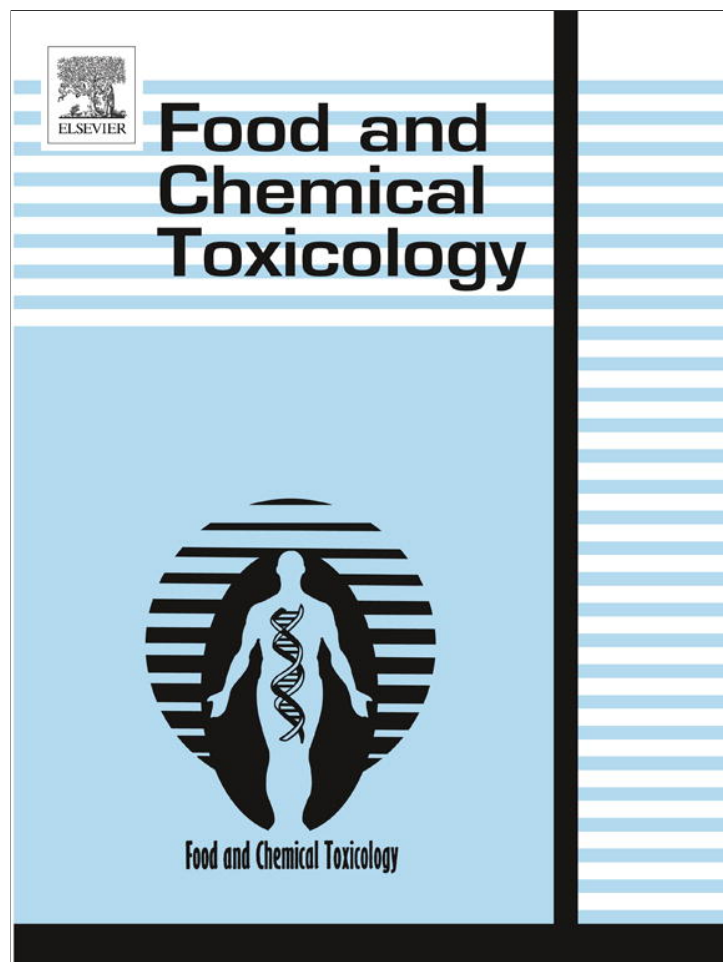


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Olive oil oleuropein has anti-breast cancer properties with higher efficiency on ER-negative cells

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ABSTRACT

Breast cancer constitutes a major health problem for women worldwide. However, its incidence varies between populations and geographical locations. These variations could be diet-related, since there are several carcinogenic compounds in the modern diet, while natural products contain various anti-cancer elements. Several lines of evidence indicate that, in addition to their clear preventive effect, these compounds could also be used as therapeutic agents. In the present report we have shown that oleuropein, a pharmacologically safe natural product of olive leaf, has potent anti-breast cancer properties. Indeed, oleuropein exhibits specific cytotoxicity against breast cancer cells, with higher effect on the basal-like MDA-MB-231 cells than on the luminal MCF-7 cells. This effect is mediated through the induction of apoptosis via the mitochondrial pathway. Moreover, oleuropein inhibits cell proliferation by delaying the cell cycle at S phase and up-regulated the cyclin-dependent inhibitor p21. Furthermore, oleuropein inhibited the anti-apoptosis and pro-proliferation protein NF- κ B and its main oncogenic target cyclin D1. This inhibition could explain the great effect of oleuropein on cell proliferation and cell death of breast cancer cells. Therefore, oleuropein warrants further investigations to prove its utility in preventing/treating breast cancer, especially the less-responsive basal-like type.

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1. Introduction

Breast cancer is the most common cancer and leading cause of cancer deaths among women worldwide (Jemal et al., 2011). The breast cancer burden is increasing in developing countries, with disproportionately high mortality (Saad, 2011). Furthermore, 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). Since ER plays a central role in the crosstalk between different signaling pathways in breast cancer, the expression of this receptor is important for the behavior of breast cancer cells and is reflected in gene expression patterns of breast tumors. ER positive cells exhibit higher proliferative capacity and distinct drug response than ER negative cells (Badve and Nakshatri, 2009; Shen et al., 2012). Importantly, ER negative cancers are generally more sensitive to chemotherapy, but associated with poor clinical outcome (Andre et al., 2008). The mode of treatment of breast can-

cer depends on various genetics, molecular and histological factors. However, breast-conserving surgery followed by whole-breast irradiation, has become the standard of care in the treatment of early-stage breast cancer (Fisher et al., 2002; Clarke et al., 2005). The implementation of adjuvant therapy either hormonal or chemical has made a major impact on disease-free survival and overall survival in both premenopausal and postmenopausal women with early-stage breast cancer. However, high proportion of treated breast cancer patients suffer from recurrence (Clarke et al., 2005), and the majority of these patients die of disseminated metastatic disease, which supports the need for more efficient and less toxic adjuvant therapeutic strategies.

The traditional Mediterranean diet is characterized by high consumption of foods of plant origin, and frequent use of olive (*Olea europaea*) and its products. This diet is rich in bioactive components, such as vitamins, flavonoids and polyphenols, which could explain the low incidence of cancer in this region of the world (Trichopoulou et al., 2000). In fact, the pharmacological properties of olive oil, the olive fruit and its leaves have been recognized since centuries (Visioli et al., 2002). Olive oil is rich of three classes of polyphenols such as hydroxytyrosol (HT), secoiridoid such as oleuropein (OL) and lignans, which exhibit remarkable antioxidant actions (Owen et al., 2000). Oleuropein is the most

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abundant of the phenolic compounds in olives (Han et al., 2009). Several studies have shown that oleuropein possesses a wide range of pharmacologic and health promoting properties including anti-atherogenic (Carluccio et al., 2003), antiviral (Micol et al., 2005), antimicrobial (Saija and Uccella, 2000), hypotensive (Khayyal et al., 2002) and antidiabetic effects (Jemai et al., 2009). Many of these properties have been described as resulting from the antioxidant character of oleuropein (Visioli et al., 2002). Therefore, oleuropein and its metabolite, hydroxytyrosol possess anti-cancer properties. Indeed, high doses of oleuropein decreased cell viability and inhibited cell proliferation in MCF-7 breast cancer cells (Han et al., 2009). To further explore the anti-breast cancer properties of oleuropein and determine the molecular mechanisms underlying its effects we investigated the effects of oleuropein on basal-like and luminal breast cancer cells and we have shown that this molecule triggers mainly apoptosis through the mitochondrial pathway and inhibits cell proliferation by delaying cell cycle at S phase. Furthermore, oleuropein inhibits NF- κ B and its major target cyclin D1.

