Research Article

Seniz Korkmaz[a], Mehmet Sarimahmut[a], Mustafa Zafer Ozel and Engin Ulukaya*

Olive leaf extract containing oleuropein modulates the cytotoxic effect of epirubicin on breast cancer cells depending on the cell line

Oleuropein içeren zeytin yaprağı ekstresi hücre hattına bağlı olarak meme kanser hücrelerinde epirubisinin sitotoksik etkilerini değiştirir

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Abstract

Objective: Epidemiologic studies showed that nutrition plays a role in incidence of cancer. However, little is known if nutrition also modulates the effect of chemotherapeutics. For this purpose, the present study investigates the cytotoxic effect of olive leaf extract and its combination with epirubicin.
Method: Cell viability was measured via ATP assay on MDA-MB-231 and MCF-7 cell lines. Apoptosis was detected by poly(ADP-ribose) polymerase (PARP) cleavage, and the expression of apoptosis-related genes. A single extract was used throughout the study.
Results: Both olive leaf extract and epirubicin resulted in cytotoxic effect in a dose-dependent manner in both cell lines. The extract further increased the cytotoxic effect of epirubicin in MDA-MB-231 cell line. However, in contrast, it abolished the cytotoxic effect of epirubicin in MCF-7 cell line. As a confirmative result, the increased expressions of *FASLG* and *HRK* following epirubicin treatment were down-regulated when epirubicin was used in combination with the extract in MCF-7 cells.
Conclusion: Olive leaf extract modulates the cytotoxic effect of epirubicin when it is in combination depending on the type of cell line. This warrants further in vivo experiments for better understanding of this intriguing result.

Keywords: Olive leaf extract; Breast cancer cell lines; Apoptosis; Cytotoxicity; Epirubicin.

Özet

Amaç: Epidemiyolojik çalışmalar beslenmenin kanser insidansında önemli bir rolü olduğunu göstermektedir. Ancak, beslenmenin kemoterapinin de etkilerini değiştirip değiştirmediğiyle ilgili az şey bilinmektedir. Bu amaçla, bu çalışma zeytin yaprağı ekstresi ve epirubisin ile kombinasyonunun sitotoksik etkilerini araştırmaktadır.
Metod: Hücre canlılığı ATP testi kullanılarak MDA-MB-231 ve MCF-7 hücre hatlarında ölçülmüştür. Apoptozis, poli-(ADP-riboz) polimeraz (PARP) kırılması ve apoptozis ilişkili genlerin ifadeleri kullanılarak saptanmıştır. Çalışma boyunca tek bir ekstre kullanılmıştır.
Bulgular: Hem zeytin yaprağı ekstresi hem epirubisin doza bağlı olarak her iki hücre hattında da sitotoksik etkiye neden olmştur. Ekstre, MDA-MB-231 hücre hattında epirubisinin sitotoksik etkisini daha da arttırmıştır. Ancak buna karşın, MCF-7 hücre hattında epirubisinin sitotoksik etkisini bozmuştur. Doğrulayıcı bir sonuç olarak, MCF-7 hücrelerinde epirubisin uygulamasıyla artan *FASLG* ve *HRK* ifadeleri, epirubisin ekstre ile kombinasyon halinde kullanıldığında düşmüştür.
Sonuç: Zeytin yaprağı ekstresi epirubisin ile kombinasyon halinde kullanıldığında epirubisinin sitotoksik etkilerini hücre hattına bağlı olarak değiştirir. Bu ilgi çekici sonuçların daha iyi anlaşılması için ileri in vivo deneylerin yapılmasını gerektirmektedir.

[a]**Seniz Korkmaz and Mehmet Sarimahmut:** These authors contributed equally to this work.
***Corresponding author: Engin Ulukaya,** Uludag University, Medical School, Department of Medical Biochemistry, Bursa, Turkey, Tel.: +90 224 295 3913, Fax: +90 224 442 8245, e-mail: eulukaya@uludag.edu.tr
Seniz Korkmaz: Uludag University, Medical School, Department of Medical Biochemistry, Bursa, Turkey
Mehmet Sarimahmut: Uludag University, Faculty of Arts and Science, Department of Biology, Bursa, Turkey
Mustafa Zafer Ozel: University of York, School of Chemistry, Heslington, York YO10 5DD, UK

Anahtar kelimeler: Zeytin yaprağı ekstresi; Meme kanseri hücre hatları; Apoptosis; Sitotoksisite; Epirubisin.

