



Inhibition of breast cancer cell proliferation by *Annurca* apple polyphenols

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Abstract

Apples are among the most consumed fruits worldwide, and several studies suggest that apple polyphenols could play a role in the prevention of degenerative diseases. The present study is aimed at evaluating the effects of *Annurca* apple polyphenol extract (APE) on proliferation on MCF-7 cells. The data indicated that apple polyphenolic compounds had significant antiproliferative action on MCF-7 cells inducing a cell cycle arrest at G2/M phase. APE was also capable of provoking morphological changes as demonstrated by nuclear condensation. The cellular, morphological, and molecular data suggested that induction of cellular apoptosis was mainly responsible for the observed antiproliferation-induced APE on MCF-7 cells. Taken together all data, it was possible to speculate that APE acts at low micromolar range against breast cancer cells and it may be considered as a promising candidate for anticancer therapy.

Introduction

The *Annurca* of Campania is defined the 'queen of the apples' thanks to its marked organoleptic qualities: flavor, taste and scent. This apple is famous for its white compact and crisp flesh, responsible for a pleasantly acidulous and fragrant flavor making it typically different from the other apple varieties (Lo Scalzo *et al.*, 2001). The *Annurca* apple has digestive features and is excellent for the diabetics as it graduates the assimilation of the glucose. Its depiction in the paintings found in the excavations of Ercolano

testifies its very ancient relationship with the Campania Felix. Surely, the pedoclimatic conditions typical of the cultivation areas in which it is exalted induce the fragrance and its characteristic taste. It is the only apple cultivar native to the Campania region in Southern Italy and forms about 60% of Campania region and 5% of national apple production, with an average of 60,000 tons produced per year (D'Angelo *et al.*, 2007). This cultivar is listed as a Protected Geographical Indication (PGI) product from the European Council [Commission Regulation (EC) No. 417/2006]; its disciplinary of production identifies 137

municipalities in Campania region as the only places of production of the so called “Melannurca Campana IGP”. One of the typical elements that surely characterized the *Annurca* is the reddening on the ground that this One of the typical elements that surely characterized the *Annurca* is the reddening on the ground that this apple undergoes in the ‘melaio’ (place where the *Annurca* trees are). In fact, the fruit is harvested in October and placed for 20–40 days in special boxes called “melai”, a layer of straw or sawdust on the land, sprayed daily with water. When the sun-exposed side turns red, the apple is rotated in order to give its opposite side the chance to turn red. This practice aims at ending the aging of the apple and chooses all handy procedures. Therefore, this phase, exalting its characteristics, gives typicality to the product (Lo Scalzo *et al.*, 2001).

These apples are extremely rich in catechin, epicatechin and chorogenic acid and display a stronger antioxidant activity compared with other varieties (Napolitano *et al.*, 2004). Previously, it has been found that polyphenols extracted by peel and flesh of *Annurca* had high antioxidant activity, regardless of the reddening of the apple (D'Angelo *et al.*, 2007) and its polyphenols defended human erythrocytes against oxidative stress (D'Angelo *et al.*, 2015). There are *in vitro* and *in vivo* evidences for chemopreventive effects of apple polyphenols by modulating cell viability, apoptosis, and reactivating tumor suppressor genes and these polyphenols prevent exogenous damage to both human gastric epithelial cells *in vitro* and rat gastric mucosa *in vivo* (Graziani *et al.*, 2005); polyphenols extracted from *Annurca* apple showed chemopreventive properties in colorectal cancer cells (Fini *et al.*, 2011). We previously showed that the whole *Annurca* apple polyphenol extract (APE) has *in vitro* chemopreventive properties by modulating cell viability, apoptosis in human HaCaT (D'Angelo *et al.*, 2012).

On the basis of these observations, in this study we aimed to evaluate the antiproliferative effects of APE on MCF-7 human breast adenocarcinomas cell line.

Materials and methods

Chemicals and reagents

RPMI medium, fetal calf serum (FCS), nonessential amino acids, and phosphate-buffered saline (PBS) tablets were purchased from Gibco Life Science Technologies. Folin–Ciocalteu reagent, MTT, sodium carbonate, propidium iodide (PI) and trypan blue 0.5% were purchased from Sigma Chemical Co.

Apple samples

Annurca apple (*Malus pumila* Miller cv. *Annurca*) fruits (each weighing about 100 g) were collected in Giugliano (Napoli, Italy) in October 2016, when fruits had just been harvested (green peel). Fruits were reddened following the typical treatment 35 for about 30 days and then analysed (D'Angelo *et al.*, 2007).