2. Materials and methods

2.1. Cell lines, chemicals and cell culture

MCF-10A, MDA-MB-231 and MCF-7 were obtained from ATCC and were cultured following the instructions of the company. MCF-7 and MDA-MB231 were maintained in RPMI-1640 (GIBCO, USA), L-glutamin 1%, 10% fetal bovine serum (FBS), 1% antibiotic/anti-mycotic (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 cholera toxin, 10 μ g/ml insulin, and 500 ng/ml hydrocortisone). Cells were maintained at 37 °C in humidified incubator with 5% CO₂.

2.2. 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 0.5–1.10⁴/well and incubated overnight. The medium was replaced with fresh one containing the desired concentrations of the drug. After 20 h, 10 μ l 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 enzyme-linked immunosorbent assay (ELISA) reader at 450 nm of absorbance.

2.3. Cell proliferation assay

Cells were seeded into 96-well plates at 0.5–1.10⁴/well and incubated overnight. The medium was replaced with fresh one containing 200 μ M of oleuropein and incubated for different time intervals (0, 24, 48, and 72 h). 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.

2.4. Cellular lysate preparation

Cells were washed with phosphate-buffered saline (PBS) and then scraped in RIPA buffer (150 mM of NaCl, 1 mM of EDTA, 1%Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM of Tris-HCl (pH 7.5), supplemented with protease inhibitors. Lysates were homogenized and then centrifuged at 14,000 rpm at 4 °C for 15 min in an Eppendorf microcentrifuge. The supernatant was removed, aliquoted, and stored at –80 °C.

2.5. Immunoblotting

SDS-PAGE was performed using 12% separating minigels and equal amounts of protein extract (50 μ g) were loaded. After protein migration and transfer onto polyvinylidene difluoride membrane (PVDF), the membrane was incubated overnight with the appropriate antibodies: Cyclin D1 (2922S), Survivin (71G4B7), Cleaved caspase-3 (ASP 175) from Cell signaling, USA. NF- κ B (F-6), Bcl2 (100), Bax (B-9), GAPDH (FL-335), α -tubulin (TU-02) from Santa Cruz, CA, USA.

2.6. Apoptosis analysis by Annexin V

Confluent cells were either treated with DMSO and used as control or challenged with different agents, whereupon 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.

2.7. Cell cycle analysis by flow cytometry

Cells were treated with DMSO or oleuropein, and then harvested and resuspended in 1 ml of PBS before being fixed by drop wise addition of 3 ml of 100% methanol. Fixed cells were centrifuged, resuspended in 50 μ l 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).

3. Results

3.1. Oleuropein has cytotoxic effects 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, we investigated the cytotoxic effect of oleuropein 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 oleuropein for 24 h, and then the cytotoxic effect was measured. Fig. 1 shows dose-dependent effect of oleuropein on breast cancer (MDA-MB-231 and MCF-7) and the 'normal' MCF-10A cells. While MCF-10A showed only marginal sensitivity even when challenged with high doses of oleuropein, both breast cancer cell lines exhibited significant sensitivity (Fig. 1). The median lethal concentrations (LC₅₀) for MCF-7 and MDA-MB-231 cells were 110 and 160 μ M, respectively. However, at 200 μ M of oleuropein, MDA-MB-231 showed much higher sensitivity than MCF-7 (Fig. 1). Indeed, the proportion of survived cells dropped to 3% for MDA-MB-231 cells while it remained close to 35% for MCF-7, showing a 10-fold difference in survival between both cell lines (Fig. 1). Interestingly, the killing effect of the drug increased only slightly in response to concentrations higher than 200 μ M for both cell lines (Fig. 1).

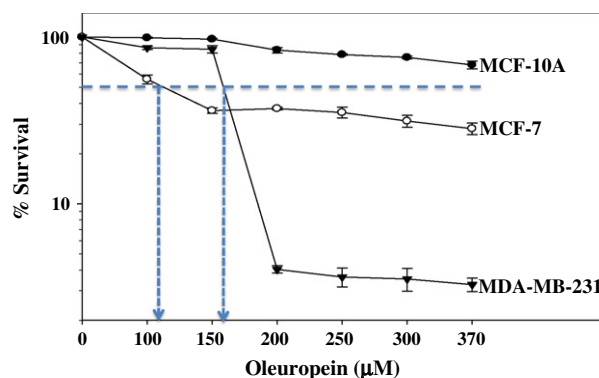


Fig. 1. Cytotoxic effects of oleuropein on breast cancer cells. Exponentially growing cells were cultured in 96-well plates and treated with the indicated oleuropein concentrations for 24 h. Cell death was analyzed using the WST-1 assay. The arrows indicate the LC₅₀. Error bars represent standard deviations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

3.2. Oleuropein triggers apoptosis through the mitochondrial pathway in breast cancer cells

To confirm the cytotoxic nature of oleuropein 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 oleuropein for 3 days, and then were stained and sorted. Fig. 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 Annexin V and PI (Annexin V⁺/PI⁺), 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 after deduction of the proportion of spontaneous apoptosis. Fig. 2A confirms the cytotoxicity of oleuropein against breast cancer cells with minimal effect on normal cells. Importantly, oleuropein triggered both apoptosis and necrosis in both cell lines MCF-7 and MDA-MB-231. This effect increased in a dose-dependent manner with a higher effect against MDA-MB-231 than MCF-7 cells (Fig. 2B). Oleuropein (200 μM) triggered apoptosis in more than 50% MDA-MB-231 cells, while less than 5% of MCF-7 were apoptotic in response to the same dose (Fig. 2B). This 10-fold difference parallels the cytotoxic result depicted in Fig. 1. The proportion of apoptotic cells reached 25% of

MCF-7 cells when treated with 300 μM of oleuropein. Similarly, the proportion of oleuropein-related necrosis reached 20% in MDA-MB-231 cells and was only 5% in MCF-7 cells (Fig. 2C). This proportion reached 30% of MCF-7 cells in response to 300 μM of oleuropein (Fig. 2C). This shows that oleuropein triggers cell death in breast cancer cell lines, but with more potent effect on the basal-like cells (MDA-MB-231) than on the luminal cells (MCF-7). Next, MDA-MB-231 and MCF-7 cells were treated with 200 μM oleuropein for different periods of time. The maximum proportions of cell death were reached after 72 h of treatment for MDA-MB-231 (Fig. 2D). Oleuropein (200 μM) triggered apoptosis in more than 55% in MDA-MB-231 and less than 5% in MCF-7 cells after 72 h of treatment (Fig. 2E). Similar dose shows around 12% and 5% necrosis in MDA-MB-231 and MCF7 cells, respectively after 72 h of treatment (Fig. 2F).

