data not shown). This profile confirms the results already described in the literature (16,24).

APE inhibits cell proliferation and induces lipid peroxidation. To evaluate the effect of APE on cell growth, MCF-7 cells were incubated for 24 and 48 h with increasing concentrations of the extract ranging from 100 to 500 μM EqC corresponding to 29-145 μg EqC/ml, and cell proliferation was then assessed by MTT assay. The cells appeared to be quite resistant to APE treatment. The results obtained evidenced that up to 100 μM EqC no appreciable changes in cell viability were observed at any time of incubation. On the other hand, at higher doses, the treatment with APE significantly reduced cell proliferation in a dose- and time-dependent

Figure 3. APE-induced morphological changes in MCF-7 cells examined by phase-contrast microscopy. Not treated MCF-7 cells (A) and MCF-7 cells treated with APE 100 μM EqC (B), 250 μM EqC (C), 500 μM EqC (D). Original magnification, x100. Bar, 20 μm.

manner (Fig. 1). It has to be noted that after 24 h incubation, 250 μM EqC and 500 μM EqC APE exerted only a poor cell growth inhibitory effect reducing cell proliferation to approximately 98 and 83%, respectively, compared to control cells. In contrast, a prolonged incubation of 48 h resulted in a severe loss of cell viability that decreases to 43.5%, a value near the IC$_{50}$, in the presence of APE 500 μM EqC. Parallel direct cell counting provided similar results (data not shown). Based on these findings, we selected for further investigations an incubation time of 48 h.

Free radicals are involved in cell death induction in a number of systems and membrane phospholipids represent
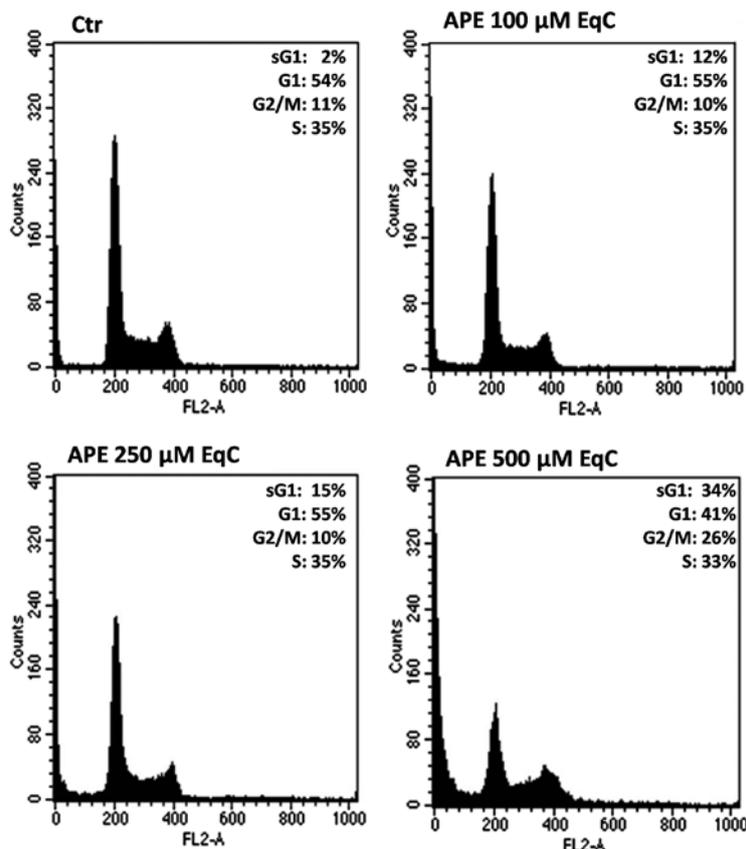
Figure 4. Effects of APE on cell cycle in MCF-7 cells. Cells were cultured for 48 h in medium supplemented or not (Ctr) with APE 100, 250 and 500 μM EqC. FACS analysis was then performed. Representative FACS histograms of PI-stained MCF-7 cells are shown. The percentage of hypodiploid sub-G1 and of each cell cycle phase is indicated. Data represent the average of three independent experiments.

one of the major target of oxidative stress in cells. It has been shown that lipid hydroperoxides as well as lipid peroxidation initiators, such as ROS, are involved in signaling pathways that control cell proliferation, differentiation, maturation, and apoptosis (1,27). Moreover, it has been proposed that lipid peroxidation may represent a protective mechanism in breast cancer (27).

