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Apoptotic and antiproliferative activity of olive oil hydroxytyrosol on breast cancer cells

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Breast cancer is a major worldwide public health concern for women. It still remains the second most prevalent form of cancer which is terminal despite the advances made in the therapeutic approach. Polyphenols in olive oil and leaves have been known for their medicinal properties. The objective of the present study was to investigate the anti-cancer effect of hydroxytyrosol and to determine the mechanisms underlying its effects. The cytotoxic and antiproliferative effects of hydroxytyrosol were determined in SK-BR-3 and T-47D breast cancer cell lines using the WST-1 assay. Apoptosis was analysed using Annexin V, and cell cycle was investigated by flow cytometry. The key findings of the present study showed that hydroxytyrosol, a pharmacologically safe natural product of olive oil, has potent anti-breast cancer properties. Indeed, it exhibits specific cytotoxicity against SK-BR-3 and T-47D breast cancer cells. Furthermore, hydroxytyrosol triggered apoptosis that showed a dose-dependent increase in both cell lines. Moreover, hydroxytyrosol inhibited cell proliferation by delaying the cell cycle at G2/M phase. Therefore, hydroxytyrosol warrants further investigations to prove its utility in preventing/treating breast cancer.

Key words: Breast cancer, hydroxytyrosol, apoptosis, cytotoxicity, cell proliferation.

INTRODUCTION

Breast cancer remains a major public health problem across the globe. The burden of breast cancer in developing countries demands adaptive strategies which can improve upon presentation of the disease in the stage when the prognosis is good (Smith et al., 2006). The incidence of breast cancer is increasing all over the world due to changes in the dietary habits (Key et al., 2004). Breast cancer is a heterogeneous disease with distinct clinical behavior and molecular properties, in particular estrogen receptor (ER) positive and ER negative cancers are the two most distinct subtypes (Rouzier et al., 2005). Approximately 60 to 70% of all breast tumors express ER, and its presence or absence remains an important prognostic indicator (Brinkman and El-Ashry, 2009). ER-negative tumors have an overall more aggressive clinical course with increased recurrence rates and decreased overall survival (Dworkin et al., 2009), they are also insensitive to antiestrogen treatment (Duffy, 2006; Rusiecki et al., 2005).

Another important oncogen in breast cancer is human epidermal growth factor receptor 2 (HER2), it is one of the most commonly analyzed proto-oncogenes in human

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cancer studies as it plays a pivotal role in oncogenic transformation, tumorigenesis and metastasis (Neve et al., 2001; Yarden, 2001; Menard et al., 2003). HER2 accounts for approximately 20 to 30% of the invasive breast cancers and it is closely associated with an unfavorable prognosis, shorter relapse time, decreased overall survival, and acquired chemoresistance (Ross et al., 2009).

Recently, phytotherapy has become popular as a cure for many diseases and their associated complications. Among the myriad of natural antioxidants, the olive tree has been widely accepted as one of the species with the highest antioxidant activity via its oil, fruits, and leaves. Olive oil is the major source of fats in the Mediterranean diet and is considered to be responsible for the health benefits associated with this diet (Warleta et al., 2011). There are diverging opinions on the association of lower cancer risk and the dietary intake of olive oil. Olive oil intake may have a potential role in lowering the risk of some human neoplasms (La Vecchia, 2004; Gallus et al., 2004; Perez-Jimenez et al., 2005; Colomer and Menendez, 2006). There are several studies reporting the biological activities of the phenolic compounds naturally present in olive oil, such as, anti-inflammatory, cardioprotective, antioxidative and chemopreventive effects in breast and other types of cancers (Cicerale et al., 2009). Phenolic compounds in olive oil have proven to be highly bioavailable, reinforcing their potential health promoting properties (Puel et al., 2008; Tuck and Hayball, 2002; Visioli et al., 2000a). It has been established that olive oil and leaf extracts have antioxidant compounds, such as, oleuropein, hydroxytyrosol, oleuropein aglycone, and tyrosol (Jemai et al., 2008a, 2008b). Hydroxytyrosol has been proven to be a potent scavenger of superoxide anion and hydroxyl radical (Fragopoulou et al., 2007). It also has antithrombotic, antiatherogenic, and anti-inflammatory properties (Carrasco-Pancorbo et al., 2005). It has been shown that oleuropein and hydroxytyrosol inhibit proliferation of human MCF-7 breast cancer cells (Goulas et al., 2009). The role of hydroxytyrosol in protecting DNA from oxidative damage *in vitro* in breast cancer cell lines was documented (Warleta et al., 2011) and has also been reported *in vivo* (Weinbrenner et al., 2004).