To confirm the induction of apoptosis by oleuropein in breast cancer cells and determine the apoptotic route, MDA-MB-231 cells were treated with 200 μM of oleuropein and harvested after different time periods (0, 24, 48, and 72 h). Whole cell extracts were prepared and 50 μg of extracted proteins were used to evaluate the levels of pro- and anti-apoptotic proteins using the immunoblotting technique and specific antibodies. GAPDH was used as internal control. Interestingly, oleuropein up-regulated the active form of caspase-3 to a level 14.8-fold higher after 72 h of treatment (Fig. 3A), confirming the induction of apoptosis in these cells. To investigate the possible involvement of the mitochondrial pathway

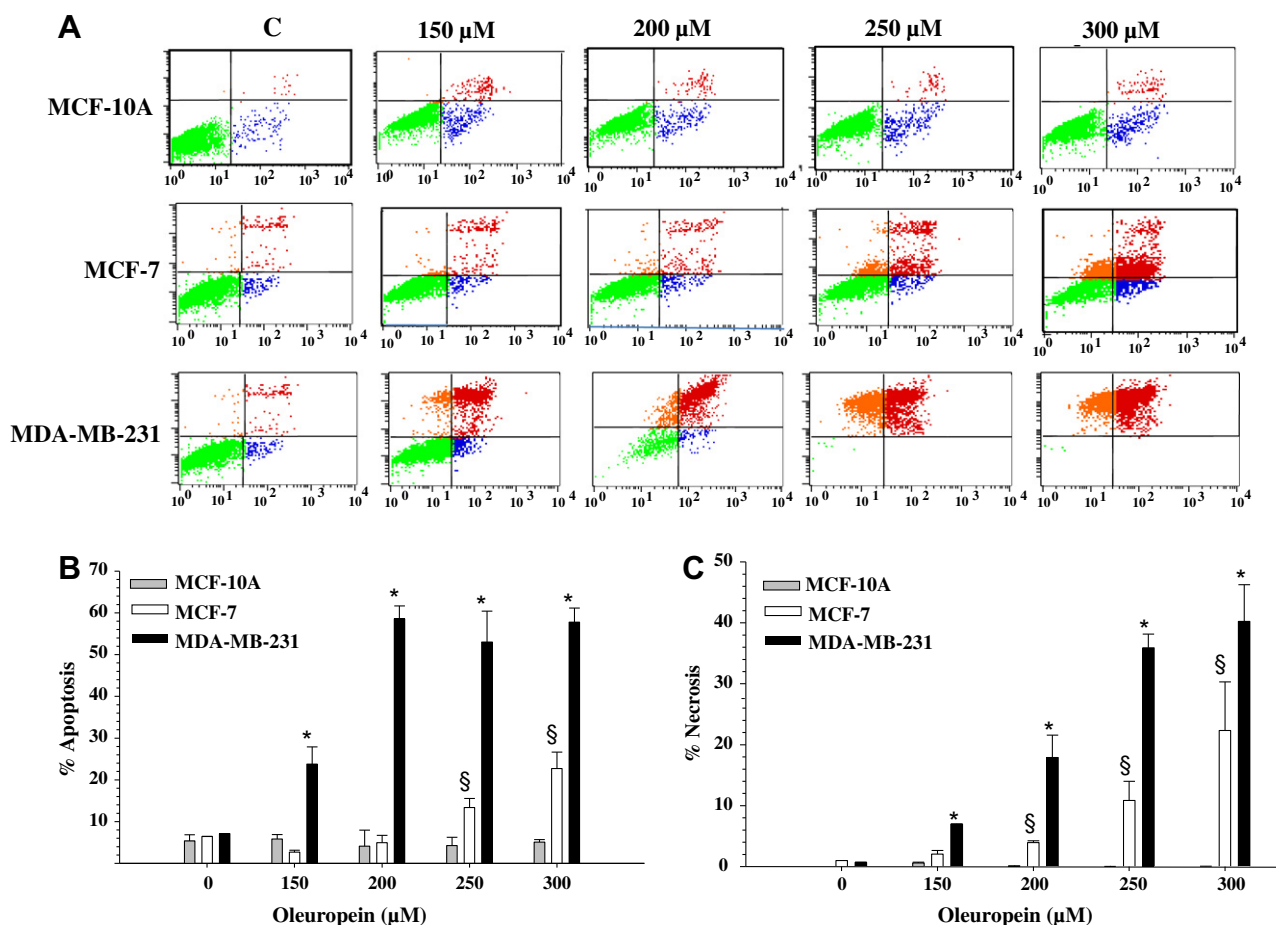


Fig. 2. Oleuropein triggers apoptosis in breast cancer cells. Sub-confluent cells were either mock-treated or challenged with the indicated concentrations of oleuropein for 72 h and then cell death was analyzed using the Annexin V/PI flow cytometry assay. (A) Charts, indicating the proportion of apoptotic and necrotic cells. (B) Histogram showing the proportions of apoptotic cells. (C) Histogram showing the proportions of necrotic cells. (D) Cells were treated with oleuropein (200 μM) for the indicated periods of time and cell death was analyzed as in A. (E, F) Histograms showing the proportions of apoptosis and necrosis, respectively. Error bars represent standard deviations of at least three different experiments. § and * indicate statistically significant differences as compared to MCF-10A.