In order to investigate whether an oxidative stress could be produced under our experimental conditions, we evaluated lipid peroxidation by measuring the extent of lipid degradation products such as, malondyaldehyde and other aldehydes reactive to thiobarbituric acid after 48 h incubation of MCF-7 cells in the presence of increasing amounts of APE. It is interesting to note that, in analogy with the effect exerted on cell viability, APE is able to cause lipid peroxidation only at concentration >100 μM EqC inducing, at 250 μM EqC and 500 μM EqC concentration, respectively, an increase of TBARS cell content of 3- and 6-fold higher than the untreated cells (Fig. 2). These results indicate that, at elevated concentrations, apple polyphenols may act as pro-oxidants and may induce growth inhibition probably enhancing intracellular ROS oxidative stress.

APE induces morphological changes in MCF-7 cells. MCF-7-cells were incubated for 48 h with 100, 250, and 500 μM EqC APE, and the morphology of treated cells was then analyzed with a phase-contrast microscope and compared with the untreated cells (Fig. 3). The results obtained indicated that the cells treated with APE 100 μM EqC (Fig. 3B) maintained the characteristic epithelial morphology and prolific growth as a monolayer, quite similar to untreated MCF-7 cells (Fig. 3A). On the contrary, the cells treated with APE 250 μM EqC (Fig. 3C) and 500 μM EqC (Fig. 3D) displayed morphological alterations such as, shrinkage and cytoplasmic condensation, cell rounding, poor adherence, and cell detachment. Altogether the evidence suggests that APE is able to commit MCF-7 cells to a type of death that mimics apoptosis.

APE induces cell cycle arrest at G2/M by downregulation of cyclin D1 and upregulation of p21 and p53. Recent literature data have highlighted the chemopreventive and anticancer properties of polyphenolic compounds and polyphenol-rich nutritional sources. Moreover, several recent investigations have elucidated the mechanisms by which polyphenols are able to modulate some cellular events such as, cell cycle arrest by decreasing cyclin levels, and apoptosis induction (28).