It was reported that, the potential molecular mechanisms responsible for the chemopreventive effects of olive oil phenols on breast cancer cells could be due to their ability to interfere with estrogen signaling, since the aromatic ring present in hydroxytyrosol could be a feature common to estrogens (Casaburi et al., 2013).

As ER and the HER2 are the two key biomarkers in breast cancer (Pinhel et al., 2012), the aim of this study is to investigate the anti-cancer effect of hydroxytyrosol on two breast cancer cell lines, the ER negative, HER2 positive SKBR3 and T-47D which is ER positive and HER2 negative. The cytotoxic, antiproliferative and apoptotic effects of the compound on these cells were studied.

MATERIALS AND METHODS

Cell lines, chemicals and cell culture

SK-BR-3 and T47D were obtained from ATCC and maintained in RPMI-1640 (GIBCO, USA), L-glutamin 1%, 10% fetal bovine serum (FBS), 1% antibiotic/antimycotic (penicillin/streptomycin) (Sigma Aldrich, USA). MCF10A cells were cultured in universal medium (1:1 mixture of Dublecos Modified Eagles Medium (DMEM) and Ham's F12 medium (GIBCO) supplemented with 5% FBS, 1% antibiotic antimycotic, 20 ng/ml epidermal growth factor (EGF), 100 ng/ml choleratoxin, 10 µg/ml insulin, and 500 ng/ml hydrocortisone). Cells were maintained at 37°C in humidified incubator with 5% CO_2.

Cytotoxicity assay

Cytotoxicity was measured by the tetrazolium salt WST-1 colorimetric assay, as recommended by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany) (Liu et al., 1995; Takenouchi and Munekata, 1995). Briefly, cells were seeded into 96-well plates at 0.5 to 1.10^4/well and incubated overnight. The medium was replaced with fresh one containing the desired concentrations of the drug. After 20 h, 10 µl of the WST-1 reagent was added to each well and the plates were incubated for 4 h at 37°C. The amount of cleaved tetrazolium salts to formazan, which directly correlates to the number of metabolically active cells in the culture, was quantified using ELISA reader at 450 nm of absorbance.

Cell proliferation assay

Cells were seeded into 96-well plates at 0.5 to 1.10^4/well and incubated overnight. The medium was replaced with fresh one containing 250 µM of hydroxytyrosol and incubated for different time intervals (0, 24, 48, and 72 h). The WST-1 reagent was added to each well. Except for the 0 h, the plates were then incubated for 4 h at 37°C. The amount of formazan was quantified using ELISA reader at 450 nm of absorbance.

Apoptosis analysis by Annexin V

Confluent cells were either treated with dimethyl sulfoxide (DMSO) and used as control or challenged with hydroxytyrosl, where upon cells were incubated in medium with supplements. Detached and adherent cells were harvested 72 h later, centrifuged and resuspended in 1 ml of PBS. Cells were then stained by propidium iodide (PI) and Alexa Fluor 488 Annexin V, using Vibrant Apoptosis Assay kit #2 (Molecular probe, Eugene, OR). Stained cells were analyzed by flow cytometry. The percentage of cells was determined by the FACS cadibur apparatus and the Cell Quest Pro software from Becton Dickinson (San Jose, CA). For each cell culture, three independent experiments were performed.