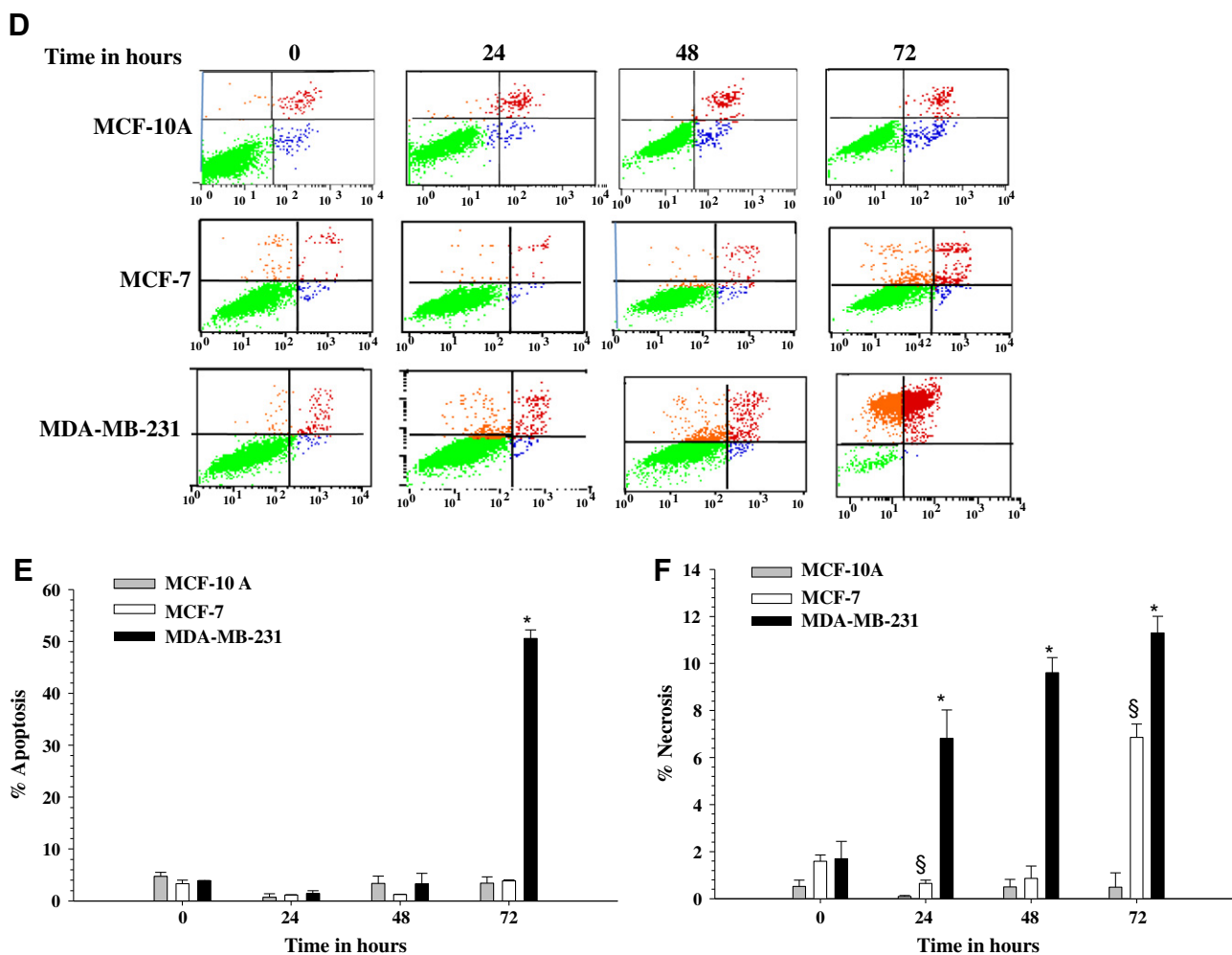


Fig. 2. (continued)

in this process, we assessed the effect of the drug on the level of Bax and Bcl-2 proteins. After 72 h of treatment, the level of the pro-apoptotic Bax protein increased, while the level of the anti-apoptosis Bcl-2 protein decreased (Fig. 3A). This led to a significant increase in the Bax/Bcl-2 ratio to a level 7-fold higher (Fig. 3B), which indicates the implication of the internal apoptosis pathway in the oleuropein-dependent induction of apoptosis in breast cancer cells. Furthermore, we studied the effect of oleuropein on the level of survivin, an anti-apoptosis protein (Karczmarek-Borowska et al., 2005; Altieri, 2008). Thereby, treatment with 200 μ M of oleuropein led to high decrease in the survivin level after 72 h of treatment, reaching a level 60% lower as compared to the basal level (Fig. 3A), which confirms the induction of apoptosis with oleuropein.

3.3. Oleuropein inhibits breast cancer cell proliferation by delaying the cell cycle at S phase

Since cancers are cell cycle/proliferation-related diseases, we investigated the effect of oleuropein on breast cancer cell proliferation using the WST-1 cell proliferation assay. MDA-MB-231 and MCF-7 cells were seeded in triplicates into microtiter plates at 0.5–1.10⁴/well and were treated with oleuropein (200 μ M) for various periods of time, and then cellular proliferation was measured by the WST-1 assay. Fig. 4A shows that while the control non-treated cells continue proliferating in a time-dependent

manner, the number of oleuropein-treated MDA-MB-231 cells decreased sharply after only 24 h of treatment. However, oleuropein was less effective on MCF-7 cells. Their proliferation was inhibited during the first 24 h, then they resumed proliferating in a rate similar to that of the non-treated cells (Fig. 4A). Therefore, like for cytotoxicity, the effect of oleuropein was more pronounced on MDA-MB-231 cells than on MCF-7 cells.

After showing the inhibitory effect of oleuropein on cell proliferation we sought to investigate the effect of this agent on the cell cycle. To this end, MDA-MB-231 and MCF-7 cells were either sham-treated or challenged with oleuropein (200 μ M) for different time intervals, and then cells were fixed, stained with PI, and cell cycle was analyzed by flow cytometry. Fig. 4B shows oleuropein-dependent accumulation of cells in the S phase of the cell cycle, reaching a maximum level of more than 30% after 16 h of incubation. Interestingly, while this effect was sustained up to 24 h of treatment for MDA-MB-231 cells, it disappeared in MCF-7 cells (Fig. 4B), reflecting the effect on cell proliferation described in Fig. 4A. This shows that oleuropein inhibits cell proliferation by delaying the cell cycle during DNA replication phase.

