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Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells

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Abstract Olive oil intake has been shown to induce significant levels of apoptosis in various cancer cells. These anti-cancer properties are thought to be mediated by phenolic compounds present in olive. These beneficial health effects of olive have been attributed, at least in part, to the presence of oleuropein and hydroxytyrosol. In this study, oleuropein and hydroxytyrosol, major phenolic compound of olive oil, was studied for its effects on growth in MCF-7 human breast cancer cells using assays for proliferation (MTT assay), cell viability (Guava ViaCount assay), cell apoptosis, cellcycle (flow cytometry). Oleuropein or hydroxytyrosol decreased cell viability, inhibited cell proliferation, and induced cell apoptosis in MCF-7 cells. Result of MTT assay showed that 200 $\mu\text{g/ml}$ of oleuropein or 50 $\mu\text{g/ml}$ of hydroxytyrosol remarkably reduced cell viability of MCF-7 cells. Oleuropein or hydroxytyrosol decrease of the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis. Also hydroxytyrosol and oleuropein exhibited statistically significant block of G_1 to S phase transition manifested by the increase of cell number in G_0/G_1 phase.

Keywords MCF-7 cells • Oleuropein • Hydroxytyrosol • Apoptosis

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Introduction

The incidence of cancer in Mediterranean countries is lower than in the rest of European countries and the United States (Keys et al. 1981). This is mostly described by the lower rate of the large bowel, breast, endometrial, and prostate cancers by a number of epidemiological studies, and the major reason for this, apart from possible genetic factors, attributed to the dietary practices. The traditional Mediterranean diet is characterized by high consumption of foods of plant origin, relatively low consumption of red meat, and high consumption of olive oil and its products. There are a number of studies on health beneficial effects of olive oil. Several studies have been reported that olive oil is more favorable against cancer than other forms of added lipids due to its high content of monounsaturated fatty acids (Owen et al. 2000; Visioli et al. 2001). In recent studies, diet containing 15% olive oil could significantly reduce pre-cancerous lesions in rat breast and colon (Martin-Moreno et al. 1994; Corona et al. 2007; Paula et al. 2007). However, similar amount of soy oil didn't have such a protective effect as was expected (La Vecchia et al. 1998). Furthermore, the incidence of breast cancer was 70% less in the rats group fed olive oil than in the rats group fed safflower oil (Owen et al. 2000). This data suggests that cancer preventive effect of olive oil is not only attributed to the "good" fat content. Recently, growing evidence show that minor compounds in olive oil may take a part in cancer protection as well, and more attention is paid to its phenolic compounds.

The phenolic compounds of olive oil and leaf are a complex mixture of compounds that include 3,4-dihydroxyphenylethanol (hydroxytyrosol), 4-hydroxyphenylethanol (tyrosol), 4-hydroxyphenylacetic acid, protocatechuic acid, caffeic acid and *p*-coumaric acid, among others (Litridou et al. 1997; Caponio et al. 1999). The concentration of the phenolic fraction is several times more in olive leaf than in oil and varies depending on the cultivar, climate (Table 1, 2) (Servili et al. 1999; Ryan et al. 2002; Abaza et al 2005). *In vivo* and *in vitro* studies suggest these bioactive compounds exhibit powerful antioxidant activity (Visioli et al. 2000; Le Tutour et al. 1992). One of various phenolic compounds hydroxytyrosol seems to be among the most important. It is present in free form and as a constituent of complex molecules such as oleuropein in leaves and fruits. Both hydroxytyrosol and oleuropein have been shown to possess anti-inflammatory, bactericidal and bacteriostatic activities (Yang et al. 2007). Some *in vivo* studies on olive leaf have shown that its extract can decrease blood pressure and dilate the coronary arteries surrounding the heart (Tuck et al. 2002). Moreover, hydroxytyrosol has been shown to have anti-cancer effect on