To verify whether APE caused cell cycle perturbation in MCF-7 cells, we evaluated the cell distribution in the cell cycle phases by flow cytometric analysis. In addition, the severe morphological changes observed in APE-treated MCF7-cells under the inverted phase-contrast microscope prompted us to further examine whether the APE-induced cell death was a consequence of activation of apoptosis. Therefore, we also looked at the proportion of cells with hypodiploid DNA content (sub-G1 population), characteristic of cells having undergone DNA fragmentation, a biochemical hallmark of apoptosis.
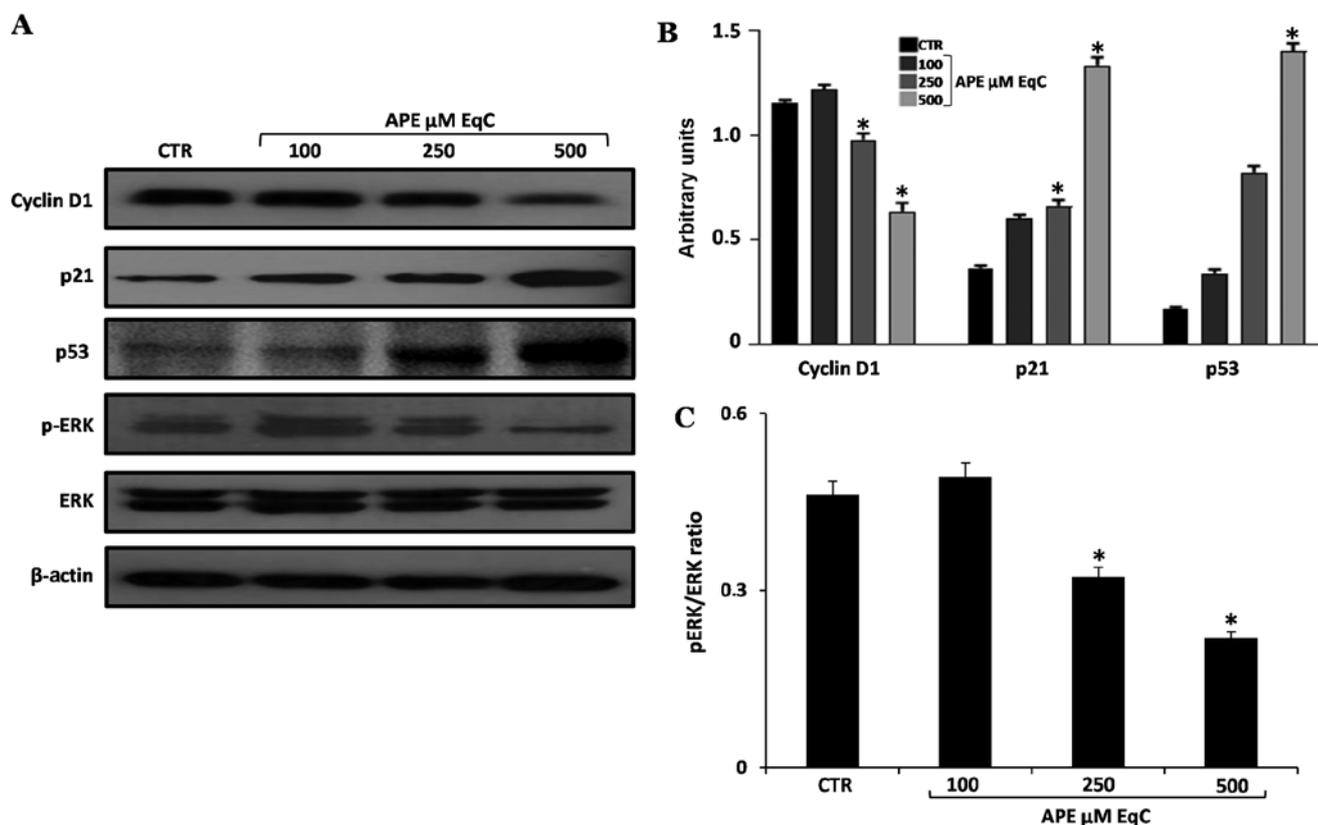
Figure 5. Effects of APE on the levels of some relevant cell cycle- and proliferation-regulating proteins in MCF-7 cells. Cells were cultured in medium supplemented or not (Ctr) with APE 100, 250 and 500 μM EqC for 48 h. Then, 30 μg of cell extracts were subjected to SDS-PAGE and blotted with antibodies against the indicated proteins. β-actin was used as a standard for the equal loading of protein in the lanes. (A) Western blot assay for the expression of cyclin D1, p21, p53, ERK1/2 and p-ERK1/2. (B) Densitometric quantification of cyclin D1, p21, and p53. (C) The graphs show the densitometric intensity of pERK/ERK band ratio in different conditions. The intensity of signals was expressed as arbitrary units. *P<0.05 versus control untreated cells. Data represent the average of three independent experiments.

MCF-7 cells were exposed to increasing concentrations of APE (100, 250, and 500 μM EqC), harvested at 48 h, and examined for DNA content after proper staining with PI. Fig. 4 shows that the treatment of cells with 100 and 250 μM EqC APE does not cause any evident effect on cell proliferation while 500 μM EqC APE induces a cell cycle arrest at G2/M, as evidenced by the significant increase of cell percentage in this phase of the cell cycle (26%) respect to untreated cells (11%). Moreover, a dose-dependent increase of sub-G1 population can be observed that becomes clearly evident at 500 μM EqC APE (34%) suggesting that the cytotoxic activity of APE in MCF7-cells occurs via apoptosis.

