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Anticancer effect of the letrozole-quercetin combination mediated by FOXOs and estrogen receptors in breast cancer cells

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ABSTRACT: Breast cancer is the most common malign tumor among women in the world and is the main lineage of cancer deaths due to its susceptibility to metastasis. Letrozole is used as an aromatase inhibitor and as anti-estrogenic, utilized to cure the breast cancer. Quercetin is a powerful antioxidant known as free radical scavenger, an important flavonoid. The current study was performed to examine the mechanism of action of the combination letrozole and quercetin in human breast cancer cells and to investigate the efficacy of letrozole and quercetin in breast cancer cells. Human breast cancer cells (MCF-7 and MDA-MB-231) were incubated with letrozole (10nM) and/or quercetin (5ng/mL). Cell proliferation of letrozole and quercetin in breast cancer cell lines was measured by MTT. Western blotting and qPCR were practiced to measure the protein and gene expression levels of ER-α, ER-β, Bax, Bcl-2, Foxo1, Foxo3, VEGF, Akt. Results from the study showed that letrozole and quercetin combination inhibited cell growth in MCF-7 and MDA-MB-231 cells and induced mitochondrial apoptosis. Letrozole and quercetin combination therapy may provide contributions to the development of new therapeutic drugs as an effective treatment approach for breast cancer patients.

KEYWORDS: Breast cancer; letrozole; quercetin; apoptosis; FOXOs; estrogen.

1. INTRODUCTION

Breast cancer is regarded as significant and commonly occurring malignancy in women that caused high death rates in women throughout the world [1,2]. Breast cancer cases are increasing day by day and this increase now constitutes 25% of the total cancers [3,4]. It is reported that every 8th woman is the patient of breast cancer. Also, it was estimated that the survival rate of 5 year locally invasive breast cancer is 98.1%. Life style, environment and genetic factors are considered main factors for the breast cancer and studies has confirmed that there is approximately 30% heritability of breast cancer [5–8].

Various strategies have been developed to mitigate the breast cancer; estrogen deprivation therapy is one of the emerging methodologies which inhibits estrogen production in the breast cancer patients [9]. Estrogen production can be inhibited by applying various aromatase inhibitors and first- to the third-generation inhibitors, has been developed. Among the various inhibitors, letrozole is a third-generation aromatase inhibitor with a high selectivity range. Peripheral tissues are main cause of estrogen production in postmenopausal women and >99% inhibition in the activity of these cell has been reported due to application of Letrozole. Earlier studies found letrozole much effective as compared to estrogen receptor (ER) antagonist tamoxifen and now preferably used for the therapy of patients with advanced breast cancer [10]. On the other hand, plant extract and their purified compounds maintained their importance for the human being and considered as the backbone of cancer chemotherapeutics [11]. Among the various anticancer drugs, flavonoids are the most studied ones and quercetin (3,3',4',5,7-pentahydroxyflavone) is one of the most important polyphenolic flavonoids commonly used for the cancer treatment. Green leafy vegetables, onions, citrus fruits, red grapes, cherries, and apples are main source of quercetin [12]. In this study, we aimed to investigate whether the combination of quercetin and letrozole has an effect on breast cancer cells.

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2. RESULTS

2.1. Effects of quercetin and letrozole on cell viability on MCF-7 and MDA-MB-231 cells

Cell viability of MCF-7 and MDA-MB-231 cell lines decreased as quercetin concentration increased. Rate of cell viability did not change considerably in the control group which quercetin-free (Figure 1a). Time dependent cell viability of quercetin was decreased as the incubation time increased compared to the control group in MCF-7 and MDA-MB-231 cells (Figure 1b). Similarly, rate of cell viability did not change considerably in the control group which letrozole-free (Figure 1c). Letrozole was decreased the cell viability as the incubation time increased compared to the control group in MCF-7 and MDA-MB-231 cells (Figure 1d). The IC50 of letrozole were found to be 86.5 nM and 178.3 nM of MCF-7 and MDA-MB-231 cells for 24 h, respectively. The IC50 of quercetin were found to be 66.3 ng/mL and 42.4 ng/mL of MCF-7 and MDA-MB-231 cells for 24 h, respectively.

The interactions of the letrozole and quercetin were determined with using the combination index (CI) method (median-effect principle) using the Chou-Talalay method [13]. CI values of letrozole and quercetin were calculated using CompuSyn free software. The synergistic response exhibited in MCF-7 and MDA-MB-231 cell lines to the combination has been depicted in Table 1. CI of MCF-7 ranges from 0.494 to 0.947, which is <1, indicating clear synergism, whereas that of MDA-MB-231 ranges from 0.614 to 1.662, which is ⩽1, indicating additive effect.