Introduction

Breast cancer represents a major health problem, with more than 1.6 million new cases and over 500,000 deaths yearly worldwide [1]. New therapies against breast cancer are introduced for clinical use; however, there is still need for improved therapeutic approaches. Leaves of olive (*Olea europaea*, Oleaceae) trees are an abundant source of raw materials in the Mediterranean basin. They contain large amounts of potentially useful phytochemicals and can play beneficial roles in health care. Epidemiologic studies show that incidence of some types of cancer, especially breast, skin and colon cancer is lower in people adapted to a Mediterranean diet [2]. Olive leaves are known for their health benefits and used for medical purposes in many places. Olive leaf and olive leaf extract is marketed to enhance the immune system, as an antibiotic and for anti-aging [3]. The active compounds found in olive leaf extracts that contribute to health are mainly oleuropein, hydroxytyrosol and other flavonoids [4]. Olive leaf extracts are shown to possess anticancer, antiproliferative and antioxidant properties [5, 6]. Previous studies have demonstrated that olive leaf extract or isolated compounds are effective in the treatments of breast, skin and bladder cancers in vitro [5, 7, 8].

Epirubicin is a semisynthetic derivative of the anthracycline, doxorubicin, but unlike doxorubicin it does not exert cardiotoxicity when used at equally effective concentrations [9]. Epirubicin is a well-established chemotherapeutic agent in breast cancer treatment and there is a rich diversity of its use in the clinical trials with recently developed anticancer agents such as the cyclin-dependent kinase inhibitor dinaciclib, microtubule stabilizer ixabepilone and other traditional chemotherapeutics as well [10–12]. In this study, we investigated the cytotoxic effects of olive leaf extract (OLE) alone and in combination with epirubicin on triple-negative breast cancer cell line, MDA-MB-231 and estrogen receptor-positive breast cancer cell line, MCF-7.

Method

Preparation of olive leaf extract

Commercially obtained OLE was produced by ethanol distillation and contains 18% oleuropein (Kale Natural Plant Products, Turkey). The olive leaves were collected from the Edremit region of Turkey. The material was cleaned, washed and dried at 35–40°C. Then, the material was ground and sifted through a 100 mesh sieve. Ethanol (70%) extract of the material was prepared after 24 h extraction. The solvent was evaporated with a rotary evaporator until the solid/liquid ratio became 1/5 and then dried in a spray dryer unit. A sample was used for HPLC analysis. The peak at 43.41 min was identified as oleuropein which corresponds to 18% (w/w) of the sample (supplemental data).

Chemicals, treatment, cell culture

Epirubicin hydrochloride (10 mg/vial, Carlo Erba, Italy) was obtained from the pharmacy of Uludag University Hospital. The stock and working concentrations of OLE and epirubicin were prepared in culture medium. Six different concentrations for epirubicin were used and defined as test drug concentrations (TDC). TDC was determined by pharmacokinetic/clinical information and clinical evaluation data [13]. One hundred percentage TDC value for epirubicin was defined as 0.5 μg/mL. Drug concentrations used for in vitro experiments were 200, 100, 50, 25, 12.5 and 6.25% of TDC. The results represent the mean of three independent experiments run in triplicates. Breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in RPMI 1640 supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL) and 5% fetal bovine serum (Invitrogen, UK) at 37°C in a humidified atmosphere containing 5% CO_2.

The ATP viability assay

The ATP assay is based on the highly sensitive "firefly" reaction to determine the level of cellular ATP as a surrogate marker for the number of live cells [13]. The amount of ATP was determined according to the manufacturer's recommendations (DCS Innovative Diagnostika-Systeme, Germany). Briefly, ATP was extracted from the cells and then luciferin-luciferase was added. Luminescence was determined in a luminometer (Bio-Tek, USA).