human colon adenocarcinoma HT-29 cells and human promyelocytic leukemia HL-60 cells (Fabiani et al. 2002, 2006), have anti-melanogenesis activity, whereas oleuropein inhibited cell growth of LN-18, poorly differentiated glioblastoma; TF-1a, erythroleukemia; 786-O, renal cell adenocarcinoma; T-47D, infiltrating ductal carcinoma of the breastpleural effusion; RPMI-7951, malignant melanoma of the skin-lymph node metastasis; and LoVo, colorectal adenocarcinoma cells (Hamdi et al. 2005). Even though anticancer properties of oleuropein and hydroxytyrosol were confirmed *in vitro* with different cell lines, studies of their protective effect from breast cancer have not been demonstrated. Since many epidemiological studies suggest the possible correlation between olive products consumption and incidence of breast cancer here we tried to elucidate the possible effect of the main phenolic compounds hydroxytyrosol and oleuropein of olive leaf on breast cancer using human breast cancer cell line MCF-7.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-(4, 5-dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Wako (Osaka, Japan). Oleuropein and hydroxytyrosol were purchased from Funakoshi (Tokyo, Japan). ViaCount Assay reagent, propidium iodide, sulforhodamine -valyl-alanyl-aspartyl-fluoromethyl-ketone and 7-amino-actinomycin D were purchased from GE Healthcare (Wisconsin, USA).

Olive leaf extract preparation

Fresh green olive leaves were collected from the olive tree grown in Tunisia. Dry olive leaves (10 g) were extracted with 70% ethanol (100 ml) for 2 weeks. Extracted solution (10% (w/v)) was filtered with the filter unit (Millipore, pore size; 0.22 μ m).

Cell culture

MCF-7 cells were obtained from American Type Culture Collection (ATCC), and maintained in DMEM, supplemented with 10% FBS (Sigma), 1% 5000 units/ml penicillin, 5000 μ g/ml

streptomycin (Sigma). The cells were incubated in an atmosphere of 5% CO₂ at 37 °C. All cells used in this study were between passages 5 and 15.

Growth inhibition assay

MCF-7 cells were seeded in 96-well plates at 2×10^3 cells/well in 100 μ l of DMEM with 10% FBS. After 24 h the cells were treated with olive leaf extract (0.001, 0.01, 0.02, 0.1% (w/v)) for 12 h. Also MCF-7 cells were seeded in 96-well plates at 2×10^3 , 2×10^4 , 2×10^5 cells/well in 100 μ l of DMEM with 10% FBS. After 24 h the cells were treated with oleuropein (25, 50, 100, 200 μ g/ml) or hydroxytyrosol (6.25, 12.5, 25, 50 μ g/ml) for 12 h. The treated cells were washed with PBS and 10 μ l of MTT solution (5 mg/ml MTT in PBS) was added to each well. Cells were incubated for 12 h at 37 °C at the end of which 100 μ l 10% SDS was added and incubated further for 6 h at 37 °C. Optical densities at 570 nm were measured using a plate reader (Powerscan HT; Dainippon Pharmaceutical, Osaka, Japan). The relative cell viability in percentage was calculated as $(A_{570} \text{ of treated samples} / A_{570} \text{ of untreated samples}) \times 100$.

Cell proliferation assay

Cells were regularly seeded into 6-well plates at 2×10^4 cells/well and incubated at 37 °C for 24 h. Cells were treated in fresh medium with Cells were incubated with oleuropein (200 μ g/ml) or hydroxytyrosol (50 μ g/ml) for 3, 6, and 12 h. At the end of each time period, the cells were trypsinized to produce a single cell suspension, and the viable cell number in each well was counted using the ViaCount Assay (Guava Technologies, Hayward, CA).

Multicaspase assay by flow cytometry

Apoptosis was assessed using a flow cytometry based multicaspase assay kits (Guava Technologies, Burlingame, CA). The multicaspase assay is based on measurement of caspase enzymes activated during apoptosis. MCF-7 cells (2×10^5 cells/plate) were plated on tissue culture dishes (100 mm) in 10% FBS medium. Cells were incubated with oleuropein (200 μ g/ml) or hydroxytyrosol (50 μ g/ml) for 3, 6 and 12 h. Cells were trypsinized, washed in PBS and stained with sulforhodamine-valyl-alanyl-aspartyl-fluoromethyl-ketone (SR-VAD-FMK), and 7-amino-

actinomycin D (7-AAD) according to the manufactures instructions. Cell populations were quantified using Guava personal cytometer (Guava Technologies, Hayward, CA).

Cell cycle analysis

MCF-7 cells (2×10^5 cells/plate) were exposed to concentrations of oleuropein (100 $\mu\text{g/ml}$) or hydroxytyrosol (25 $\mu\text{g/ml}$) for 24 h and the treated cells were collected by trypsinisation. The cells were fixed with 70% ethanol and stored at $-20\text{ }^\circ\text{C}$. On the day of analysis, the cells were washed with PBS and suspended in 250 μl of PBS. To the cell suspension 1.0 ml of phosphate-citrate buffer was added and the suspension was incubated at room temperature for 5 min to facilitate the extraction of low molecular weight DNA. Following centrifugation the cells were resuspended in 500 μl DNA staining solution (20 $\mu\text{g/ml}$ propidium iodide, 200 $\mu\text{g/ml}$ DNase free RNase and 0.1% Triton X-100) and incubated in the dark at room temperature for 30 min. Cell cycle distribution was determined by fluorescence-activated cell sorting analysis of propidium iodide-stained ethanol-fixed cells using a Guava EasyCyte (Guava Technologies, Hayward, CA).