Cell cycle analysis by flow cytometry

Cells were treated with DMSO or hydroxytyrosol, and then harvested and resuspended in 1 ml of PBS before being fixed by

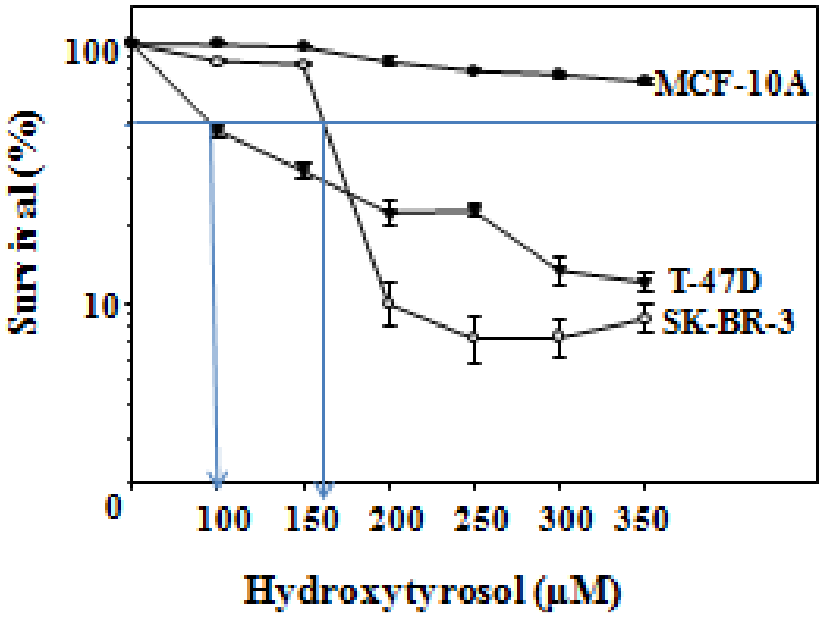

Figure 1. The cytotoxic effect of hydroxytyrosol on breast cancer cells. Exponentially growing cells were cultured in 96-well plates and treated with the indicated hydroxytyrosol concentrations for 24 h. Cell death was analyzed using the WST-1 assay. The arrows indicate the LC50. Error bars represent standard deviations of at least 3 different experiments.

drop wise addition of 3 ml of 100% methanol. Fixed cells were centrifuged, resuspended in 50 µl of RNase (1 mg/ml) and incubated for 30 min at room temperature, followed by addition of 1 ml of 0.1 mg/ml of PI. Cells were analyzed for DNA content by flow cytometry (Becton Dickinson). The percentage of cells in various cell-cycle phases was determined by using Cell Quest software (Becton Dickinson).

RESULTS

Hydroxytyrosol has cytotoxic effect on breast cancer cells

The main feature of anti-cancer agents is their ability to trigger cell death specifically in cancer cells avoiding normal ones. Therefore, this study investigated the cytotoxic effect of hydroxytyrosol on normal and different breast cancer cell lines using the WST-1 assay. Cells were seeded in triplicates into microtiter plates and treated with increasing concentrations of hydroxytyrosol for 24 h, and then the cytotoxic effect was measured. Figure 1 shows dose-dependent effect of hydroxytyrosol on breast cancer (SK-BR-3 and T-47D) and the normal MCF-10A cells. While MCF-10A showed only marginal sensitivity even when challenged with high doses of hydroxytyrosol, both breast cancer cell lines exhibited high sensitivity (Figure 1). The median lethal concentrations (LC_{50}) for SK-BR-3 and T-47D cells were 160 and 100 µM, respectively. However, at 250 µM of hydroxytyrosol, both SK-BR-3 and T-47D cells were highly sensitive (Figure 1). Indeed, the proportion of survived cells dropped to 7 and 20% for SK-BR-3 and T-47D cells, respectively (Figure 1). Interestingly, the cytotoxic effect of the drug increased and decreased only slightly in response to concentrations higher than 250 µM in SK-BR-3 and T-47D cell lines, respectively (Figure 1).