Table 1. CI values of the letrozole and quercetin combinations.

	MCF-7			MDA-MB-231		
	Fa	CI	DRI	Fa	CI	DRI
1 nM letrozole + 5 ng/mL quercetin	0.5	0.947	0.718	0.5	1.120	1.053
10 nM letrozole + 5 ng/mL quercetin	0.5	0.494	6.216	0.5	0.614	3.401
10 nM letrozole + 1 ng/mL quercetin	0.5	0.822	1.140	0.5	1.662	0.820

Whether the effect of the compounds on the cells changed morphology was examined under the microscope (Figure 2). MCF-7 and MDA-MB-231 cells were incubated with letrozole (10 nM), quercetin (5 ng / mL) and a combination of these two compounds for 24 hours. After incubation, when the cells were controlled under white light under inverted microscope, it was observed that the cells lost their morphology and moved away from each other in letrozole and quercetin combinations.

2.2. Effects of quercetin and letrozole on ER-α and ER-β protein and gene expression on MCF-7 and MDA-MB-231 cells

MCF-7 and MDA-MB-231 cells were incubated for 24 hours with letrozole (10 nM), quercetin (5 ng / mL) and a combination of these two compounds. Protein expression levels of ER-α and ER-β were measured. Protein expression of ER-α and ER-β via western blotting in human breast cancer cell lines was shown in Figure 3. The MCF-7 cell expressed a slight increase in the expression of ER-α protein due to letrozole as compared to the group, but this increase was non-significant (Figure 3a). A significant increase in the expression of ER-α protein due to quercetin was observed as compared to the control group (*** $p<0.001$). In the letrozole-quercetin combination, ER-α protein expression showed an increase compared to control, but there was no significant change (Figure 3b). Although the expression of ER-β protein in letrozole increased compared to control, this increase was not found significant. A significant increase in the expression of ER-β protein in quercetin was observed as compared to control (** $p<0.01$). Although the expression of ER-β protein increased compared to control in the combination of quercetin and letrozole, it was not found significant (Figure 3c). Although the expression of ER-α protein in MDA-MB-231 cell was increased in the combination of quercetin, letrozole and quercetin-letrozole compared to control, this increase was not significant (Figure 3d- 3e). Although ER-β protein expression in quercetin increased and letrozole decreased in MDA-MB-231 cell compared to control, this increase was not found significant (Figure 3f). There was no significant change in ER-β protein expression in combination therapy with quercetin and letrozole.

The changes in ER-α and ER-β gene expression levels in MCF-7 and MDA-MB-231 cells of the combination of quercetin and letrozole were showed in Figure 4. No significant change was detected in the MCF-7 cells with the application of letrozole, quercetin and combination of letrozole-quercetin at the ER-α

(Figure 4a) and ER-β (Figure 4b) gene expression levels compared to the control group. Similarly, significant changes were not found in ER-α (Figure 4c) and ER-β (Figure 4d) gene expression levels in MDA-MB-231 cells with treated quercetin and/or letrozole.

2.3. Effects of quercetin and letrozole on Foxo1 and Foxo3 protein and gene expression on MCF-7 and MDA-MB-231 cells

Protein expression of Foxo1 (Forkhead box protein O1) and Foxo3 (Forkhead box protein O3) in human breast cancer cells was shown in Figure 5. Although, an increased expression of Foxo1 protein was observed in MCF-7 due to letrozole, it was non-significant. Foxo1 protein expression in quercetin shows a significant increase compared to the control group (*** $p<0.001$). In the combination of quercetin and letrozole, Foxo1 protein expression shows a significant increase compared to the control group (** $p<0.01$) (Figure 5b).

Although Foxo3 protein expression in letrozole increased compared to control in MCF-7 cell, this increase was not found significant. Although Foxo3 protein expression in quercetin and combination of quercetin and letrozole decreased compared to control, this decrease was not found significant (Figure 5c). It was observed that Foxo1 gene expression levels increased significantly in quercetin and combination of quercetin and letrozole combination groups (Figure 5d) in MCF-7 cells. Foxo1 protein expression was enhanced in letrozole as compared to control in MDA-MB-231 cells, still the increment was non-significant. In quercetin, Foxo1 protein expression shows a significant increase compared to the control group (** $p<0.01$). Although the expression of Foxo1 protein increased in the combination of quercetin and letrozole as compared to the control group, it was non-significant (Figure 5e). According to Figure 5f, although the expression of Foxo3 protein in letrozole in MDA-MB-231 cell increased as compared to control, still the increment was non-significant. Foxo3 protein expression in quercetin shows a significant increase as compared to the control group (*** $p<0.001$) in MDA-MB-231 cells. Foxo3 protein expression was increased in the combination of quercetin and letrozole compared to the control group, this increase was not found significant (Figure 5g). It was observed that Foxo1 gene expression levels increased significantly in quercetin and combination of quercetin and letrozole combination groups (Figure 5h) in MDA-MB-231 cells.