Results

Olive leaf extract has cytotoxic effect

For the screening of anticancer effect of olive leaf we used olive leaf extract to treat breast cancer MCF-7 cells. MTT assay results have shown that after 48 h treatment with the extract at the dosage of 0.1% cancer cell proliferation was inhibited to 60% compared to the vehicle treated control cells (Fig. 1). The rest of treatment concentrations did not show cytotoxic effect. Since the major phenolic compounds of olive leaf are oleuropein and hydroxytyrosol, in subsequent anticancer assays we assumed that this cytotoxic effect could be mainly due to presence of these compounds and tried to elucidate their tumor suppressive effects.

Hydroxytyrosol and oleuropein inhibit cell proliferation

The time-dependent change in MCF-7 cell viability was determined using the MTT assay. As shown in Fig. 2, there was a reduction in cell viability in a time- and concentration-dependant manner after an exposure to hydroxytyrosol and oleuropein. Initial cell number had a great

influence in cytotoxic effects of these compounds. 200 µg/ml of oleuropein and 50 µg/ml of hydroxytyrosol treatment had the greatest cytotoxic effects in 2×10^3 cells/well incubation, after 12 h inducing cell growth arrest in almost 90% of treated cells respectively to compare to the controls. Whereas in doubled and tripled cell number treatment the effect has diminished but the compounds still could induce cell growth arrest. The results of flow cytometry analysis showed the same trend as with MTT assay. As shown in Fig. 4, the percentage of dead cells increased significantly after 12 h of treatment, and the inhibition rate of cell proliferation was similar with MTT assay results in both compounds. However, it was reported that non-toxic concentration of oleuropein and hydroxytyrosol were evaluated by using the primary cell such as normal human gingival GN61 fibroblasts and normal human neutrophils. Oleuropein didn't show the cytotoxicity at a 100mM concentration. Also, hydroxytyrosol didn't show the cytotoxicity at a 200mM concentration (Babich et al. 2003; Yvonne et al. 2004).

Morphology change of MCF-7 cells

During our trials with cell cytotoxicity assays we observed significant morphological changes in the cells (2×10^3 cells/well) incubated with 200 µg/ml oleuropein. Cell shrinkage, chromatin condensation and formation of apoptotic bodies were clearly defined. The same pattern was also observed in cells incubated with 50 µg/ml hydroxytyrosol for 12 h, suggesting apoptotic cell death of MCF-7 cells (Fig. 3). Since morphological features of apoptosis were well noticed during above mentioned concentration ranges we tried to use these ranges throughout the study, unless it is stated.

Hydroxytyrosol and oleuropein induce cell apoptosis

Since caspase activation is the classic feature of apoptosis we performed multicaspase assay using fluorochrome conjugated caspase inhibitor sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK). This inhibitor is cell permeable and non-cytotoxic and binds to multiple caspases activated during apoptosis. Also the cell impermeant dye 7-aminoactinomycin D (7-ADD) was included in the assay to detect dead cells from apoptotic ones (Georg et al. 2007). From the multicaspase assay we could observe that caspases were activated soon after treatment with both compounds, besides, induction of the enzymes' activation was greater in oleuropein treated cells (29.11%) compare to the hydroxytyrosol treated cells (11.64%)

in the beginning of the treatment. Nevertheless, both compounds induced significant time dependant caspases activation to compare to the control cells. After 12 h the majority of the cells were apoptotic in both compound treated cells (Fig. 5).

Hydroxytyrosol and oleuropein induce G1 cell cycle arrest in MCF-7 cells

Because it has been reported that the cells undergoing apoptosis are arrested in G₁/G₀ phase of cell division phase we could verify the induction of apoptosis of breast cancer cells by hydroxytyrosol and oleuropein using cell cycle analysis. In this assay we treated MCF-7 cells with 100 µg/ml of oleuropein and 25 µg/ml of hydroxytyrosol. When cells were treated for 24 h with changed concentration ranges, the cell cycle arrest at G1 phase was evident (41.1% and 45.7% in oleuropein and hydroxytyrosol treated cells respectively), accompanying a decrease in G2/M phase when compared with the untreated control cells (27.1% in untreated cells) (Fig. 6). From this result we suggest that apoptotic cell death of MCF-7 cells after hydroxytyrosol and oleuropein treatment was accompanied with the block of cell cycle at G1 phase.