Sample preparation and chromatographic analysis

Olive leaf ethanol extract (0.1 g) was dissolved in 2 mL ethanol, filtered and then injected (1 μL of sample volume) into the GCxGC-TOF/MS system using the splitless method. The GCxGC-TOF/MS system consisted of an HP 6890

(Agilent Technologies, Palo Alto, CA, USA) gas chromatograph and a Pegasus III TOF-MS (LECO, St. Joseph, MI, USA). The first column was a non-polar BPX5 (30 m × 0.32 mm i.d. ×0.25 µm film thickness) and the second column a BPX50 (2 m × 0.10 mm i.d. ×0.10 µm film thickness). Helium was used as a carrier gas. The initial temperature of the first dimension column was 65°C for 1 min and the subsequent temperature program had a heating rate of 5°C/min until 245°C was reached and held isothermally for a further 1 min. The initial temperature of the second dimension column was 80°C for 1 min and a 5°C/min heating rate was used until 260°C was reached and held isothermally for further 1 min. Peak identification was made using TOF/MS with electron impact ionization. Mass spectra were compared against the NIST mass spectral library.

SDS-PAGE and western blotting

MDA-MB-231 and MCF-7 cells (1×10^7) were seeded in 75 cm^2 flasks and treated with 50 µg/mL OLE, 25 TDC epirubicin and with their combination for 30 h, an appropriate time point determined in our previous experiments [14]. After treatment, cells were washed in ice-cold PBS, and lysed in RIPA lysis buffer (Santa Cruz Biotechnology Inc., CA, USA) containing protease inhibitors. Cells were extracted at 4°C for 30 min, and centrifuged at 4°C for 10 min at 10,000 g. Equal amounts of protein (30 µg protein/lane) were subjected to 4–12% gradient gel electrophoresis and then transferred to nitrocellulose membrane. Western blotting was performed using rabbit monoclonal anti-poly-(ADP-ribose) polymerase (PARP) antibody (1:1000 dilution; Cell Signaling Technologies, MA, USA), mouse monoclonal anti-β-actin antibody (1:200 dilution; Santa Cruz Biotechnology Inc., CA, USA) and HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (1:5000; Amersham Biosciences, Sweden). Bound antibodies were visualized by enhanced chemiluminescence (ECL) and exposed to CL-XPosure film (Thermo Scientific, Rockford, IL, USA).

Real-time PCR assay

Cells were harvested for RNA isolation 14 h after OLE, epirubicin and combination treatment which was an optimized time point determined on the basis of our observation on morphology of cell death over time. Genes were selected among the most studied antiapoptotic and proapoptotic genes. Cellular RNA was isolated using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). One microgram of each total RNA sample was used for cDNA synthesis with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany). A single cDNA synthesis reaction using 5 µg total RNA was used as a template for each RealTime ready Human Apoptosis Panel, 96 (Roche, Mannheim, Germany). The total PCR reaction volume per well was 20 µL with the LightCycler 480 Probes Master (Roche, Mannheim, Germany). Sample setup and analysis were accomplished using the macro file for each gene panel with the Light-Cycler 480 Software 1.5.

Statistical analyses

All statistical analyses were performed using the SPSS 16.0 statistical software for Windows. The statistical significance was calculated using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. Results were expressed as mean ± SD.

Results

Growth-inhibiting effects of the olive leaf extract by the ATP assay

Cytotoxicity of OLE (3.12–400 µg/mL) on MDA-MB-231 and MCF-7 cell lines after 72 h of treatment was studied by using the ATP cell viability assay (Figure 1). IC_{50} (50% inhibitory concentration) dose was determined approximately as 50

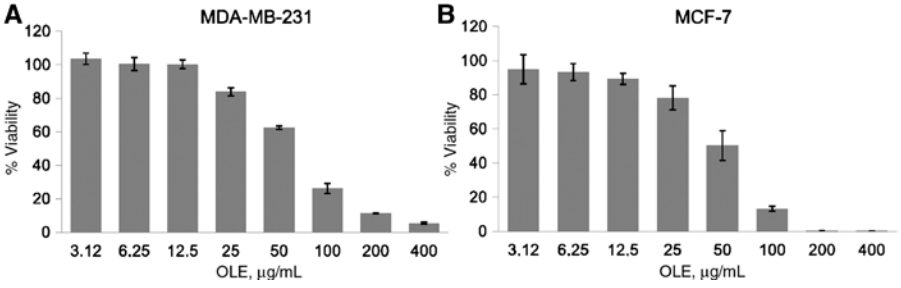

Figure 1: Treatment with OLE for 72 h on MDA-MB-231 (A) and MCF-7 (B) cell lines determined by the ATP cell viability assay.