To gain further insight into the molecular mechanism of cell cycle arrest induced by APE, we analyzed the expression level of some relevant cell proliferation- and cell cycle-regulators such as, cyclin D1, cyclin-dependent kinase inhibitor 1 (p21) and p53 transcription factor. Thus, MCF-7 cells were exposed to 100, 250, and 500 μM EqC APE for 48 h, after which cell extracts were analyzed by western blotting. As shown in Fig. 5, the treatment for 48 h with APE induced a significant dose-dependent increase in the level of p53 and p21 proteins. It has to be pointed out that the tumor suppressor p53 is a transcription factor that regulates a broad range of processes among which cell cycle arrest, senescence, and apoptosis are the best characterized. *In vitro* and *in vivo* studies have shown that polyphenols such as, epigallocatechin, resveratrol, and curcumin as well as nutritional sources of polyphenols are able to upregulate p53 protein in several cancer cell lines (28-32) and that the polyphenol-induced stabilization and expression of p53 is often associated with a G1 or G2/M phase cell cycle arrest (33). It has also been reported that tumor suppressors, like p53 and its analogs, are key molecular targets of polyphenols responsible for their pro-apoptotic effect in human and animal cancer models through the transcriptional regulation of target genes such as p21, Bax, and PUMA (32).

From the western blot analysis reported in Fig. 5 we can observe that concomitantly with the increase of p53 and p21, the level of cyclin D1 was decreased in a dose-dependent manner. In agreement with our results it has been recently reported that epigallocatechin-3-gallate, the major polyphenolic component of green tea, induces apoptosis and cell cycle arrest in colorectal cancer cells by downregulating cyclin D1 and upregulating p21 (34).

Cyclin D1 is a key factor regulating cell proliferation, this cyclin links the extracellular signals to cell cycle advancement (35). Cyclin D1 levels must be elevated during G1 phase to start DNA synthesis, but then they must be suppressed during S phase to allow DNA synthesis. If the cell has to continue to proliferate, cyclin D1 levels must be induced once again during G2 phase. This induction depends upon the activity of proliferative signaling molecules, and ensures that the extracellular environment continues to be conducive for growth (36).
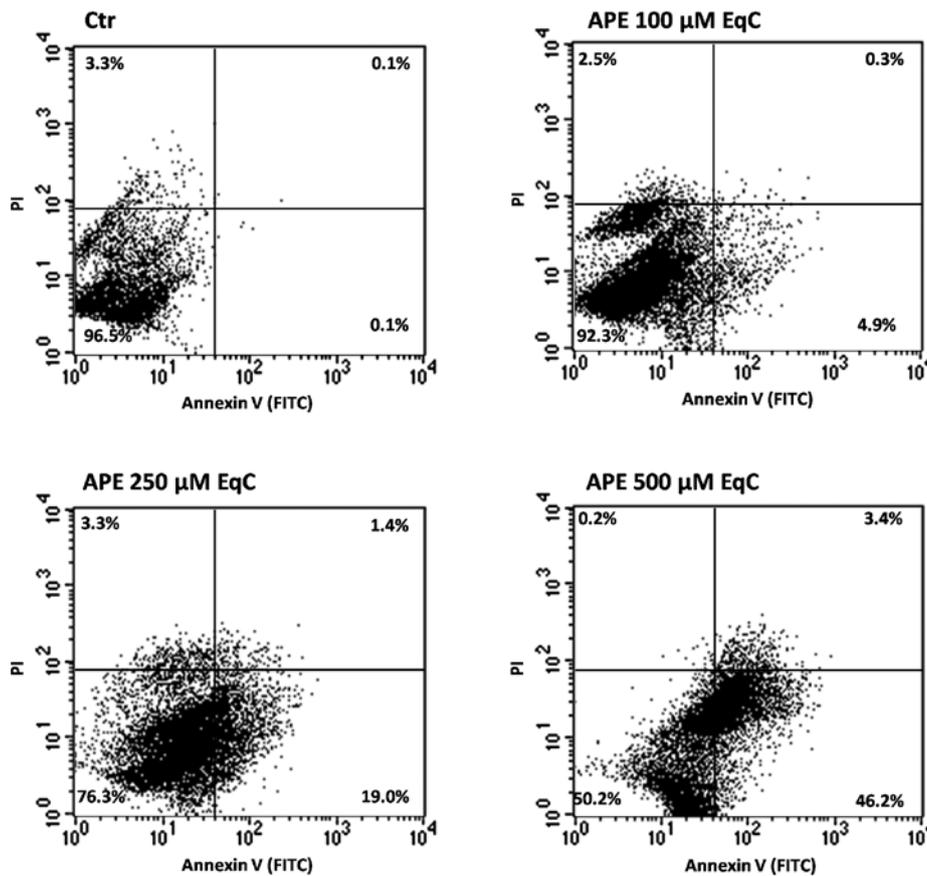
Figure 6. Evaluation of apoptosis induced by APE in MCF-7 cells. Cells were cultured for 48 h in medium supplemented or not (Ctr) with APE 100, 250 and 500 μM EqC. FACS analysis was then performed. Representative dot plots V-FITC (x-axis)/PI (y-axis) of both Annexin V-FITC and PI-stained MCF-7 cells. The percentage of cells is reported in the quadrants. Viable cells, lower left; non-viable necrotic cells, upper left; early apoptotic cells, lower right; late apoptotic cells, upper right.