Discussion

Many vegetable foods in Mediterranean diet contain substances possessing anticancer properties (Visioli et al. 1998). Among them olive products such as oil are obviously the substantially different characteristics from western diet. Unlike refined oils, olive oil has a significant content of polyphenolic compounds. Even though recent studies have demonstrated that phenolic compounds of olive oil may play a role in cancer protection the main source of these compounds is olive leaf rather than olive oil. And it is not surprising that olive leaf has a long history for its medicinal remedies. Hydroxytyrosol and the secoiridoid oleuropein are studied well for their biological activities among olive phenolics. It should be noted that while tocopherols, phenolic acids, phenolic alcohols and flavonoids are present in many fruits and vegetables belonging to several botanical families, secoiridoids are present exclusively in plants of the family of *Oleaceae*. Anticancer properties of hydroxytyrosol and oleuropein were confirmed *in vitro* on different cell lines studies. But, breast cancer cell lines have not been demonstrated.

In this study we have demonstrated that hydroxytyrosol and oleuropein of olive leave inhibit the cell proliferation of breast cancer cells and induce apoptotic cell death. The results of

MTT assay have shown that both hydroxytyrosol and oleuropein have cytotoxic effect on MCF-7 cells with the dose dependant manner. Also there was not much difference in cell growth inhibition effect between 2 compounds. Since MTT assay is based on the metabolic activity of the cells we tried to confirm the cytotoxic effect by flow cytometry analysis. Results of flow cytometric analysis also confirmed that the phenolic compounds reduced cell viability with the same trend as MTT assay results. Interestingly, the cell growth arrest was more effective at small number of initial cell seeding (Fig. 2). It is probably caused by the contact surface with hydroxytyrosol or oleuropein. The contact surface with hydroxytyrosol or oleuropein at small number of initial cell seeding is wider than the large number of initial cell seeding. This data may suggest that phenolic compounds of olive leaf have health protective effect rather than healing effect. Constant consumption of olive products such as leaf tea and oil may enhance this protective effect thus, suggesting their daily consumption is more effective than the infrequent intake. Also *in vivo* studies have shown that soon after oral administration the compounds appeared in the plasma suggesting their good absorption from the intestines, but the entire quantity administered was not found in the urine which may accumulate in organs such as breast or erythrocytes (Elisa et al. 2005).

During our trials with cell cytotoxicity assays we observed significant morphological changes in the cells (Fig. 3). Cell shrinkage, chromatin condensation and formation of apoptotic bodies were clearly observed suggesting the cell growth arrest could be due to apoptosis. Apoptosis has been well characterized and is thought to proceed through a series of conserved and well studied steps.

The caspases, cysteine proteases, are of central importance in the apoptotic signaling network which is activated in most cases of apoptotic cell death. Actually, strictly defined, cell death only can be classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity. Our multicaspase activity assay revealed that with compounds' treatment the proportion of caspase enzymes activated cells increased time-dependently, indicating the activation of these enzymes in the cells (Fig. 5).

To further elucidate our hypothesis of apoptotic cell death of breast cancer cells by hydroxytyrosol and oleuropein we tried to use other methods for its detection. Since the G1/G0 cell cycle arrest has been shown to correlates well with apoptosis determined by other means we examined sub-G1 DNA content by flow cytometry analysis following treatment with two phenolic

compounds. Our data confirmed that the cells treated with hydroxytyrosol and oleuropein exhibited statistically significant block of G₁ to S phase transition manifested by the increase of cell number in G₀/G₁ phase. Although the oleuropein notably induced less cell cycle arrest than hydroxytyrosol it was still capable to significantly promote breast cancer apoptotic cell death.