Polyphenol extraction and polyphenolic determination

APE extraction from *Annurca* apple was carried out as previously reported by D'Angelo *et al.* (D'Angelo *et al.*, 2012). The total polyphenol content of apple extract was assessed approximately by using the Folin–Ciocalteu phenol reagent as described in Singleton *et al.* (Singleton *et al.*, 1999). Because APE is a mixture of different phenolic compounds with different molecular weights, to give APE an arbitrary molar concentration, its polyphenol concentration was expressed as milligrams of catechin equivalents (EqC) /100 g of *Annurca* flash fresh weight. The chemical characterization was performed in HPLC as reported in D'Angelo *et al.* (D'Angelo *et al.*, 2007).

Cell Culture.

MCF-7 (human breast adenocarcinoma cell line) were routinely cultured in RPMI-1640 (GIBCO, UK) medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/streptomycin (pen/strep) antibiotics (Gibco, Scotland) at 37°C in 5% CO₂ under 90-95% humidity.

Evaluation of morphological changes by phase-contrast microscopy

MCF-7 cells were cultured in 6-well tissue culture plates 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;

i.e. 29, 70 and 145 μg EqC/ml) of APE and the untreated cells were used as control. The cells were cultured for 24-48 hours under standard culture conditions and their morphological changes were imaged using a phase-contrast microscope (Axiovert-10 Zeiss microscope).

Cell viability assays

The number of cells surviving to the treatment with APE was evaluated by vital cell count in Trypan Blue (0.5% solution) in a Burker chamber (D'Angelo *et al.*, 2005). The percentage of viable and dead cells per sample was calculated to determine the effect of APE on cell proliferation.

Flow cytometry analysis of cell cycle.

Cell cycle distribution was studied by PI staining and flow cytometry analysis using a FACScalibur (Becton Dickinson, CA, USA) interfaced with a Hewlett-Packard computer (model 310) for data analysis.

Results and discussion

Total polyphenolic content of the annurca flesh extract was measured by folin-ciocalteu's method. the value has been 125.2 ± 7.1 mg of catechin per 100 g of sample and this measure is similar to that evaluated in other papers. then, we have analyzed the hplc profile of the polyphenols and the data obtained in this paper were comparable to those described previously (data not shown) (d'angelo *et al.*, 2007).

MCF7-cells were incubated for 24-48 hours with increasing concentration of APE. In Fig 1, the APE effects on the cell morphology were indicated. The results obtained indicated that the cells treated with 100 μM EqC APE (Fig. B) maintained the characteristic epithelial morphology and prolific growth as a monolayer, quite similar to untreated MCF-7 cells (Fig. A). On the contrary, the cells treated with 250 μM EqC (Fig. C) and 500 μM EqC (Fig. D) APE displayed morphological alterations such as, shrinkage and cytoplasmic condensation, cell rounding, poor adherence, and cell detachment. These evidences suggested that APE is able to commit MCF-7 cells to a type of death that mimics apoptosis.

The effects of APE on MCF-7 cell viability determined by the trypan blue exclusion method were shown in Figure 2. 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 manner. It has to be noted that after 24 hours incubation, 250 μM EqC APE exerted a cell growth inhibitory effect reducing cell proliferation to about 56% compared to control cells. A prolonged incubation up to 48 hours resulted in a severe loss of

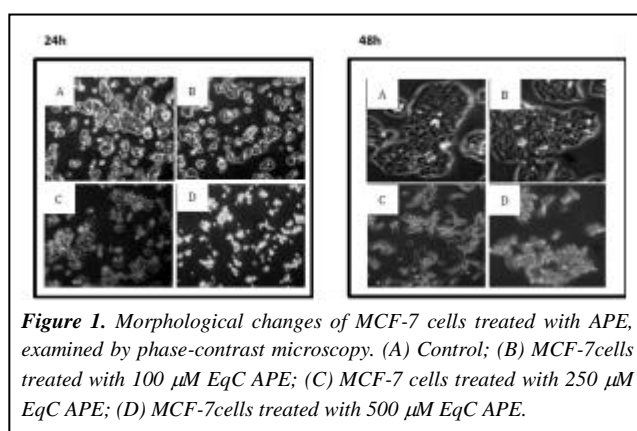


Figure 1. Morphological changes of MCF-7 cells treated with APE, examined by phase-contrast microscopy. (A) Control; (B) MCF-7 cells treated with 100 μM EqC APE; (C) MCF-7 cells treated with 250 μM EqC APE; (D) MCF-7 cells treated with 500 μM EqC APE.

cell viability that decreases to 27%. 500 μM APE caused a severe loss of cell viability at both incubation times. Based on these findings, we selected for further investigations an incubation time of 48 hours.