μg/mL. Therefore, this dose was used in combination with epirubicin to measure the cytotoxic effects of the drug combination.

Growth-inhibiting effects of epirubicin and olive leaf extract on MDA-MB-231 and MCF-7 human breast cancer cell lines

MDA-MB-231 and MCF-7 cell lines were treated with different % TDC doses (200%, 100%, 50%, 25%, 12.5%, 6.25% TDC) of epirubicin alone and in combination with OLE (50 μg/mL) for 72 h (Figure 2). The differences in % viability values were found to be significant ($p < 0.05$) for all doses of epirubicin treatment compared to epirubicin + OLE combination in the MDA-MB-231 cell line. The % viability differences in the MCF-7 cell line were found to be significant ($p < 0.05$) only for 6.25% and 12.5% TDC epirubicin treatment compared to epirubicin + OLE combination. There were no differences between the two cell lines in terms of higher doses since % viability values were at minimum levels. It was found that in comparison to epirubicin alone, epirubicin + OLE combination resulted in a higher cell death-inducing effect in the MDA-MB-231 cell line while it exhibited just an opposite effect (growth-protective effect) on the MCF-7 cell line. A combination of 25 % TDC epirubicin + 50 μg/mL OLE was selected for further studies because this is an optimal concentration since higher concentrations exert too much cytotoxicity.

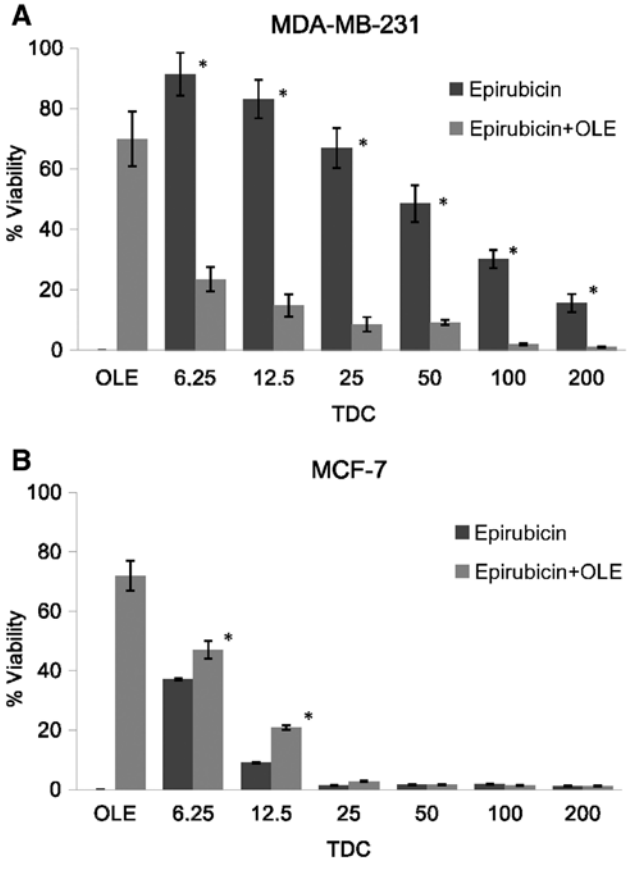

Figure 2: Cytotoxic effects of epirubicin treatment alone and its combination with a constant OLE dose (50 μg/mL) on MDA-MB-231 (A) and MCF-7 (B) cell lines for 72 h were determined by the ATP assay. Test drug concentration (TDC) values for epirubicin: 200% TDC: 1 μg/mL, 100% TDC: 0.5 μg/mL, 50% TDC: 0.25 μg/mL, 25% TDC: 0.12 μg/mL, 12.5% TDC: 0.06 μg/mL, 6.25% TDC: 0.031 μg/mL (*$p < 0.05$).

Chromatographic analysis

The percentage compositions of volatile components of OLE by using GCxGC-TOF/MS system are shown in Table 1. The most prominent volatile compound determined by this method was ethyl β-d-glucopyranoside (30.85% area).