The molecular mechanism of hydroxytyrosol and oleuropein induced apoptotic cell death is not clear. Yet, these compounds have high antioxidant activities. In a recent study, the antioxidant activity of α -tocopherol and phenolic extracts from olives and olive oil was compared over time. It was demonstrated that after initiation of radical formation the scavenger activity of α -tocopherol was higher but soon terminated whereas the extract from olives and oil contained compounds continued to reduce the concentration of these radicals and with time the extracts of the olives were much more active than α -tocopherol (Keceli et al. 2001). On the other hand, some investigators observed that phenolic compounds with high reducing ability can be not only the antioxidants but also the pro-oxidants, thus generating reactive oxygen species (ROS) (Wlodek et al. 2001). It is interesting to note that many human cancer cell types exist in highly oxidative state due to decreased antioxidant protective enzyme levels to compare to their normal tissue counterparts. Thus, cancer cells may be more sensitive to any generated ROS within the cells. Growing evidence exist that ROS may even activate apoptotic death pathways. Zhao et al. (1997) observed that vitamin E induced DNA synthesis arrest and stimulated apoptosis in MCF-7 cells (Wlodek et al. 2001). Also most chemotherapeutic agents such as radiation, alkylating agents, anthracyclines induce apoptotic death in cancer cells through generating ROS. This could be the possible mechanism of action of antioxidants for tumor suppressive effect.

In conclusion, our results have shown that main phenolic compounds of olive leaf hydroxytyrosol and oleuropein induced apoptotic cell death of human breast cancer MCF-7 cells. Our findings may support the breast cancer protective results of epidemiological studies of Mediterranean diet with correlation of olive products' consumption. Even though the cancer protective effect of the main olive oil component oleic acid in gene regulation was demonstrated further studies are needed to explore molecular mechanisms of tumor suppressive effect of other olive tree products such as olive leaf because they also could be the potent inducers of such effect if consumed frequently.

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Table1. The concentrations of major phenolic compounds in extra-virgin olive oil

(Servili et al. 1999; Ryan et al. 2002; Abaza et al 2005).

Phenolic compound	Concentration (mg/kg)
Hydroxytyrosol	14.42 ± 3.01
Oleuropein	2.04 ± 0.78
Oleuropein agycone	14.42 ± 3.01
Tyrosol	27.45 ± 4.05
Apigenin	15.80 ± 4.51

Data expressed in mg/kg SEM.

Table2. The concentrations of major phenolic compounds in olive leaf

(Servili et al. 1999; Ryan et al. 2002; Abaza et al 2005).

Phenolic compound	Concentration (mg/kg)
Hydroxytyrosol	219 ± 3
Oleuropein	2231 ± 52
Demethyloleuropein	984 ± 47
Verbascoside	27.45 ± 4.05
Rutin	15.80 ± 4.51
Luteolin 7-O-glucoside	15.80 ± 4.51

Data expressed in mg/kg SEM.

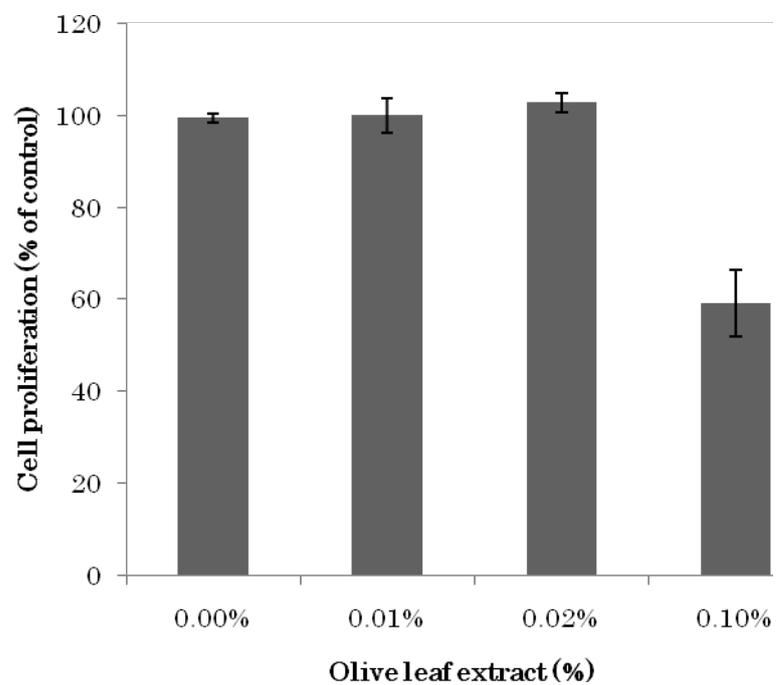


Fig. 1 Cytotoxic effect of olive leaf extract.

Influence of initial cell number of human breast cancer MCF-7 cells incubated with olive leaf extract (0.1, 0.02, 0.01, 0.001 % (w/v)). Cell viability was determined with the MTT assay. Results (\pm SD) represent the average of three independent experiments.