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 (Fantini *et al.*, 2015). To verify whether APE caused cell cycle perturbation in MCF-7 cells, we evaluated the cells distribution in the cell cycle phases by flow cytometry. Moreover, 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. MCF-7 cells were exposed to increasing concentrations of APE harvested at 48 hours, and examined for DNA content. Table 1 shows that the treatment of cells with 100 and 250 μM EqC APE didn't cause any evident effects on cell proliferation, whereas 500 μM EqC APE induced a cell

Table 1. Distribution (%) of MCF7 cells in different phases of the cell cycle

Cell phase	Control	100 μM APE (29 μg EqC/ml)	250 μM APE (70 μg EqC/ml)	500 μM APE (145 μg EqC/ml)
Sub G ₁	2	12	15	34
G ₁	54	55	55	41
G ₂ /M	11	10	10	26
S	35	35	35	33

higher than 100 μM induced apoptosis in MCF-7 cells and that the proapoptotic effect was dose-dependent. It has to be stressed, again, that the concentrations used (250-500 μM EqC i.e. 70-145 μg EqC/ml), were of the same order of magnitude of those utilized by other research groups to analyze the antiproliferative activity of polyphenolic extracts from other sources.

Conclusion

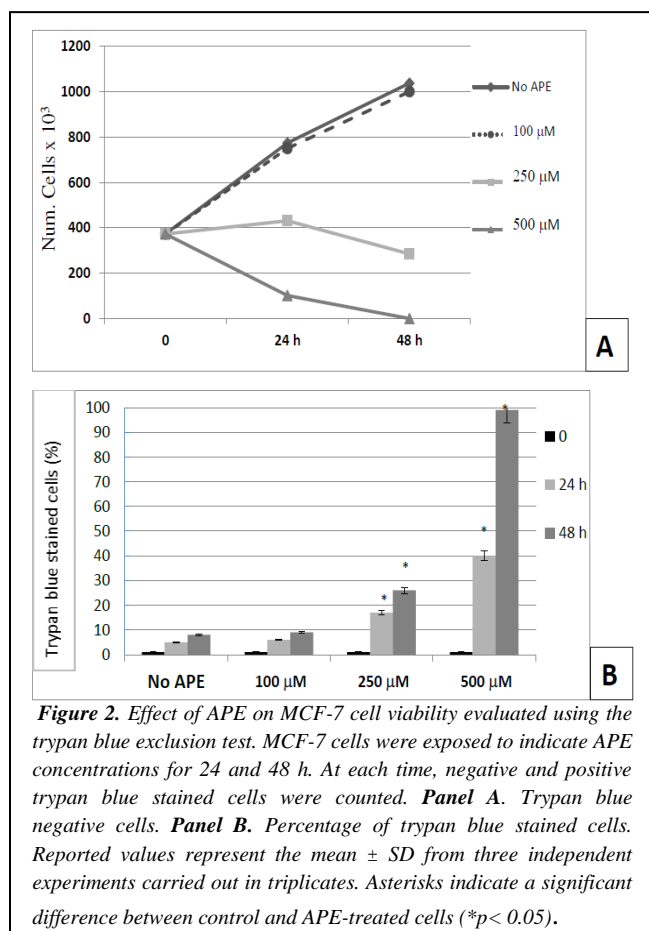
Data indicate that the *Annurca* apple is rich in food components that can markedly inhibit *in vitro* growth of human breast cancer cells and could provide natural bioactive nutraceutical compounds, with potential chemopreventive activity. Our results represent further evidence in support of the idea that *Annurca* apples are an excellent source of bioactive phenolic phytochemicals that may provide special nutraceutical benefits compared to other common fruits.

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cycle arrest at G₂/M, as shown 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 was observed and it had become clearly evident at 500 μM EqC APE (34%) suggesting that the cytotoxic activity of APE in MCF7-cells occurred *via* apoptosis.

We demonstrated that the exposure of cells for 48 hours to *Annurca* apple polyphenols at concentrations

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