Hydroxytyrosol triggers apoptosis in breast cancer cells

To confirm the cytotoxic nature of hydroxytyrosol and to identify the death pathway that this agent triggers in breast cancer cells, the Annexin V/propidium iodide (PI) staining technique followed by flow cytometry was used. Sixty percent confluent cells were treated with different concentrations of hydroxytyrosol for 3 days, and then were stained and sorted. Figure 2A shows four groups of cells, viable cells that excluded both Annexin V and PI (Annexin V-/PI-), bottom left; early apoptotic cells that were only stained with Annexin V (Annexin V+/PI-), bottom right; late apoptotic cells that were stained with both AnnexinV and PI (Annexin V+/P+), top right and necrotic cells that were only stained with PI (Annexin V-/PI+), top left. The proportion of apoptosis was considered as the sum of both early and late apoptosis

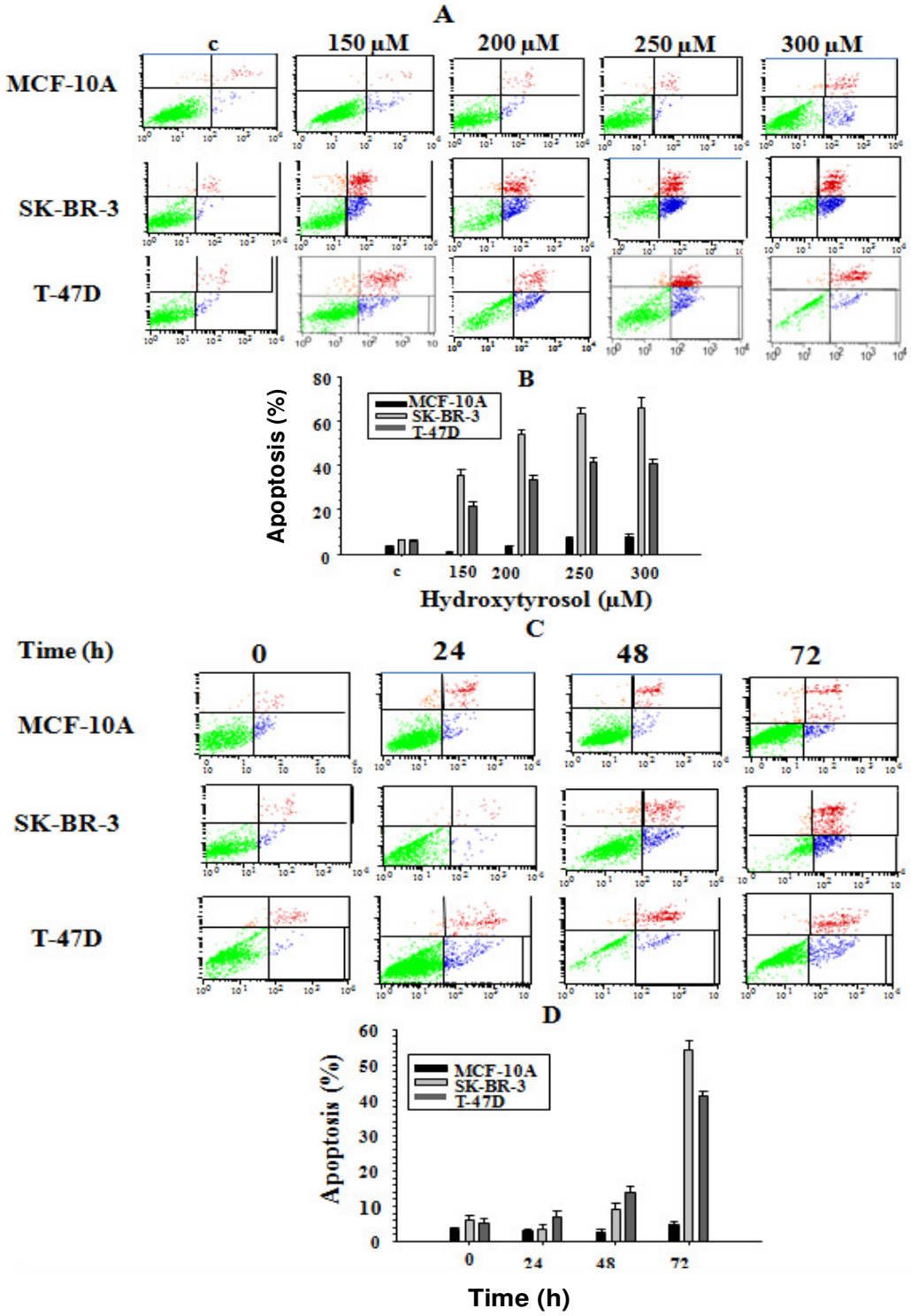

Figure 2. The apoptotic effect of hydroxytyrosol on breast cancer cells. Sub-confluent cells were either mock-treated or challenged with the indicated concentrations of hydroxytyrosol for 72 h and then cell death was analyzed using the Annexin V/PI flow cytometry assay. (A) Charts, indicating the proportion of apoptotic cells. (B) Histogram showing the proportions of apoptotic cells. (C) Cells were treated with hydroxytyrosol (250 µM) for the indicated periods of time and cell death was analyzed as in A. (D) Histograms showing the proportions of apoptosis. Error bars represent standard deviations of at least 3 different experiments.