2.4. Effects of quercetin and letrozole on VEGF and Akt protein expression on MCF-7 and MDA-MB-231 cells

Protein expression of vascular endothelial growth factor (VEGF) and protein kinase B (Akt) in human breast cancer cells was shown in Figure 6. In the MCF-7 cells, VEGF protein expression in the combination of letrozole, quercetin and letrozole-quercetin slightly increased as compared to control, but was non-significant (Figure 6b). Akt protein expression levels in letrozole and quercetin was not change compared to control (Figure 6c). Although Akt protein expression in the combination of quercetin and letrozole decreased compared to control, this decrease was not found significant (Figure 6d).

2.5. Effects of quercetin and letrozole on caspase-3 activity, Bax and Bcl-2 protein/gene expression on MCF-7 and MDA-MB-231 cells

Caspase-3 is an essential indication of apoptosis, and it is used as a marker in both mitochondrial and exogenous apoptosis. Caspase-3 activity was significantly increased in combination of quercetin and letrozole compared to the control group (*** $p<0.001$) (Figure 7a, 7b) in MCF-7 and MDA-MB-231 cells. Quercetin treatment alone was found to increase caspase-3 activity only in MCF-7 cells.

The expression at protein level of BCL2 associated X, apoptosis regulator protein (Bax) and B-cell lymphoma 2 protein (Bcl-2) in human breast cancer cells was shown in Figure 8. Bax protein expression level in letrozole was not changed and in quercetin was significantly increased as compared to control, (** $p<0.01$). In the combination of quercetin and letrozole, Bax protein expression level was significantly increased compared to the control group (*** $p<0.001$) (Figure 8b) in MCF-7 cells. In MCF-7 cells, Bcl-2 protein expression levels were decreased letrozole, quercetin and combination of quercetin and letrozole group compare to control (*** $p<0.001$, Figure 8c). In MDA-MB-231 cells, protein expression of Bax in letrozole was not changed and in quercetin was significantly increased compare to control (** $p<0.01$, Figure 8e). In the combination of quercetin and letrozole, Bax protein expression was significant increased compare to control group (*** $p<0.001$). In MDA-MB-231 cells, Bcl-2 protein expression levels were decreased letrozole, quercetin and combination of quercetin and letrozole group compare to control (*** $p<0.001$, Figure 8f).

It was observed that Bax gene expression levels increased significantly in quercetin and letrozole combination groups in MCF-7 (Figure 9a) and MDA-MB-231 cells (Figure 9c). Bcl-2 gene expression levels were decreased significantly in quercetin and letrozole combination groups in MCF-7 (Figure 8b) and MDA-MB-231 cells (Figure 8d).

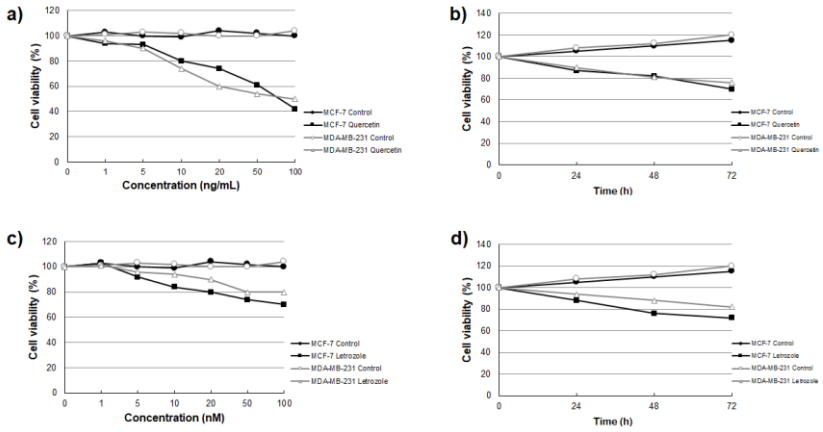

Figure 1. Time and dose-dependent cell viability effect of quercetin or letrozole on MCF-7 and MDA-MB-231 cells. The cell viability levels of a) different concentrations (0-100 ng/ml) and b) time dependent quercetin (5 ng/mL) on MCF-7 and MDA-MB-231 cells. The cell viability levels of c) different concentrations (0-100 nM) and d) time dependent letrozole (10 nM) on MCF-7 and MDA-MB-231 cells.