Western blotting

PARP cleavage was determined as an indicator of apoptotic cell death by Western blotting. PARP cleavage was not observed in MDA-MB-231 after 30 h of treatment with OLE, epirubicin and epirubicin + OLE treatments. OLE did not also cause PARP cleavage in MCF-7 cell line. However, epirubicin alone and epirubicin + OLE combination treatments resulted in PARP cleavage suggesting apoptotic cell death in MCF-7 cells (Figure 3).

Determination of gene expression level changes by Real-Time PCR Assay

More than 3-fold increases in gene expression levels are accepted as significant. In MDA-MB-231 cell line, the negative expression levels of *HRK* (proapoptotic) and *FASLG* (proapoptotic) in epirubicin-treated cells were reversed in favor of apoptosis following the combinatorial treatment, which is in accordance with the cytotoxicity data (Figure 4). In MCF-7 cells, significant expression level changes from highest to lowest are in *FASLG* (proapoptotic), *BCL2L10* (antiapoptotic), *HRK* (proapoptotic) and *BAX* (proapoptotic) genes after epirubicin treatment. However, the epirubicin + OLE combination dramatically decreased the expression level of *FASLG* (approximately 100-fold decrease) (Figure 4B). This decrease was again in accordance with the decrease in cytotoxicity in the MCF-7 cell line when epirubicin was combined with OLE (Figure 2B).

Table 1: Percentage compositions of volatile components of olive leaf extract using GCxGC-TOF/MS system.

Compound[a]	RI[b]	% Area[c]
2,3-Butanedione	593	3.72
Acetic acid	600	3.05
1-Hydroxy-2-propanone	674	8.34
Acetic acid, 1-methylethyl ester	768	4.54
Hexanal	801	0.28
Furfural	828	0.19
2-Methyl-2-cyclopenten-1-one	848	3.19
2-Furanmethanol	866	0.80
1,3-Dihydroxy-2-propanone	932	6.23
Benzaldehyde	960	8.13
2-Cyclopenten-1-one	975	0.13
Benzyl Alcohol	1026	0.25
3-Methyl-benzaldehyde	1067	0.21
2-Methoxy-phenol	1086	0.45
Phenylethyl alcohol	1118	0.71
1,2-Benzenediol	1197	0.68
Benzoic acid	1276	1.37
γ-Butyrolactone	1299	0.74
2-Methoxy-4-vinylphenol	1313	1.57
2,6-Dimethoxy-phenol	1357	0.16
2-Methoxy-4-propyl-phenol	1365	0.43
4-Formyl-benzoic acid methyl ester	1385	11.49
4-Hydroxy-benzene ethanol	1451	0.70
p-Coumaric acid	1486	0.80
α-Zingiberene	1494	0.82
β-Curcumene	1514	1.00
β-Sesquiphellandrene	1560	0.38
Tetradecanoic acid	1720	0.12
Ethyl β-d-glucopyranoside	1796	30.85
Hexadecanoic acid	1984	0.81
Hexadecanoic acid ethyl ester	2103	0.35
Unknown		7.52

RI, Kovats retention index.

The results are the mean of the four experiments.

[a]As identified by GCxGC-TOF/MS software; names according to NIST mass spectral library, and by comparing their Kovats retention indices.

[b]Kovats retention indices of each component was collected from the literature for column BPX5.

[c]Percentage of each component is calculated as peak area of analyte divided by peak area of total ion chromatogram times 100 (in the case of multiple identification, the areas of the peaks that belong to one analyte were combined to find the total area for this particular analyte).

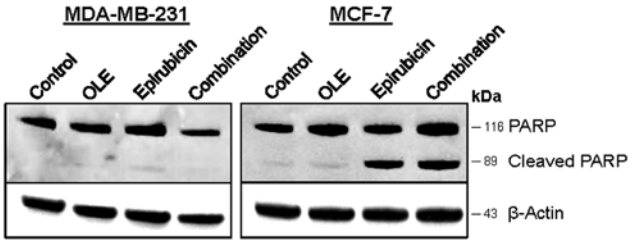

Figure 3: Cleavage of PARP was determined by Western blot after 30 h of OLE (50 μg/mL), epirubicin (25% TDC) and their combination [epirubicin (25% TDC) + OLE (50 μg/mL)] treatments. β-Actin was used as loading control.