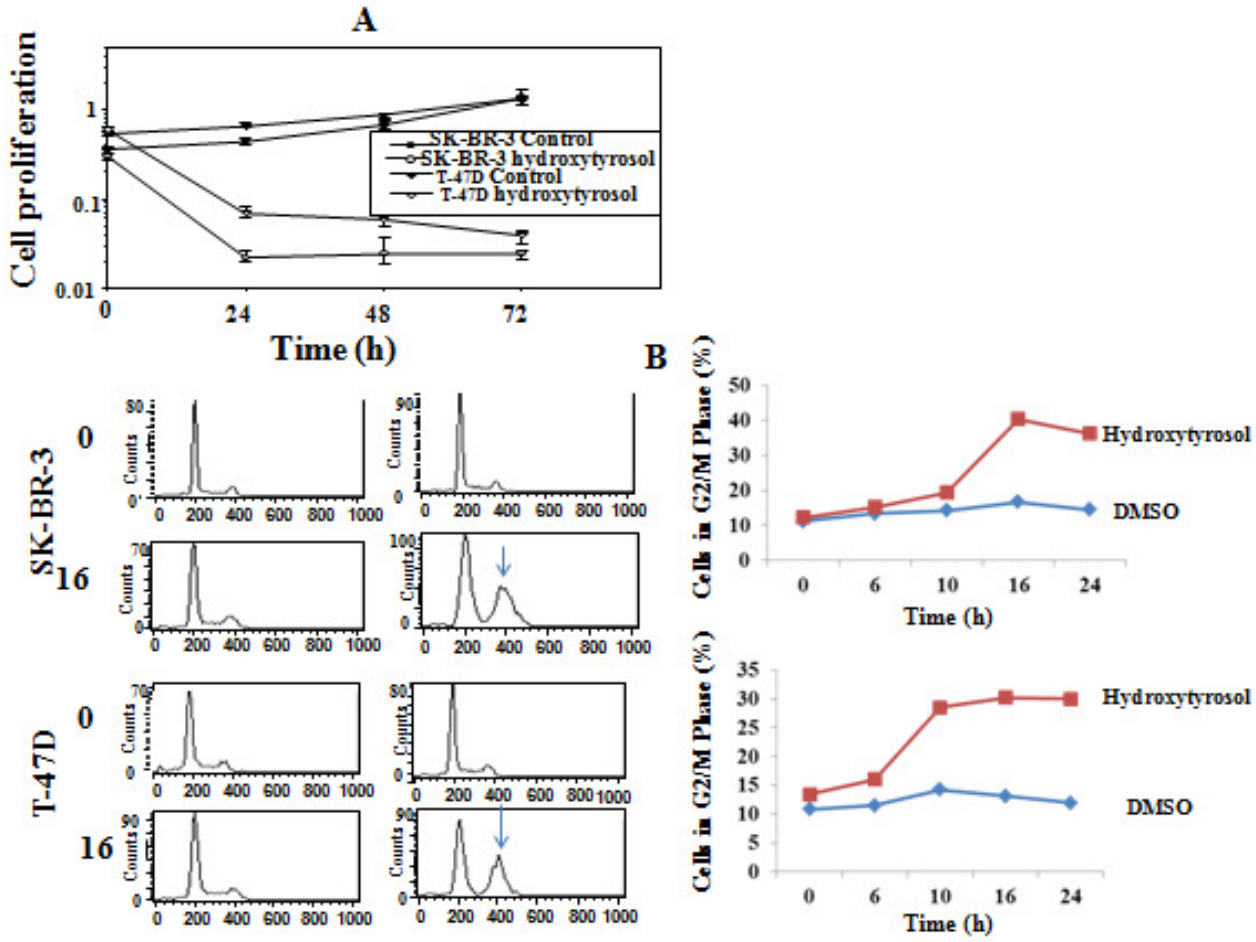

Figure 3. The effect of hydroxytyrosol on the cell cycle of breast cancer cells. (A) Cells were cultured in 96 wells plates and challenged with hydroxytyrosol (250 µM) for the indicated periods of time, and then cell proliferation was assessed by the WST-1 assay. (B) Cells were either mock-treated or challenged with hydroxytyrosol (250 µM) for different time intervals. The cell cycle status was analyzed by flow cytometry. The arrows indicate the accumulated cells at G2/M phase. The curves show the proportions of cells in G2/M phase.

after deduction of the proportion of spontaneous apoptosis. Figure 2A confirms the cytotoxicity of hydroxytyrosol against breast cancer cells with minimal effect on normal cells. Importantly, hydroxytyrosol triggered apoptosis in both cell lines SK-BR-3 and T-47D. This effect increased in a dose-dependent manner in both cell lines (Figure 2B). Hydroxytyrosol (250 µM) triggered apoptosis in more than 60% SK-BR-3 and around 40% T-47D cells (Figure 2B). This shows that this agent triggers cell death in breast cancer cell lines. Next, SK-BR-3 and T-47D cells were treated with 250 µM hydroxytyrosol for different periods of time. The maximum proportions of cell death were reached after 72 h of treatment for both SK-BR-3 and T-47D (Figure 2C). Hydroxytyrosol (250 µM) triggered apoptosis in more than 50% SK-BR-3 and around 40% T-47D cells after 72 h of treatment (Figure 2D).

Hydroxytyrosol inhibits breast cancer cell proliferation by delaying the cell cycle at G2/M phase