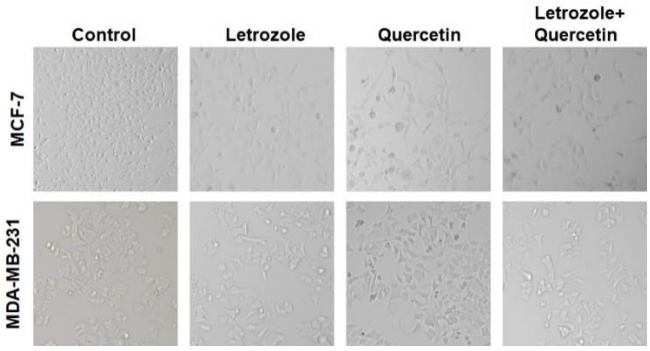

Figure 2. Microscopic image of MCF-7 and MDA-MB-231 cells treated with letrozole (10 nM), quercetin (5 ng/mL) for 24 hours.

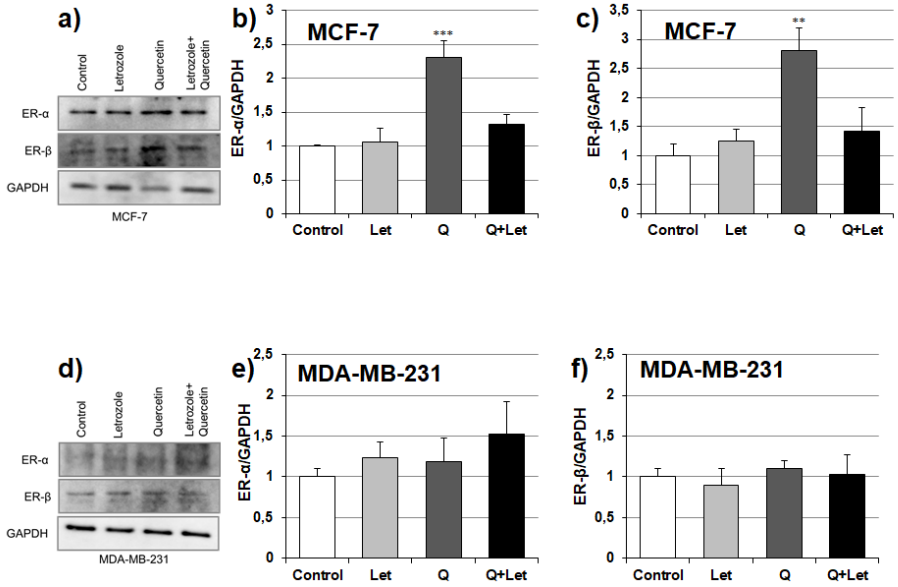

Figure 3. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on ER-α and ER-β protein expression in MCF-7 and MDA-MB-231 cells a) Western blot band image of ER-α and ER-β protein expression on MCF-7 cells. Densitometric analysis of the ER-α (b) and ER-β bands (c) of MCF-7 cells treated with letrozole and quercetin. d) Western blot band image of ER-α and ER-β protein expression on MDA-MB-231 cells. Densitometric analysis of the ER-α (e) and ER-β bands (f) of MDA-MB-231 cells treated with letrozole and quercetin. Significance was indicated as *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

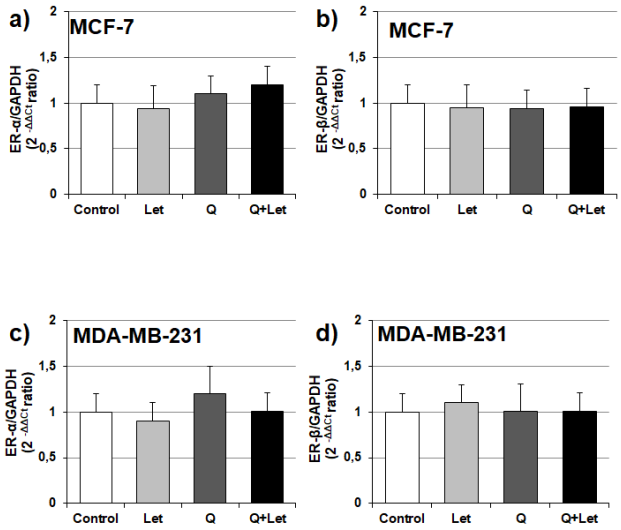

Figure 4. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on ER-α and ER-β gene expression in MCF-7 and MDA-MB-231 cells a) ER-α and b) ER-β gene expression on MCF-7 cells. c) ER-α and d) ER-β gene expression on MDA-MB-231 cells. Significance was indicated as *** p<0.001, ** p<0.01, * p<0.05.