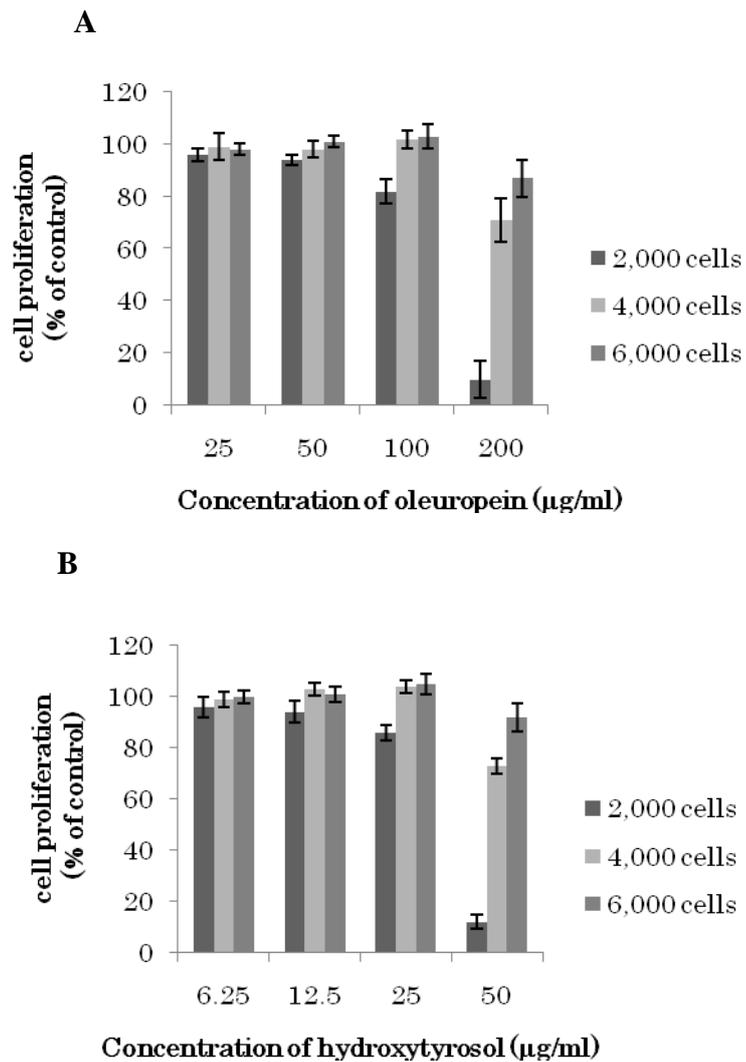


Fig. 2 Inhibition of MCF-7 cell proliferation

Influence of initial cell number on the concentration-dependent decrease in viability of human breast cancer MCF-7 cells incubated with oleuropein (A) and hydroxytyrosol (B). Cell viability was determined with the MTT assay. Results (\pm SD) represent the average of three independent experiments.