3.4. Oleuropein down-regulates NF- κ B and cyclin D1 but activates p21

After showing the effect of oleuropein on both cell death and cell proliferation, we wanted to identify the molecular pathway responsible for this dual effect. NF- κ B is one of the most important

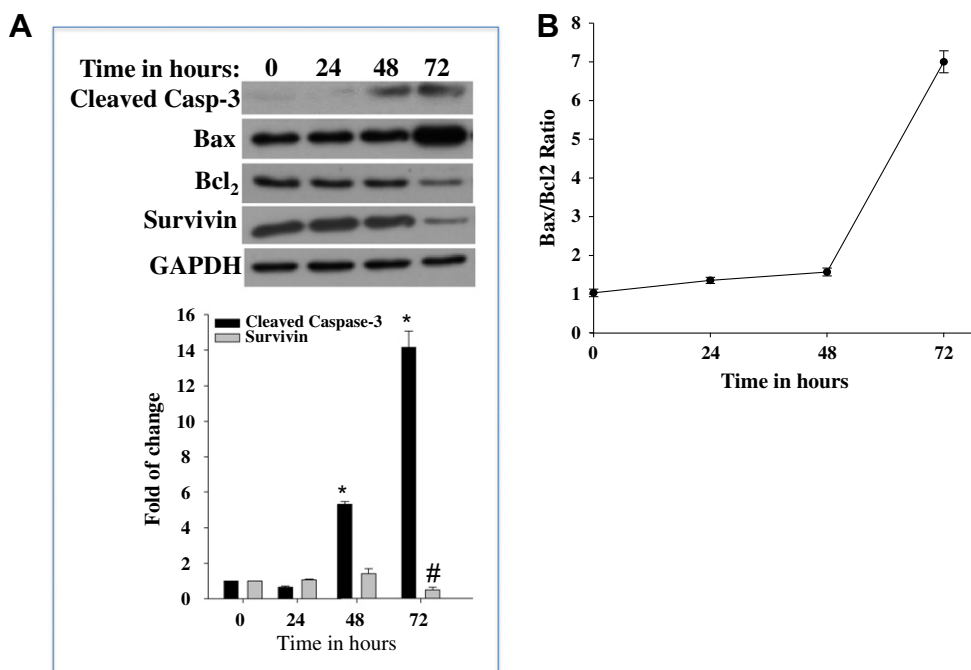


Fig. 3. Oleuropein triggers apoptosis through the internal pathway. MDA-MB-231 cells were treated with oleuropein (200 μ M) for the indicated periods of time, and then cell extracts were prepared and used for immunoblotting analysis using the indicated antibodies. (A) Upper panel: Immunoblots, Lower panel: histogram showing fold of change in expression level as compared to basal level (time 0). # And * indicate statistically significant differences. (B) Graph showing the Bax/Bcl-2 ratio. Error bars represent standard deviations of three different experiments.

pro-carcinogenic molecules, which enhances cell proliferation and inhibits cell death (Haffner et al., 2006). Therefore, we started by assessing the effect of oleuropein on the expression of NF- κ B. MDA-MB-231 cells were treated with oleuropein (200 μ M) for different periods of time (0–24 h), and then cellular lysates were prepared and used for Western blot analysis using specific antibodies and α -tubulin as internal control. Fig. 5A and B show that the expression level of the NF- κ B protein was reduced in a time-dependent manner, reaching a level 10-fold lower after 24 h of incubation. To confirm the inhibitory effect of oleuropein on NF- κ B, we studied the effect on the main NF- κ B downstream effector cyclin D1. Importantly, oleuropein decreased cyclin D1 level 5-fold after 24 h of treatment (Fig. 5B). On the other hand, the level of the versatile cyclin-dependent kinase inhibitor p21 increased 25-fold after 8 h of treatment with oleuropein (Fig. 5A and B). This indicates that while oleuropein inhibits oncogenes, it up-regulates p21, one of the important tumor suppressor genes.

4. Discussion

The Mediterranean diet, rich in fruits, vegetables, and fish, has been associated with a lower incidence of diseases and an overall improvement in health. These findings were attributed to the high consumption of olive oil and olive leaves (*Olea europaea* L. Oleaceae) (Kimura and Sumiyoshi, 2009). In the present report, we present clear evidence that oleuropein, the major active element in olive oil and leaf extracts, could constitute a potential therapeutic agent for basal-like breast tumors for the following reasons:

First, we have shown that oleuropein has only marginal cytotoxicity against “normal” epithelial cells *in vitro* (Figs. 1 and 2). In fact, it has been previously shown that oleuropein has an outstanding safety profile in rodents. Indeed, a single dose as high as (100 mg/kg) of oleuropein was given to rats with no side effects (Del Boccio et al., 2003). In another study, olive leaf extract (1000 mg/kg) was administered orally twice daily for 30 weeks to

inhibit skin carcinogenesis and tumor growth in mice, and this high dose was found to be safe and significantly effective (Kimura and Sumiyoshi, 2009). In a recent study it has been shown that oleuropein concentration in olives is highly variable. The authors assumed a usual consumption of 20 olive fruits (Throuba Thassos, which contains the highest quantities of oleuropein) per day, corresponds to a daily intake of 25 mg (46.2 μ M) of oleuropein (Zoidou et al., 2010). This dose can be considered as safe for human use, since it can be found in the usual diet. In our experiments 200 μ M of oleuropein was used in the different experiments, which corresponds to approximately 4 times the daily intake of oleuropein (80 olive fruits).

Second, oleuropein triggers mainly apoptosis in breast cancer cells through the mitochondrial pathway (Figs. 2 and 3). Interestingly, this effect was more pronounced on ER-negative breast cancer cells than ER-positive cells. Indeed, the pro-apoptotic effect of oleuropein (200 μ M) was more than 10 times higher against MDA-MB-231 than against MCF-7 cells. This could suggest that oleuropein is more effective in cells that do not express the estrogen receptor. The other possibility is that MCF-7 cells are highly resistant to oleuropein owing to the overexpression of the multidrug resistance-related protein, P-glycoprotein (Wosikowski et al., 1995), which may reduce the effect of oleuropein in these cells.