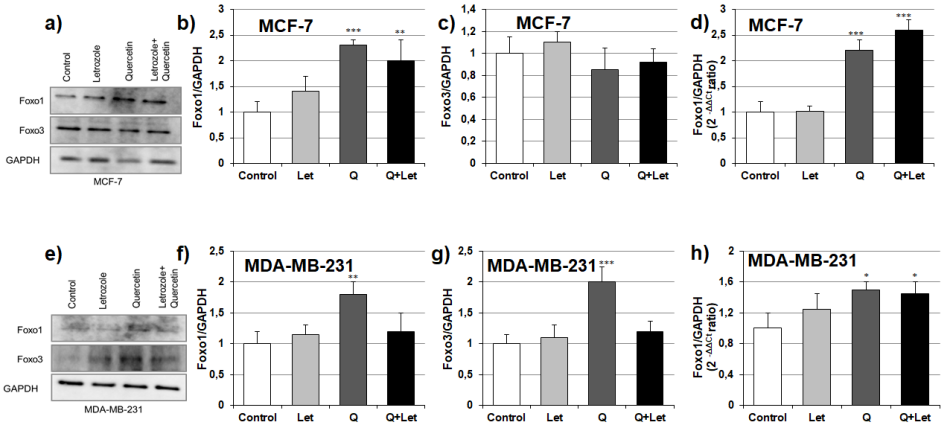

Figure 5. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on Foxo1 and Foxo3 expression in MCF-7 and MDA-MB-231 cells a) Western blot band image of Foxo1 and Foxo3 protein expression on MCF-7 cells. Densitometric analysis of the Foxo1 (b) and Foxo3 bands (c) of MCF-7 cells treated with letrozole and quercetin. d) Foxo1 gene expression on MCF-7 cells e) Western blot band image of Foxo1 and Foxo3 protein expression on MDA-MB-231 cells. Densitometric analysis of the Foxo1 (f) and Foxo3 bands (g) of MDA-MB-231 cells treated with letrozole and quercetin. h) Foxo1 gene expression on MDA-MB-231 cells Significance was indicated as *** p<0.001, ** p<0.01, * p<0.05.

3. DISCUSSION

Breast cancer is considered as the most common form of cancer in women throughout the world. Cancer cells are weakened and destroyed with the application of different types of agents. Aromatases are enzymes responsible for converting androgens to estrogen. Aromatase inhibitors suppress this transformation, reducing estrogen levels and inhibiting cell growth they are also used in cancer treatment. Drugs such as anastrazole and letrozole are used in the treatment of breast cancer patients who are positive for the estrogen receptor and reduce the death due to breast cancer [16,17]. Letrozole is a triazole derivative and phase III studies are used in case of progression of postmenopausal metastatic breast cancer disease [17]. It has been found that letrozole significantly increased the lifespan by stopping the growth of cancer cells [18]. On the other hand, cancer treatment is the goal of many studies, and many studies focus on plant-derived compounds with healing potential. Quercetin is one of the most abundant natural flavonoids and has been found to be effective against many types of cancer cells [19]. At the same time, quercetin and other flavonoids have been shown to prevent atherosclerotic plaque formation, have antiviral and cariostatic properties, prevent platelet aggregation and promote cardiovascular smooth muscle relaxation [20].

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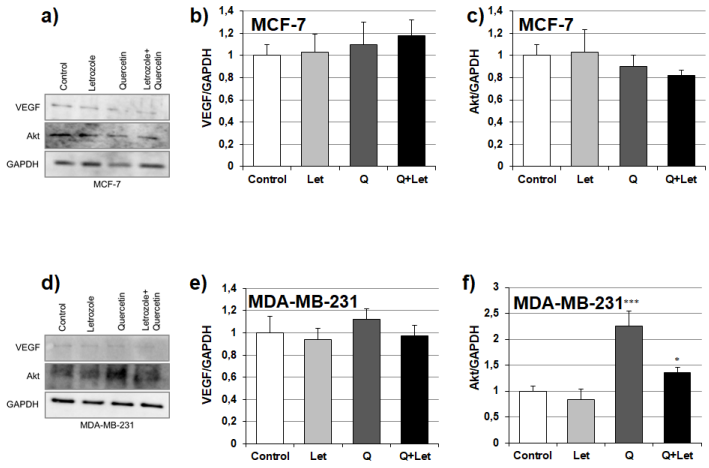

Figure 6. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on VEGF and Akt protein expression in MCF-7 and MDA-MB-231 cells a) Western blot band image of VEGF and Akt protein expression on MCF-7 cells. Densitometric analysis of the VEGF (b) and Akt bands (c) of MCF-7 cells treated with letrozole and quercetin. d) Western blot band image of VEGF and Akt protein expression on MDA-MB-231 cells. Densitometric analysis of the VEGF (e) and Akt bands (f) of MDA-MB-231 cells treated with letrozole and quercetin. Significance was indicated as *** p<0.001, ** p<0.01, * p<0.05.