Since cancers are cell cycle/proliferation-related diseases, this study investigated the effect of hydroxytyrosol on breast cancer cell proliferation using the WST-1 cell proliferation assay. SK-BR-3 and T-47D cells were seeded in triplicates into microtiter plates at 0.5 to 1.10^4/well and were treated with hydroxytyrosol (250 µM) for various periods of time, and then cellular proliferation was measured by the WST-1 assay. Figure 3A shows that while the control non-treated cells continue proliferating in a time-dependent manner, the number of hydroxytyrosol-treated SK-BR-3 and T-47D cells decreased sharply after only 24 h of treatment. Therefore, like for cytotoxicity, the effect of hydroxytyrosol was pronounced on both SK-BR-3 and T-47D cells. After

seeing the inhibitory effect of hydroxytyrosol on cell proliferation, the research team sought to investigate the effect of this agent on the cell cycle. To this end, SK-BR-3 and T-47D cells were either sham-treated or challenged with hydroxytyrosol (250 µM) for different time intervals, and then cells were fixed, stained with PI, and cell cycle was analyzed by flow cytometery. Figure 3B shows hydroxytyrosol-dependent accumulation of cells in the G2/M phase of the cell cycle, reaching a maximum level of around 40 and 30% after 16 h of incubation in SK-BR-3 and T-47D cells, respectively. Interestingly, this effect was sustained up to 24 h of treatment in T-47D and started to decrease slightly in SK-BR-3 (Figure 3B), reflecting the effect on cell proliferation described in Figure 3A. This shows that hydroxytyrosol inhibits cell proliferation by delaying the cell cycle during the G2/M phase.