It is also possible that the fact MCF-7 does not express caspase-3 due to a deletion, which introduces a premature stop codon and leads to a complete absence of caspase-3 protein and activity (Jänicke et al., 1998), makes these cells relatively more resistant than MDA-MB-231 cells. However, T47-D cells are caspase-3 proficient, yet they showed high resistance to oleuropein (data not shown), suggesting that the MCF-7 resistance may not be due to caspase-3 deficiency. Therefore, oleuropein could be used to consolidate the adjuvant treatment of the clinically aggressive ER-negative breast cancer patients, whose prognosis is still poor.

Third, oleuropein is a potent inhibitor of cell proliferation by delaying the cell cycle at S phase. This effect was also more pronounced on ER-negative breast cancer cells than ER-positive cells

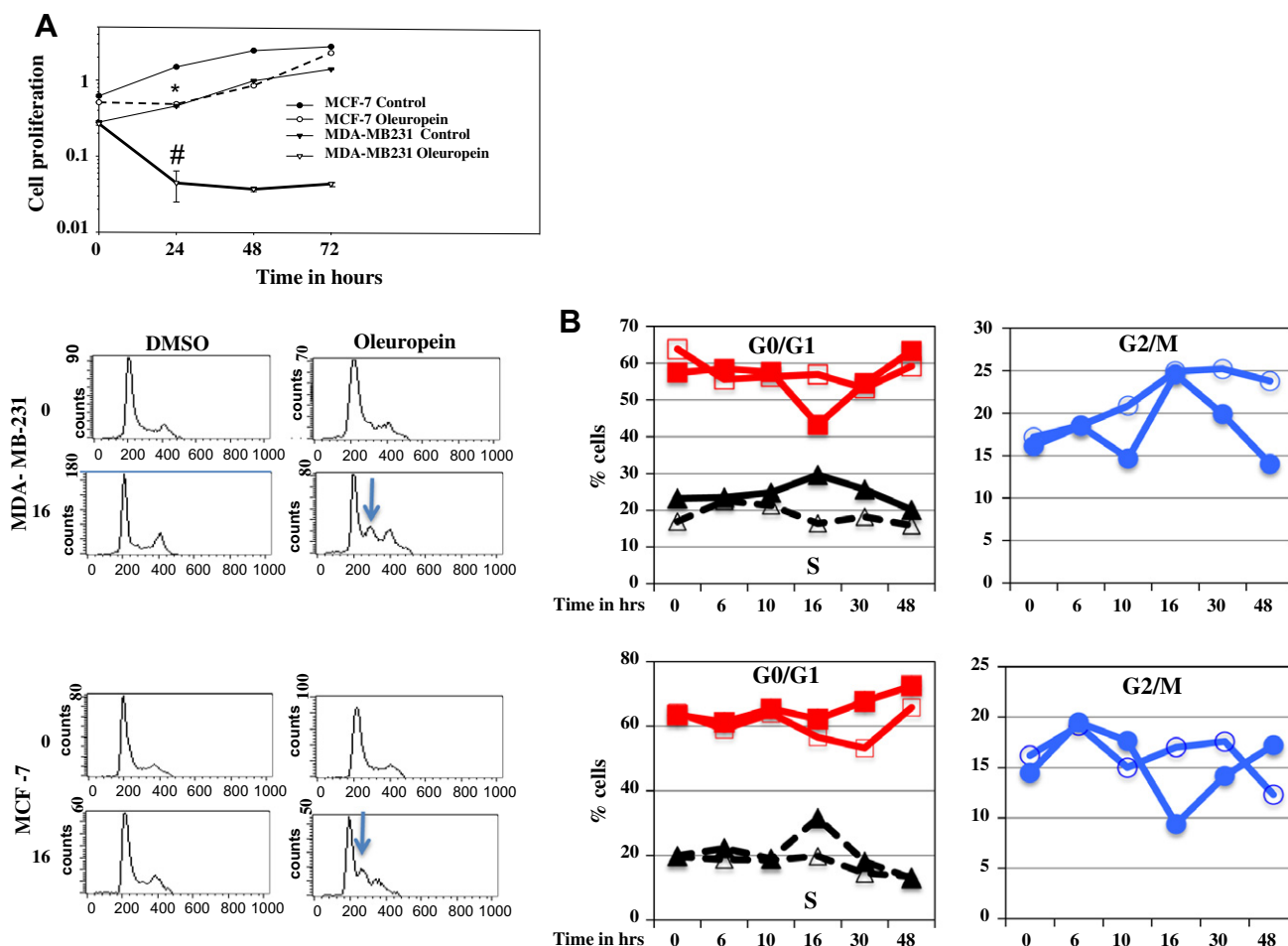


Fig. 4. Oleuropein inhibits the proliferation of breast cancer cells by delaying cell cycle at S phase. (A) Cells were cultured in 96 well plates and were either mock-treated (DMSO) or challenged with oleuropein (200 μ M) for the indicated periods of time, and then cell proliferation was assessed by the WST-1 assay. Error bars represent standard deviations of at least three different experiments, # And * indicate statistically significant differences. (B) Cells were either mock-treated or challenged with oleuropein (200 μ M) for different time intervals. The cell cycle status was analyzed by flow cytometry. The arrows indicate the accumulated cells at S phase. The curves show the proportions of cells in various phases of the cell cycle as indicated. Open symbols: DMSO, closed symbols: Oleuropein.

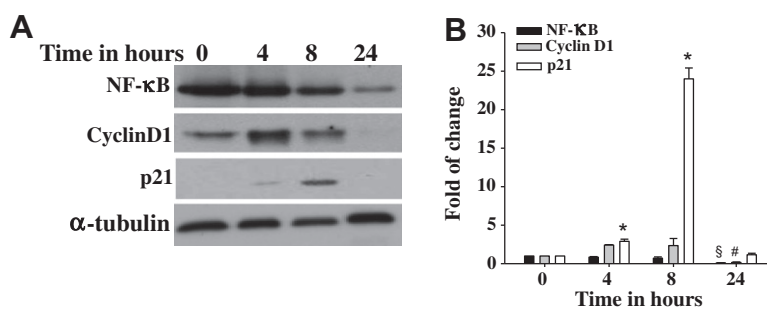


Fig. 5. Oleuropein down-regulates NF- κ B and cyclin D1 but up-regulates p21. Oleuropein (200 μ M) treated MDA-MB-231 cells were incubated for the indicated periods of time and proteins were extracted and used for immunoblot analysis using the indicated antibodies. (A) Immunoblots. (B) Histogram showing fold of change in expression level as compared to basal level (time 0). Error bars represent standard deviations of at least three different experiments, §, # And * indicate statistically significant differences.