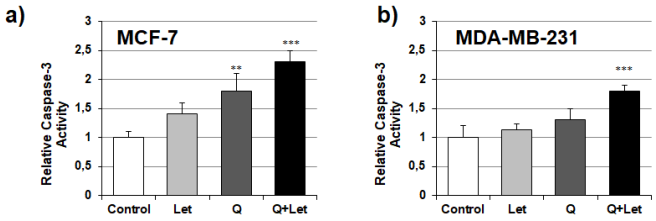

Figure 7. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on Caspase-3 activity in a) MCF-7 and b) MDA-MB-231 cells. Significance was indicated as ** p<0.01, * p<0.05.

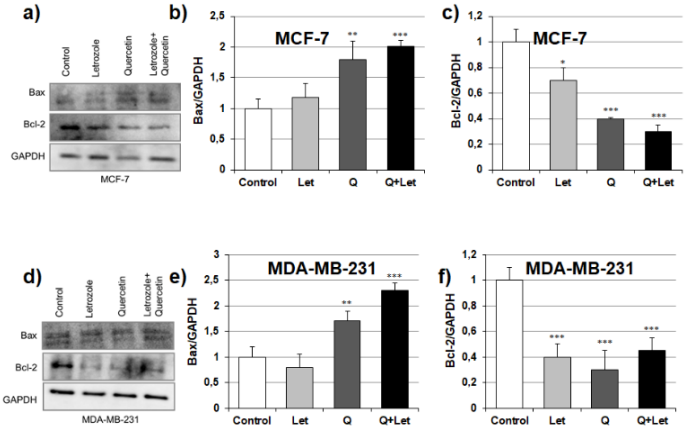

Figure 8. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on Bax and Bcl-2 protein expression in MCF-7 and MDA-MB-231 cells a) Western blot band image of Bax and Bcl-2 protein expression on MCF-7 cells. Densitometric analysis of the Bax (b) and Bcl-2 bands (c) of MCF-7 cells treated with letrozole and quercetin. d) Western blot band image of Bax and Bcl-2 protein expression on MDA-MB-231 cells. Densitometric analysis of the Bax (e) and Bcl-2 bands (f) of MDA-MB-231 cells treated with letrozole and quercetin. Significance was indicated as *** p<0.001, ** p<0.01, * p<0.05.

In vitro and *in vivo* studies using quercetin have shown many possible mechanisms of action for killing cancer cells [21]. In particular, the antitumor activity of quercetin, which belongs to the flavanol group of flavonoids on MCF-7 and MDA-MB-231 cell lines, has been previously documented [22]. More controlled, randomized human research studies are needed to confirm the effectiveness, magnitude and optimal dose of quercetin. Despite the uncertainty of quercetin 's mechanism of action on human cells, this promises for

flavonoid cancer treatment and guarantees larger-scale clinical trials to date [23]. The idea of quercetin to identify multiple cell death paths and mechanisms related to cell killing will provide useful information for quercetin 's potential application in cancer treatment [24].

Choi et al., measured the anti-proliferative effect of quercetin on MCF-7 and MDA-MB-231cell lines with the help of a colony formation assay due to 10 days. At the end of the 10-day incubation, quercetin has been observed to greatly reduce the survival rate in cells and has been reported to be used as an adjuvant in cancer therapy [25]. Chou et al provided a detailed understanding of the effects of quercetin about the apoptosis pathway induction in MCF-7 human breast cancer cells. Cell cycle distribution and apoptosis in MCF-7 cells were studied when treated with quercetin of different doses (10-175 µM) for 24 and 48 hours. It has been reported that, quercetin increased p53 and p57 proteins, CDK2 reduced the protein expression of cyclin A and B, causing the S phase to stop [26] and induced mitochondrial apoptotic cell death [27]. In another study, quercetin was induced cytosolic Ca + levels and reduced mitochondrial membrane potential and promoted activation of apoptosis-inducing factor (AIF), caspase-3, -8 and -9 in MDA-MB-231 cells [28]. In our study, we showed that the combination of lower doses of quercetin and letrozole is more effective. The mitochondrial apoptotic effect of quercetin showed a stronger effect with letrozole combination.