DISCUSSION

Synthetic antioxidants have been reported to be carcinogenic in some cases (Imaida et al., 1984), thereby triggering an interest in phenolic compounds particularly the naturally occurring antioxidants as an alternative to the synthetic ones (Latte and Kolodziej, 2004). Olive (*Olea europea L.*) leaves have been extensively exploited for the prevention or the treatment of many diseases. Hydroxytyrosol is a phenolic compound present in the olive oil and leaf extract. It has powerful antioxidant activity (Visioli et al., 2000c), and offers other health benefits (Granados-Principal et al., 2011). Studies on bioavailability have demonstrated that hydroxytyrosol in olive oil is dose-dependently absorbed in animals and humans after olive oil ingestion (Visioli et al., 2000b) and accumulated in the body which finally exerts biological effects, systemically (Weinbrenner et al., 2004). On the basis of these considerations, this study focused on the effects that hydroxytyrosol, an important phenolic in olives, may exert on breast cancer cells. In the present study, it is evident that hydroxytyrosol, an active element in olive oil, could constitute a potential therapeutic agent for breast tumors for the following reasons.

First, it has been shown that hydroxytyrosol has high cytotoxicity against SK-BR-3 and T-47D cells; while it has marginal cytotoxicity against “normal” epithelial cells *in vitro* (Figures 1 and 2). The median lethal concentrations (LC_{50}) for SKB-R-3 and T-47D were 160 and 100 µM, respectively. However, the physiological concentration of hydroxytyrosol after olive oil ingestion was suggested to be between 10 and 100 µM (Quiles et al., 2002). Hydroxytyrosol exposure also reduced cell viability in the SK-BR-3 breast cancer cell line (Menendez et al., 2007). It has been previously reported that, hydroxytyrosol decreased cell viability in MCF-7 cells (Han et al., 2009). Olive leaf extracts were also documented to decrease breast cancer cell viability on SK-BR-3, MCF-7 and JIMT-1breast cancer cells and this effect was attributed to the phenolics identified in olive leaves (Fu et al., 2010). In fact, it has been previously shown that hydroxytyrosol has an outstanding safety profile in rodents. Hydroxytyrosol, at 16 mg/kg body weight exhibited a pronounced hypoglycemic and hypolipidemic effects, and enhanced the antioxidant defense system in rats (Jemai et al., 2009). The safety of olive extracts was confirmed by another study which reported a dose as high as 1000 mg/kg to be safe and significantly effective in mice (Kimura and Sumiyoshi, 2009). These promising results have already led to ongoing pilot clinical trials.

Second, hydroxytyrosol triggers apoptosis in breast cancer cells. Interestingly, the effect was highly pronounced on both SK-BR-3 and T-47D breast cancer cells (Figure 2). Although the results of this study are in accordance with those achieved in MCF7 (Han et al., 2009), HL60 cells (promyelocytic leukemia) (Fabiani et al., 2006) and HT29 (colon adenocarcinoma) (Guichard et al., 2006), they are in contrast with the findings that hydroxytyrosol had no apoptotic effect of MDA-MB-231 and MCF-7 (Warleta et al., 2011). This discrepancy could be due to the different apoptosis assays used by the researchers (Granados-Principal et al., 2010) or it could be explained on the basis that, cell lines are prone to genotypic and phenotypic drift during their continual culture. It has been previously reported that, this type of drift is particularly common in the more frequently used cell lines, especially those that have been deposited in cell banks for many years (Burdall et al., 2003). Many discrepancies were also found in the most commonly used breast cancer cell line, namely MCF-7, obtained from different laboratories (Osborne et al., 1987). The karyotypic variation of established cell lines in culture has been demonstrated in both MDA-MB-231 (Watson et al., 2004) and MCF-7 (Bahia et al., 2002). In general, there are inherent problems with the use of cell lines especially those derived from tumours. The phenomenon of intraspecies cross-contamination of established cell cultures appears to be widespread and may account for some misrepresentation of data (MacLeod et al., 1999).