For its prominence, cyclin D1 is an intriguing target in anti-cancer agent development. Many anticancer drugs induce cyclin D1 degradation in several tumor cell lines. Moreover, increased cyclin D1 degradation in cancer cells, is achieved also by various natural compounds such as polyphenols (37). Altogether our results indicate that cyclin D1 degradation and p53 and p21 overexpression contribute to APE-induced growth inhibition in MCF-7 cells.

APE inhibits ERK1/2 phosphorylation. MAPK cascade is a major cell signaling pathway triggered by ROS, leading to activation of numerous transcription factors which control DNA repair, cell cycle, apoptosis, or the immune system, among other processes. Major MAPKs can be grouped into four subfamilies: extracellular signal-regulated kinases (ERKs), c-Jun amino terminal kinase (JNK), p38 MAPK, and big-1 kinases (BMAPK-1). The whole pathway is regulated by various extracellular signals and, in this manner, it regulates distinct and even opposing cellular phenomena, such as prolif-eration, differentiation, survival and apoptosis (38). Moreover, in human hepatocellular carcinoma cell lines, multiple anti-cancer effects such as inhibition of cellular proliferation as well as induction of cell cycle arrest and apoptosis have been achieved by blocking ERK signaling (39).

In order to investigate whether the antiproliferative effect induced by APE in MCF-7 cells was related to ERK, we analyzed the expression and activation (phosphorylation) of ERK by western blotting. As reported in Fig. 5, APE caused a strong dose-dependent inhibition of ERK phosphorylation and a decrease of pERK/ERK ratio while no variations in the total amount of ERK1/2 protein levels occur at any of the concentrations tested. The obtained results suggest that ERK inactivation may contribute to the G2/M cell cycle arrest and to the antiproliferative activity of APE even if the detailed molecular mechanisms underlying the APE-induced ERK signaling modulation needs to be further investigated. In agreement with our data it has been reported that red wine polyphenols inhibit the proliferation of colon carcinoma cells by modulating MAPK intracellular signal transduction path-ways (40) and that quercetin may induce apoptosis in HepG2 cells by direct activation of caspase cascade and by inhibiting survival signaling (41).