(Fig. 4). It has been previously shown that oleuropein inhibits cell proliferation of MCF-7 breast cancer cells (Han et al., 2009). However, the dose used in that study was 200 μ g/ml, which corresponds to 370 μ M of oleuropein. Another study showed no growth inhibition of MCF-7 cells treated with oleuropein at 100 μ g/ml (Bulotta et al., 2011).

Fourth, oleuropein down-regulated two major breast cancer-related onco-proteins namely, NF- κ B and cyclin D1 (Fig. 5). NF- κ B up-regulation is implicated not only in tumour growth

and progression, but also in the resistance to chemo- and radio-therapies. Several studies have documented the elevated activity of this protein in breast cancer cells (Cao and Karin, 2003; Haffner et al., 2006), and a number of metastasis-promoting genes are known to be under the control of NF- κ B, which makes it an excellent target for cancer therapy (Van Waes, 2007). One of the most important targets of NF- κ B is the cyclin D1, which plays important roles in cell proliferation and survival (Ouyang et al., 2006; Liu et al., 2008). However, the kinetic of action of oleuropein on cyclin

D1 was different than that on NF- κ B. Cyclin D1 expression increased during the first 8 h of treatment while NF- κ B level decreased in a time-dependent manner (Fig. 5). It is possible that cyclin D1 responds to a kind of oleuropein-induced stress that up-regulates cyclin D1 before the negative action of NF- κ B. Cyclin D1 is an oncogene that is overexpressed in about 50% of all breast cancer cases (Mulloy et al., 2003), and its down-regulation is an important target in breast cancer therapy (Yang et al., 2006). Therefore, oleuropein-related down-regulation of NF- κ B and its common downstream target cyclin D1 could have a great inhibitory effect on breast cancer growth. Furthermore, oleuropein had a strong inhibitory effect on the two major apoptosis inhibitor proteins Bcl-2 and survivin (Fig 3), which are both related to breast cancer pathology and therapeutic outcome (Tanaka et al., 2000; Callagy et al., 2006; Altieri, 2008). Survivin is a potent anti-apoptosis protein that is differentially expressed in cancer and therefore constitutes an important anti-cancer target (Karczmarek-Borowska et al., 2005; Li and Ling, 2006; Altieri, 2008). On the other hand, oleuropein up-regulated p21 in a p53-independent manner, since this occurred in MDA-MB-231 cells, which are p53-defective (Lacroix et al., 2006). Together, these various effects could explain the oleuropein-dependent suppression of cell proliferation and the induction of apoptosis in breast cancer cells.

Collectively, these data show that oleuropein has potent anti-breast cancer properties and thereby warrants further investigations for its potential use as chemotherapeutic agent against basal-like breast cancers.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