Discussion

Anticancer, antioxidative, anti-inflammatory and antimicrobial effects of olive leaf extract were demonstrated in various studies [15–18]. Olive leaf extracts, as a natural source of polyphenols and especially oleuropein, are regarded as potentially helpful for health. In addition, oleuropein and hydroxytyrosol, the main phenolics found in olive oil, have been shown to possess anti-proliferative effects in breast cancer cell lines (MCF-7 and SKBr3) [7, 19, 20]. Polyphenols as antioxidants protect the cells against oxidative damage and act as chemopreventive agents [21–23]. The most abundant of the phenolic compound in olives, oleuropein, inhibits cell growth, motility and invasiveness and it also exerts direct anti-tumor effects which enable it to be considered as a new class of anticancer compounds that targets multiple steps in cancer progression [24]. A single extract was used throughout the study. Our commercially obtained olive leaf extract contains 18% oleuropein.

In our study, cytotoxic effects of OLE combination with epirubicin which is a chemotherapeutic drug currently used in the treatment of breast cancer were evaluated in human breast cancer cell lines, MDA-MB-231 and MCF-7. We observed decreased tumor cell viability after OLE treatment in a dose-dependent manner in MDA-MB-231 and MCF-7 human breast cancer cell lines with ATP viability assay. The difference between epirubicin treatment alone and its combination with OLE was significant ($p < 0.05$) in the MDA-MB-231 cell line. This finding might be important in preventing the adverse effects of chemotherapeutic drugs since relatively lower doses of epirubicin + OLE combination had cytotoxic effects. This result becomes especially significant if the invasive characteristic of the MDA-MB-231 cell line is taken into account. On the other hand, we found increased tumor cell viability in the MCF-7 cell line after epirubicin + OLE combination treatment, implying a detrimental (cytotoxicity-inhibiting) effect on the MCF-7 cell line. Of course, this is not a desirable effect. But it is important in terms of understanding the effects of natural products on chemotherapeutics. This detrimental effect was confirmed in gene expression studies. OLE treatment increased the expressions of *FASLG* and *HRK* genes in the MDA-MB-231 cell line while epirubicin had no significant effects on these genes. However, OLE + epirubicin combination dramatically decreased *FASLG*

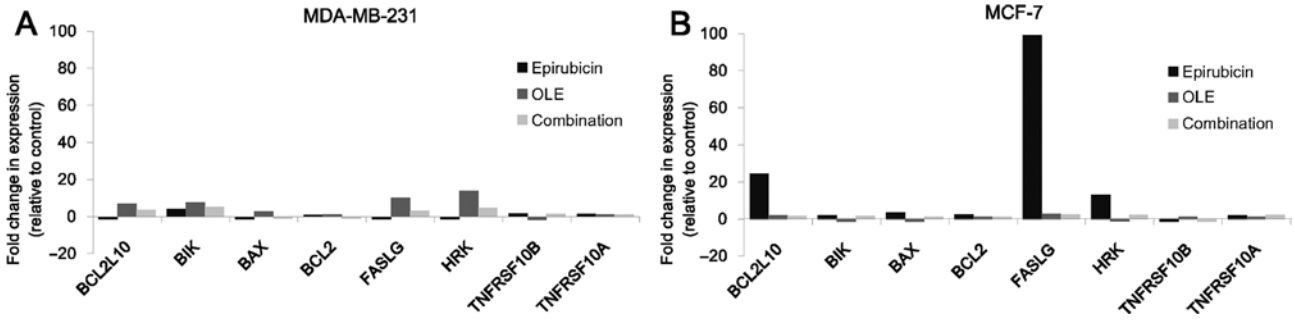

Figure 4: Gene expression level changes of proapoptotic and antiapoptotic genes in MDA-MB-231 (A) and MCF-7 (B) cell lines after 14 h treatment determined by RT-PCR.

expression that was increased after epirubicin in MCF-7 cells. It seems that the cell type plays a role in the effect of OLE + epirubicin treatment. Thus, cell type-specific cytotoxic effect of epirubicin + OLE combination might be attributed to the changes in the expression levels of *FASLG* and *HRK* genes. However, this intriguing result needs to be explored in more detail.