APE induces apoptosis through the mitochondrial pathway. The occurrence of apoptosis in MCF-7 cells upon APE treat-ment was assessed by FACS analysis after double labeling of cells with Annexin V and PI. Representative pictures are shown in Fig. 6 together with the percentage of apoptotic cells. The exposure of cells for 48 h to *Annurca* apple polyphenols caused a dose-dependent increase of apoptotic cells respect to the control. A low percentage of early apoptotic cells (4.9%) is already evident after exposure to 100 μM EqC APE. This
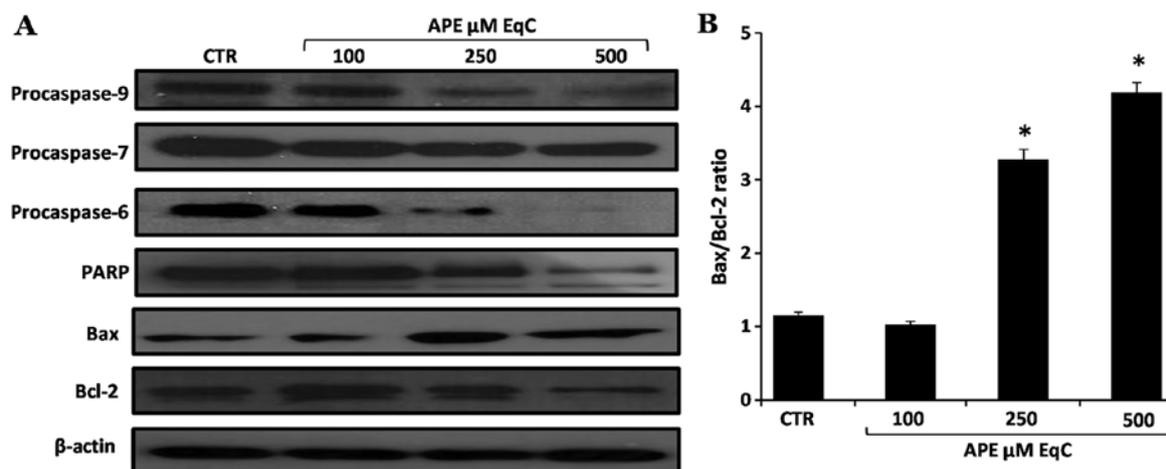
Figure 7. Effects of APE on the levels of some relevant apoptosis regulating proteins in MCF-7 cells. Cells were cultured in medium supplemented or not (Ctr) with APE 100, 250 and 500 μM EqC. Then, 30 μg of cell extracts were subjected to SDS-PAGE and blotted with antibodies against the indicated proteins; the housekeeping protein β-actin was used as loading control. (A) Western blot assay for the expression of pro-caspase-6, -7, -9, PARP, Bax, and Bcl-2. The graph shows the densitometric intensity of Bax/Bcl-2 band ratio in different conditions. The intensities of signals were expressed as arbitrary units. *P<0.05 versus control cells. All results were obtained from at least three independent experiments.

value significantly increases in the cells treated with APE 250 μM EqC (19%) and 500 μM EqC (46.2%).

The APE-induced lipid peroxidation and the highly increased expression levels of p53 after cell incubation with APE led us to hypothesize that the apoptotic process in MCF-7 cells occurs through a ROS-dependent mitochondrial pathway.

The balance of the expression of anti- and pro-apoptotic members of the Bcl-2 gene family is one of the major mechanisms that regulate this type of apoptotic process in mammalian cells (42). Therefore, to determine whether APE-induced apoptosis in MCF-7 cells was associated with the modulation of members of this protein family, we examined the expression of Bcl-2 and Bax proteins. The results obtained, reported in Fig. 7, show that APE treatment resulted in the increased expression of pro-apoptotic Bax while the level of the anti-apoptotic Bcl-2 was decreased. We then tested the effect of APE on the cascade of caspases that are crucial initiators or effectors in this cell death pathway. Western blot analysis shows that the increased Bax/Bcl-2 ratio coincides with the activation of the initiator caspase-9 and of the executioner caspase-6 and -7 and with the increased PARP cleavage (Fig. 7). Altogether these results indicate that the activation of the mitochondrial pathway is involved in the apoptosis of MCF-7 cells induced by APE.