- Altieri, D.C., 2008. Survivin, cancer networks and pathway-directed drug discovery. *Nat. Rev. Cancer* 8 (1), 61–70.
- Andre, F., Broglio, K., Roche, H., Martin, M., Mackey, J.R., Penault-Llorca, F., Hortobagyi, G.N., Pusztai, L., 2008. Estrogen receptor expression and efficacy of docetaxel-containing adjuvant chemotherapy in patients with node-positive breast cancer: results from a pooled analysis. *J. Clin. Oncol.* 26 (16), 2636–2643.
- Badve, S., Nakshatri, H., 2009. Oestrogen-receptor-positive breast cancer: towards bridging histopathological and molecular classifications. *J. Clin. Pathol.* 62 (1), 6–12.
- Bulotta, S., Corradino, R., Celano, M., D'Agostino, M., Maiuolo, J., Oliverio, M., Procopio, A., Iannone, M., Rotiro, D., 2011. Antiproliferative and antioxidant effects on breast cancer cells of oleuropein and its semisynthetic peracetylated derivatives. *Food Chem.* 127 (4), 1609–1614.
- Callagy, G.M., Pharoah, P.D., Pinder, S.E., Hsu, F.D., Nielsen, T.O., Ragaz, J., Ellis, I.O., Huntsman, D., Caldas, C., 2006. Bcl-2 is a prognostic marker in breast cancer independently of the Nottingham Prognostic Index. *Clin. Cancer Res.* 12 (8), 2468–2475.
- Cao, Y., Karin, M., 2003. NF- κ B in mammary gland development and breast cancer. *J. Mammary Gland Biol. Neoplasia* 8 (2), 215–223.
- Carluccio, M.A., Siculella, L., Ancora, M.A., Massaro, M., Scoditti, E., Storelli, C., Visioli, F., D'Amico, A., De Caterina, R., 2003. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler. Thromb. Vasc. Biol.* 23 (4), 622–629.
- Clarke, M., Collins, R., Darby, S., Davies, C., Elphinstone, P., Evans, E., Godwin, J., Gray, R., Hicks, C., James, S., MacKinnon, E., McGale, P., McHugh, T., Peto, R., Taylor, C., Wang, Y., 2005. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 366 (9503), 2087–2106.
- Del Bocco, P., Di Deo, A., De Curtis, A., Celli, N., Iacoviello, L., Rotilio, D., 2003. Liquid chromatography-tandem mass spectrometry analysis of oleuropein and its metabolite hydroxytyrosol in rat plasma and urine after oral administration. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 785 (1), 47–56.
- Fisher, B., Anderson, S., Bryant, J., Margolese, R., Deutsch, M., Fisher, E., Jeong, J., Wolmark, N., 2002. Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. *Engl. J. Med.* 347, 1233–1241.
- Haffner, M.C., Berlato, C., Doppler, W., 2006. Exploiting our knowledge of NF- κ B signaling for the treatment of mammary cancer. *J. Mammary Gland Biol. Neoplasia* 11 (1), 63–73.
- Han, J., Talorete, T., Yamada, P., Isoda, H., 2009. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. *Cytotechnology* 59 (1), 45–53.
- Jänicke, R.U., Sprengart, M.L., Wati, M.R., Porter, A.G., 1998. Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J. Biol. Chem.* 273 (16), 9357–9360.
- Jemai, H., El Feki, A., Sayadi, S., 2009. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. *J. Agric. Food Chem.* 57 (19), 8798–8804.
- Jemal, A., Bray, F., Center, M.M., et al., 2011. Global cancer statistics. *CA Cancer J. Clin.* 61 (2), 69–90.
- Karczmarek-Borowska, B., Filip, A., Wojcierowski, J., Smoleń, A., Pilecka, I., Jabłonka, A., 2005. Survivin antiapoptotic gene expression as a prognostic factor in non-small cell lung cancer: in situ hybridization study. *Folia Histochem. Cytobiol.* 43 (4), 237–242.
- Key, T.J., Schatzkin, A., Willett, W.C., Allen, N.E., Spencer, E.A., Travis, R.C., 2004. Diet, nutrition and the prevention of cancer. *Public Health Nutr.* 7 (1A), 187–200.
- Khayyal, M.T., el-Ghazaly, M.A., Abdallah, D.M., Nassar, N.N., Okpanyi, S.N., Kreuter, M.H., 2002. Blood pressure lowering effect of an olive leaf extract (*Olea europaea*) in L-NAME induced hypertension in rats. *Arzneimittelforschung* 52 (11), 797–802.
- Kimura, Y., Sumiyoshi, M., 2009. Olive leaf extract and its main component oleuropein prevent chronic ultraviolet B radiation-induced skin damage and carcinogenesis in hairless mice. *J. Nutr.* 139 (11), 2079–2086.
- Lacroix, M., Toillon, R., Leclercq, G., 2006. p53 and breast cancer, an update. *Endocrine-Related Cancer* 13 (2), 293–325.
- Li, F., Ling, X., 2006. Survivin study: an update of "what is the next wave". *J. Cell Physiol.* 208 (3), 476.
- Liu, S., Tan, W.Y., Chen, Q.R., Chen, X.P., Fu, K., Zhao, Y.Y., Chen, Z.W., 2008. Daintain/AIF-1 promotes breast cancer proliferation via activation of the NF- κ B/cyclin D1 pathway and facilitates tumor growth. *Cancer Sci.* 99 (5), 952–957.
- Liu, S.Q., Saijo, K., Todoroki, T., Ohno, T., 1995. Induction of human autologous cytotoxic T lymphocytes on formalin-fixed and paraffin-embedded tumour sections. *Nat. Med.* 1 (3), 267–271.
- Micol, V., Caturla, N., Pérez-Fons, L., Más, V., Pérez, L., Estepa, A., 2005. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicemia rhabdovirus (VHSV). *Antiviral Res.* 66 (2–3), 129–136.
- Mulloy, R., Salinas, S., Phillips, A., Hipskind, R.A., 2003. Activation of cyclin D1 expression by the ERK5 cascade. *Oncogene* 22 (35), 5387–5398.
- Ouyang, W., Li, J., Ma, Q., Huang, C., 2006. Essential roles of PI3K/Akt/IKK β /NF- κ B pathway in cyclin D1 induction by arsenite in JB6 Cl41 cells. *Carcinogenesis* 27 (4), 864–873.
- Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Würtele, G., Spiegelhalter, B., Bartsch, H., 2000. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol.* 1, 107–112.
- Rouzier, R., Perou, C.M., Symmans, W.F., Ibrahim, N., Cristofanilli, M., Anderson, K., Hess, K.R., Stec, J., Ayers, M., et al., 2005. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin. Cancer Res.* 11 (16), 5678–5685.
- Saad, E.D., 2011. Endpoints in advanced breast cancer: methodological aspects & clinical implications. *Indian J. Med. Res.* 134 (4), 413–418.
- Sajja, A., Uccella, N., 2000. Olive biophenols: functional effects on human wellbeing. *Trends Food Sci. Technol.* 11 (9–10), 357–363.
- Shen, K., Rice, S.D., Gingrich, D.A., Wang, D., Zhibao Mi, Z., Tian, C., et al., 2012. Distinct genes related to drug response identified in ER positive and ER negative breast cancer cell lines. *PLoS ONE* 7 (7), e40900.
- Takenouchi, T., Munekata, E., 1995. Trophic effects of substance P and beta-amyloid peptide on dibutyryl cyclic AMP-differentiated human leukemic (HL-60) cells. *Life Sci.* 56 (26), PL479–484.
- Tanaka, K., Iwamoto, S., Gon, G., Nohara, T., Iwamoto, M., Tanigawa, N., 2000. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin. Cancer Res.* 6 (1), 127–134.
- Trichopoulou, A., Lagiou, P., Kuper, H., Trichopoulos, D., 2000. Cancer and Mediterranean dietary traditions. *Cancer Epidemiol. Biomarkers Prev.* 9 (9), 869–873.
- Van Waes, C., 2007. Nuclear factor- κ B in development, prevention, and therapy of cancer. *Clin. Cancer Res.* 13 (4), 1076–1082.
- Visioli, F., Poli, A., Gall, C., 2002. Antioxidant and other biological activities of phenols from olives and olive oil. *Med. Res. Rev.* 22 (1), 65–75.
- Wosikowski, K., Regis, J.T., Robey, R.W., Alvarez, M., Buters, J.T., Gudas, J.M., Bates, S.E., 1995. Normal p53 status and function despite the development of drug resistance in human breast cancer cells. *Cell Growth Differ.* 6 (11), 1395–1403.
- Yang, C., Trent, S., Ionescu-Tiba, V., Lan, L., Shioda, T., Sgroi, D., Schmidt, E.V., 2006. Identification of cyclin D1- and estrogen-regulated genes contributing to breast carcinogenesis and progression. *Cancer Res.* 66 (24), 11649–11658.
- Zoidou, E., Melliou, E., Gikas, E., Tsaropoulos, A., Magiatis, P., Skaltsounis, A.L., 2010. Identification of Throuba Thassos, a traditional Greek table olive variety, as a nutritional rich source of oleuropein. *J. Agric. Food Chem.* 58 (1), 46–50.