Hydroxytyrosol was reported to be an inhibitant to HER2 expression in SK-BR-3 breast-cancer cells in addition; it induced cytotoxic activity in these cells (Menendez et al., 2007). In addition, hydroxytyrosol has proven to be powerful inhibitor of the fatty-acid enzyme synthase, an enzyme that is deeply involved in carcinogenesis in SKBR3 and MCF7 cells, which overexpress the receptor HER2 (Menendez et al., 2008).

Third, hydroxytyrosol is a potent inhibitor of cell proliferation by delaying the cell cycle at G2/M phase (Figure 3). Hydroxytyrosol showed a dose-dependent reduction of cell proliferation in both MDA-MB-231 and MCF-7 cells at concentrations starting from 200 µM (Warleta et al., 2011). It has been previously reported that

hydroxytyrosol inhibits cell proliferation of MCF-7 cells (Han et al., 2009; Sirianni et al., 2010). Lack of inhibition of cell proliferation in SK-BR-3 and MCF7 was documented by Menendez et al. (2007), using hydroxytyrosol treatments in the range of 6.25 to 100 µM. The reason for such discrepancy is unclear but it may be due to the low doses used as compared to those used in our study. Seeding of cells at high concentration may also reduce the growth inhibition action of hydroxytyrosol. Han et al. (2009) found that hydroxytyrosol showed a more pronounced cell growth arrest on MCF7 when cells were seeded at low number. Growth inhibition of MCF-7 cells due to the cell cycle arrest in the G0/G1 phase was shown when the cells were treated with hydroxytyrosol, this effect was induced by downregulation of Pin1 which in turn decreased the level of Cyclin D1 (Bouallagui et al., 2011). A significant block of G1 to S phase transition in MCF7 using hydroxytyrosol was also reported (Han et al., 2009); however, the antiproliferative and cell cycle effects of hydroxytyrosol on tumor cells remain completely unknown (Fabiani et al., 2008). Our findings are in agreement with results in human promyelocytic leukaemia HL60 cells with a detectable antiproliferative effect coupled with cell cycle arrest shown by hydroxytyrosol (Fabiani et al., 2002). In a study carried out by Warleta et al. (2011), hydroxytyrosol was reported as a preventive agent for oxidative DNA damage in breast cancer cells. Moreover, several human studies have been conducted with olive extracts or its polyphenolics showing no adverse effects (Visioli and Galli, 2001; Gonzalez et al., 2002). A Mediterranean diet rich in olive oil supplies around 10 to 20 mg of polyphenols per day and, although the bioavailability of different phenolic compounds varies, it appears that those from olive oil are well absorbed in humans (Visioli et al., 2000c; Vissers et al., 2002) mainly in the small intestine, the absorption process is followed by metabolism of these phenols. Also, *in vivo* studies have shown that soon after oral administration, olive oil phenolic compounds appeared in the plasma suggesting their effective absorption from the intestines, but the entire quantity administered was not found in the urine which may suggest accumulation in organs such as breast or erythrocytes (Tripoli et al., 2005).

The overall evidence from *in vitro* assays, animal and human studies support the antioxidant effect of olive polyphenols (Raederstorff, 2009). Hydroxytyrosol was suggested to prevent the initiation of a chain of reactions to transform normal cells into cancer cells due to the probability of its prevention to oxidative stress in normal breast cells (Warleta et al., 2011). It has been suggested that consumption of virgin olive oil, which is particularly rich in phenolic antioxidants, such as hydroxytyrosol, should provide considerable protection against breast cancer by inhibiting oxidative stress (Owen et al., 2000). Protective effect of hydroxytyrosol was detected against basal and H_2O_2-induced DNA damage regardless of the breast cellular type (Warleta et al., 2011).

In conclusion, our results support the hypothesis that hydroxytyrosol may exert a protective effect against breast cancer by reducing cell viability, arresting the cell cycle and inducing apoptosis in these cells. The accessibility of olive oil and leaf extracts to cancer cells should be carefully addressed in animal models and pilot human clinical studies. Further, molecular studies are necessary to elucidate the anti-breast cancer properties of hydroxytyrosol.

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