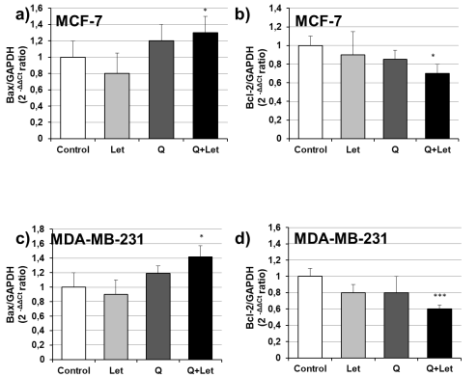

Figure 9. Combined effects of letrozole (10 nM) and quercetin (5 ng/mL) on Bax and Bcl-2 gene expression in MCF-7 and MDA-MB-231 cells a) Bax and b) Bcl-2 gene expression on MCF-7 cells. c) Bax and d) Bcl-2 gene expression on MDA-MB-231 cells. Significance was indicated as *** p<0.001, ** p<0.01, * p<0.05.

When studies on estrogen receptors and mechanisms are examined, it is suggested that quercetin acts as a phytoestrogen in some breast cancer studies [29]. On the contrary, in another study, it was suggested that it exerts an anti-cancer effect by inducing apoptosis via ER-β [30]. In a study using MCF-7 and MDA-MB-231 cancer cells, it was reported that the anti-apoptotic effect would be more effective, especially in cells with positive estrogen receptors [31]. In this study, we found that quercetin induces ER1 and ER2 receptors in MCF-7 cells at the protein level, but did not change gene expression. Neither Letrozole nor quercetin made any changes in MDA-MB-231 cells. We found that letrozole alone or in combination with quercetin did not cause any changes on estrogen receptors. This is an indication that quercetin has an indirect effect on estrogen sensitive cells. According to these findings, the use of quercetin together with estrogen-focused chemotherapies can help increase the effects of drugs. *In vitro* studies show that the use of phytoestrogens in the menopausal period suppresses the effect of aromatase inhibitors in breast cancer. It has been shown that genistein, a phytoestrogen such as quercetin, suppresses the action of letrozole in MCF-7 cells [32]. Another study showed that genistein reduced catalytic aromatase inhibitor as fadrozole in H295R and MCF-7 cells [33].

FOXO group members are transcription factors and are related to different signaling pathways and pathological processes in the cell [34]. Especially in breast cancer, FOXO members are suggested to be associated with Akt and estrogen signaling pathways [35]. It has been reported in many publications that Foxo1 and Foxo3 are tumor suppressors [36]. There was studies suggesting that Foxo1 and Foxo3 are effective and suppressed on estrogen receptors and modulate to ER-DNA binding in breast cancer [37]. It is suggested that this mechanism is involved in the Akt signal molecule. Studies have shown that Foxo3 inactivates estrogen receptors [38]. There was opinions that Foxo1 suppresses the stimulating effect of estrogen receptors and that Foxo1 levels should be increased in breast cancer [39]. In our study, we found that the combination of quercetin and letrozole increased Foxo1 protein and gene expression and this effect was more dominant in MCF7 cells, which are estrogen receptor positive cells. On the other hand, the combination of **letrozole** and quercetin was found to be more effective on Foxo3 protein expression in MDA-MB-231 cells. The increase in transcription factors such as Foxo1 and Foxo3 with quercetin and its combination is an important indicator that it can activate anti-cancer mechanisms.

Akt is a protein associated with many signaling pathways and may have different effects on cell signaling. Studies have shown that AKT and VEGF are interrelated in the cancer mechanism [40,41]. Akt activation has been shown to cause VEGF induction in breast cancer metastasis [42]. In our results, we showed that the combination of quercetin and **letrozole** did not alter VEGF but caused a decrease in Akt signal in breast cancer cells. The fact that there were no major changes between the groups suggests that it may be effective on different pathways in the active signal pathway. It needs to be phosphorylated to be fully activated. Protein kinase B, encoded by the Akt1 and Akt2 genes, is directly effective in apoptosis and acts as an inhibitor on the caspase-9 with the pro-apoptosis BAD protein, while generating an antiapoptotic response with NF-κB stimulation. Quercetin has been shown to decrease VEGF levels in the animal model of mammary carcinoma [43]. In addition, in another study on choroid-retinal endothelial cells, VEGF levels were shown to be reduced by quercetin administration [44]. In a study on the Akt pathway, it was shown that quercetin suppresses Akt related signals in metastatic breast cancer cells [45]. However, it has been shown that quercetin inhibits the act / PTEN pathway on chemosensitivity of breast cancer cells to doxorubicin [46]. Different mechanisms of action of quercetin on cancer cells are beneficial in drug and combined therapy. There are essential opinions about the need to elucidate quercetin 's cellular role in the interaction of proteins in combined therapies [47].