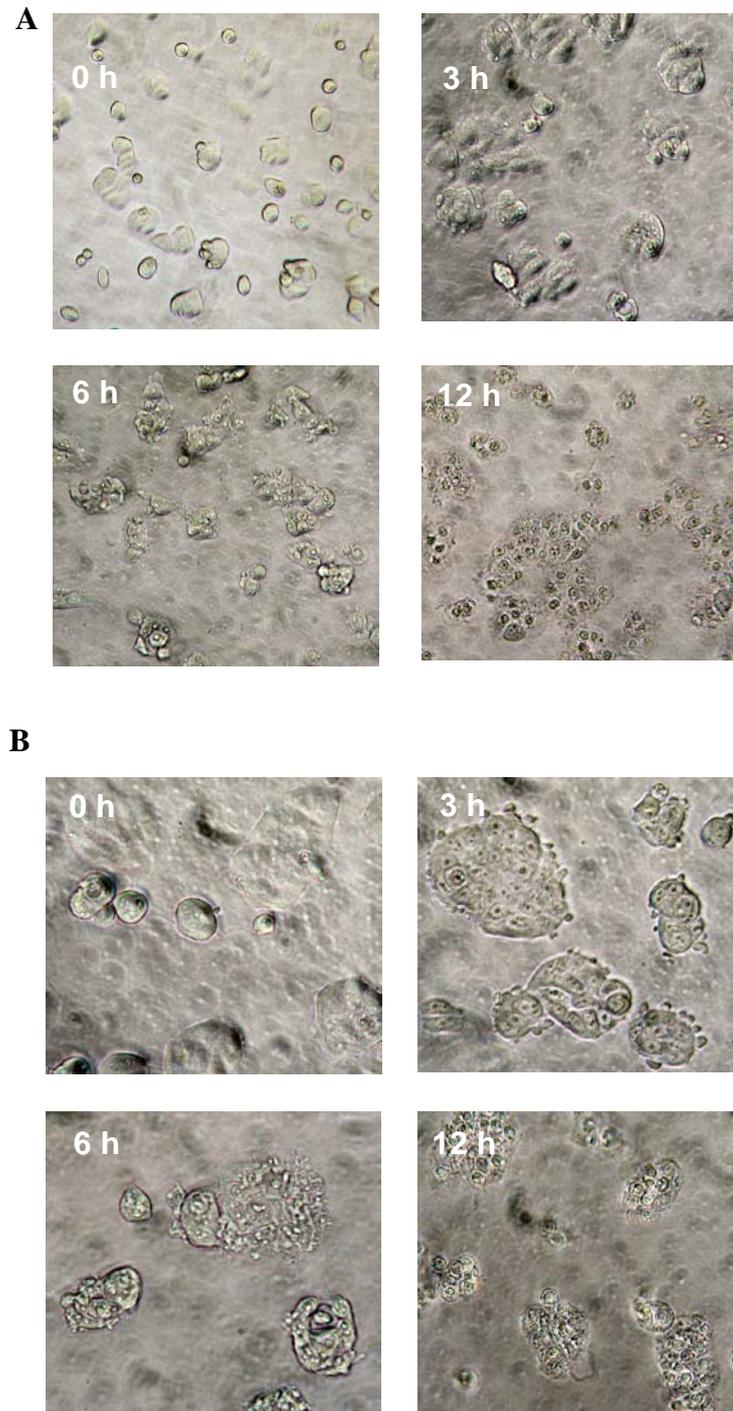
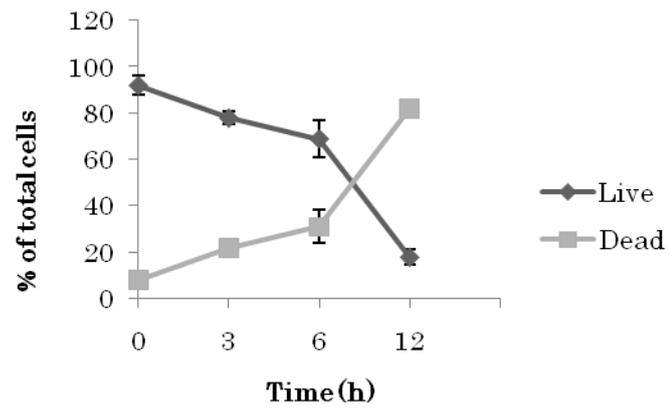


Fig. 3 Effect of oleuropein and hydroxytyrosol on MCF-7 cells morphology.

MCF-7 cells (2×10^3 cells / well) incubated with 200 µg/ml oleuropein (A) or with 50 µg/ml hydroxytyrosol (B) for 3 , 6 , 12 h.

A



B

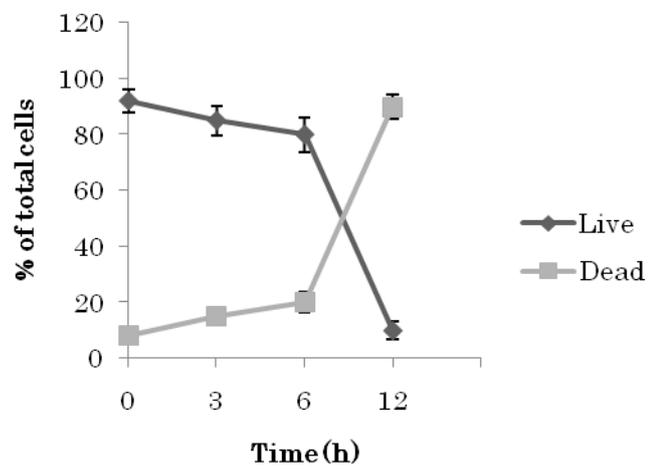
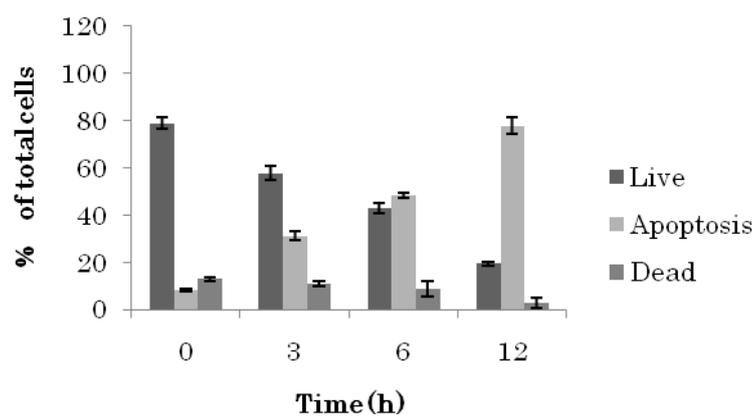


Fig. 4 Effect of oleuropein and hydroxytyrosol on the viability of MCF-7 cells.