Discussion

The potential of dietary components, in particular polyphenols, as antiproliferative agents has been evidenced in the literature, and growing scientific interest is focused on identifying the biological mechanisms and the signal transduction pathways related to the chemopreventive activities of these compounds. Polyphenols, a heterogeneous class of phytochemicals with a wide range of pharmacological properties, appear quite intriguing molecules because of their antioxidant/pro-oxidant effects. As antioxidants, they are able to act as scavengers of ROS. Conversely, under some conditions, they can act as pro-oxidant, generating ROS and causing cellular oxidative

stress (43). Such pro-oxidant effect of polyphenols seems to be responsible for apoptosis induction in cancer cells. It is speculated that the malignant cells which have an increased level of oxidative stress would be more vulnerable to further ROS attack. Thus, the conventional anticancer therapeutic strategies are based on increased ROS generation treatments which trigger apoptotic damage (44).

In the current study we show that *Annurca* apple polyphenol extract induces lipid peroxidation and inhibits the growth of human breast cancer MCF-7 cells by altering cell cycle kinetics and by inducing apoptosis. We also show that APE exerts its antiproliferative effect by targeting p53 and ERK1/2 signaling pathways. The following considerations deduced from the results obtained allowed us to hypothesize that the pro-apoptotic effect of *Annurca* apple polyphenol extract is related to its pro-oxidant activity: i) APE causes MCF-7 cells to arrest in G2/M phase of the cell cycle, and this is accompanied by a marked decrease of cyclin D1. It is well known that cyclin D1 is downregulated in presence of ROS causing a cell cycle arrest in G2/M and it has been also reported that cyclin D1 depletion is a defense mechanism to reduce ROS-induced genotoxic damages (45) and that ROS affect the proteasomal degradation of cyclin D1 (46); ii) APE induces a marked increase of p53. It is well known that in presence of oxidative stress, the tumor suppressor p53 has a dual action increasing the synthesis of pro- or anti-oxidant enzymes depending to high or low levels of ROS, respectively. In response to low levels of oxidative stress, p53 exhibits anti-oxidant activities and assists the survival and repair of cells with minor injuries while in response to high levels of ROS p53 exhibits pro-oxidant activities and induces mitochondrial apoptosis (47); iii) APE causes a decrease of phosphorylated ERK1/2 protein. Because the activation of ERK1/2 is necessary for cell survival and proliferation, the inhibition of ERK1/2 by APE contributes to the increased occurrence of cell death.

The ERK signaling pathway is activated in response to certain situations of cellular stress, and it is implicated in

cellular death or survival signaling. Generally, the ERK signal transduction pathway is activated by growth factors and is important for proliferation. Nevertheless, ERK has been reported to be inactivated by p53-regulated transcription of phosphatases MKP1, PAC1 and DUSP5. It has been shown, that in response to oxidative stress, MAPKs phosphorylate and activate p53 leading to upregulation of PAC1 and DUSP5 phosphatases. The resulting inactivation of ERK1/2 in turn causes cell cycle arrest and apoptosis (48). The functional interaction between p53 and ERK 1/2 suggests the existence of a reciprocal negative regulation between these signaling pathways. ERK 1/2-dependent p53 phosphorylation induced by different stressful stimuli can lead to p53-dependent cell cycle arrest and apoptosis. On the other hand, activated p53 is able to suppress ERK1/2 signaling via the transcriptional activation of members of the dual specificity phosphatase family thus facilitating p53-induced apoptosis. On the basis of these considerations, it is possible to assume that the balance between the p53 and MAPK pathways will determine the final cell response to oxidative stress.

In conclusion, our findings suggest that APE-mediated ROS generation probably represents the central trigger for the antiproliferative activity of these compounds in MCF-7 cells and allow us to propose the *Annurca* apple polyphenol extract as a promising target for further investigations finalized to the design of innovative adjuvant therapies in breast cancer treatments.

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