Effect of OLE on cell viability was investigated in several studies. Dose dependent inhibition with MTT viability assay was observed after 72 h in OLE treatment in MCF-7, SKBr3 and JIMT-1 breast cancer cell lines. IC_{50} value for MCF-7 cells resided between 200 and 400 µg/mL [25]. In another study, MTT assay results have shown that after 48 h treatment with OLE at 100 µg/mL dose, cancer cell proliferation was inhibited up to 60% compared to the vehicle treated control cells [19]. These findings are not in parallel with our results. These differences might arise from the difference in the composition of the extracts, the assays implemented to determine the cell viability and the total incubation period with the extract.

Treatment with OLE alone did not result in the cleavage of PARP, a substrate of activated effector caspases which can be considered as another apoptosis marker for both cell lines in Western blot analysis. However, some findings in the literature suggest OLE might cause apoptosis under different conditions. Anter et al. [26] treated HL60 (human promyelocytic leukemia cells) cells with OLE and reported DNA ladder formation 5 h after treatment caused by degradation of nuclear DNA into nucleosomal fragments during apoptosis. Another report indicates that OLE obtained from a Tunisian variety was also able to trigger apoptosis and cause cell cycle arrest at the G0/G1 phase as well as at the G2/M phase after prolonged incubation on the K562 multipotent leukemia cell line [4]. Parallel to this, Bouallagui et al. [5] demonstrated that OLE induces cell cycle arrest in the G0/G1 phase after 48 h treatment on MCF-7 cells although at relatively higher doses (2000–2800 µg/mL).

Epirubicin alone and its combination with OLE resulted in PARP cleavage in MCF-7, but not in MDA-MB-231 which suggests that the cell death mode in the MCF-7 cell line is apoptosis. In a previous study, epirubicin treated MCF-7 cells were reported to display caspase-independent cell death [27]. However, PARP cleavage might occur independently from caspase activation during apoptotic cell death depending on the cell type or the agents used [28].

According to RT-PCR analysis, expression levels of antiapoptotic gene *BCL2L10* and proapoptotic genes *BIK*, *FASLG* and *HRK* increased after 14 h OLE treatment in the MDA-MB-231 cell line; however, these increases were probably not significant enough to induce apoptosis. In the MCF-7 cell line, there were no increases in expression levels of apoptosis related genes after OLE treatment. These results confirm that the cell death caused by OLE treatment was not apoptosis. Significant gene expression level changes after epirubicin treatment from highest to lowest were *FASLG* (proapoptotic), *BCL2L10* (antiapoptotic), *HRK* (proapoptotic) and *BAX* (proapoptotic), respectively. Combination of epirubicin with OLE decreased the expression levels of the same genes. Therefore, OLE interferes with the cytotoxic effect of epirubicin treatment which is in line with the results of cell viability assays (Figure 2).

Olive oil polyphenols have an aromatic ring with a hydroxyl group such as estrogen and its antagonist tamoxifen. This characteristic might be responsible for the antiestrogenic effect [29]. Oleuropein doses between 10 and 75 µM inhibited cell proliferation induced by estrogen in a dose-dependent manner which was shown by [3H]thymidine incorporation assay. Furthermore, oleuropein was shown to inhibit the proliferation of breast cancer cell lines by delaying cell cycle at S phase [30, 31]. These findings might explain how OLE decreased the cytotoxic effect of epirubicin treatment; because decreased proliferation rate makes the cells less prone to cell death. As known, anti-cancer agents are most active on cells that have a higher proliferation rate.

This idea was supported in a previous combination study performed with dry olive leaf extract and doxorubicin or temozolomide that resulted in evident antagonistic effect on B16 mouse melanoma cells in vitro [8].

Conclusions

Despite the fact that the various beneficial properties of OLE or its constituents as an antioxidant, chemopreventive or anticancer compound, it should be noted that combination of OLE with some of the clinically used anticancer drugs, particularly with the agents act on rapidly proliferating cells might have detrimental effect on the therapy depending on the cancer cell phenotype.

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Conflict of Interest Statement: The authors have no conflict of interest.

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