Viability of MCF-7 cells (2×10^4 cells/well) incubated with oleuropein (200 $\mu\text{g/ml}$) and hydroxytyrosol (50 $\mu\text{g/ml}$) for 3, 6, and 12 h as determined by the Guava ViaCount Assay. Results represent the average of two independent determinations.

A



B

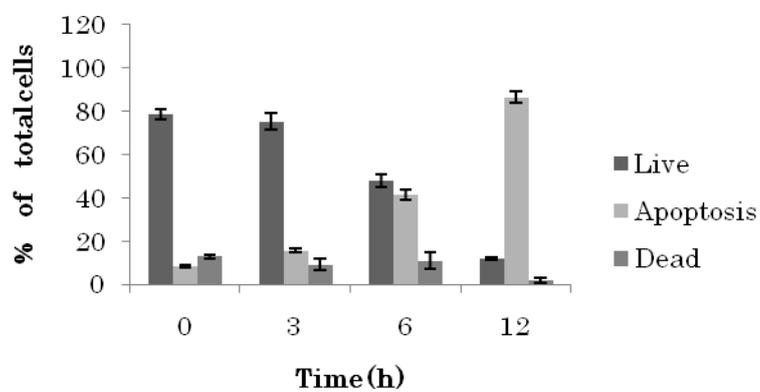
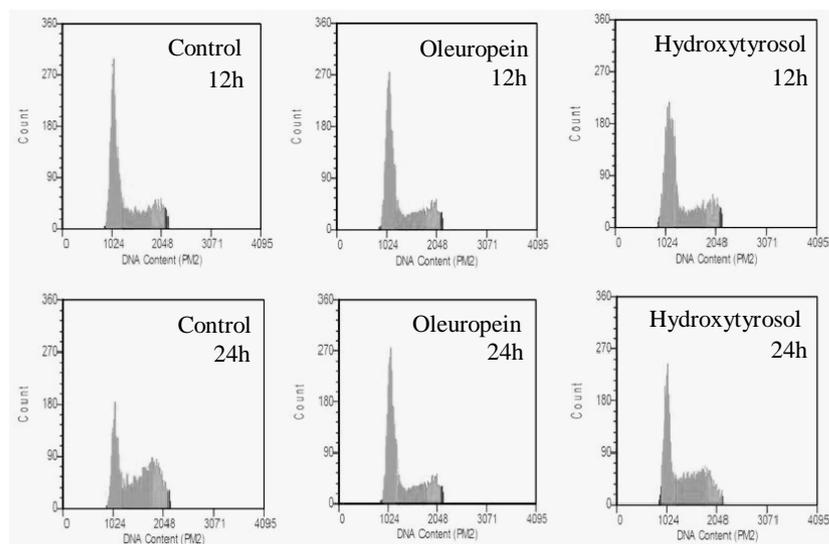


Fig. 5 Effect of hydroxytyrosol and oleuropein on induction of cell apoptosis.

Multicaspase assay of human breast cancer MCF-7 cells incubated for 3, 6 and 12 h with 200 $\mu\text{g/ml}$ oleuropein or 50 $\mu\text{g/ml}$ hydroxytyrosol. Results represent the average of two independent determinations.



	12 h			24 h		
	Control	Oleuropein	Hydroxytyrosol	Control	Oleuropein	Hydroxytyrosol
G0/G1	57.9	60.6	58.6	32.5	41.1	45.7
S	22.7	21.4	23.4	39.9	37.6	36.7
G2/M	17.6	16.3	15.7	27.1	20.7	16.4

Fig. 6 Effect of hydroxytyrosol and oleuropein on MCF-7 cell cycle.

Cell cycle kinetics of human breast cancer MCF-7 cells incubated for 12, 24 and 48 h with 100 $\mu\text{g/ml}$ oleuropein or 25 $\mu\text{g/ml}$ hydroxytyrosol. Results represent the average (%) of two independent determinations.