4. CONCLUSION

The results of this study show that the combination of letrozole and quercetin is highly effective on cell apoptosis. The antioxidant properties of quercetin also enable it to induce various ways as anti-cancer. The combination of letrozole and quercetin for breast cancer can benefit in clinical treatment and will contribute to the development of new therapeutic drugs. This shows that patients using letrozole can obtain more beneficial results in clinical practice with quercetin use.

5. MATERIALS AND METHODS

5.1. Cell cultures

The human breast cancer cell lines MDA-MB-231 and MCF-7 were commercially provided from the American Type Culture Collection (ATCC). Quercetin, letrozole, RPMI-1640, Penicilin-Streptomycin and DPBS were supplied from Sigma-Aldrich. MDA-MB-231 and MCF-7 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (Gibco 26400044), 100 units/ml penicilin/streptomycin, L-Glutamine (Gibco 10378016) and NaHCO3 in atmosphere containing 370C with 5% CO2 and 95% moisture. The cells were passaged and collected using trypsin EDTA and washed with DPBS solution. Cell culture medium was changed three times a week in cell cabinet.

5.2. Cell proliferation

The cytotoxicity test kit (MTT: Vybrant, Invitrogen) was used to determine the viability of the cells. MCF-7 and MDA-MB-231 cells were plated on 96 well plates. Six different doses of letrozole (0-100 nM) and quercetin (0-100 ng/ml) were applied to the cells growing in the plate and incubated for 24, 48, and 72 hours. After incubation, it was added to 12 mM MTT (3-(4,5-dimethylthiazol-2yl)2,5diphenyltetrazolium bromide) solution and incubated for 4 hours at 37°C in 5% CO_2. At the end of 4 hours to dissolve the purple-colored formazan crystals, 100 µL of SDS dissolved with 0.01M HCl was added and incubated in an oven containing 5% CO_2 at 37 ° C. The absorbance of the purple color formed after 4 hours was measured with ELISA plate (Epoch, Biotek) reader at 570 nm.

5.3. Western blotting

For western blot analysis [14], the cells were collected after the treatment of quercetin and/or letrozole. The cells were lysed with RIPA buffer (89900; Thermo Fisher Scientific) included protease inhibitor cocktail (Sigma P8340) and centrifuged for using supernatant. Samples containing protein were dissolved SDS-PAGE electrophoresis. After that, the proteins were transferred from the gel to the polyvinylidene fluoride membrane (PVDF, sc-3723 Santa Cruz) membrane by immunoblotting. The membrane was blocked with 3% skimmed milk powder and kept overnight on the mixer at + 4°C with primary antibodies (GAPDH (sc-25778), AKT (sc-1618), ESR-α (sc-542), ESR-β (sc-6822), Foxo1 (sc-11350), Foxo3 (sc-48348), Bax (sc-7480), BcL2 (sc-492), VGEF (sc-7269)) from Santa Cruz. Then the membrane was washed with TBST and re-incubated with secondary antibody at room temperature. After the membrane was washed again, the chemiluminescence substrate was incubated in the dark for 1 minute with the solution and the bands obtained by the Syne Gene imaging system were evaluated by densitometric analysis.

5.4. Quantitative real-time PCR

Total RNA was isolated from cells using NucleoSpin Ambion PureLink RNA Mini Kits according to the manufacturer's protocol and RNA concentration was measured using Nanodrop [15]. cDNA synthesis was performed with the Applied Biosystems commercial kit (ABI-K1622) in accordance with the procedure. The gene expression assays with qRT-PCR were performed Sybr Green Master Mix (Applied Biosytems 4309155) according to the procedure. The following primers were used in Table 2. GAPDH was used as a reference gene. Gene expression was calculated using the comparative $2^{-\Delta\Delta}$Ct method with Applied Biosytems StepOne software version 2.3.

Table 2. Primer Sequences.

Foxo1	5′-TTATGACCGAACAGGATGATCTTG-3′ (forward) 5′-TGTTGGTGATGAGAGAAGGTTGAG-3′ (reverse)
Foxo3	5′- GCAAGAGCTCTTGGTGGATCATCAA-3′ (forward) 5′- TGGGGCTGCCAGGCCACTTGGAGAG-3′ (reverse)
Bax	5'-GCCCTTTTGCTTCAGGGTTT-3' (forward) 5'-TCCAATGTCCAGCCCATGAT-3' (reverse)
BcL2	5'-GACAGAAGATCATGCCGTCC-3' (forward) 5'-GGTACCAATGGCACTTCAAG-3' (reverse)
ESR1	5′-AGATCTTCGACATGCTGCTGGCTA -3′ (forward) 5′-AGACTTCAGGGTGCTGGACAGAAA-3′ (reverse)
ESR2	5′-TGGGCACCTTTCTCCTTTAGTGGT -3′ (forward) 5′-TCTTGCTTCACACCAGGGACTCTT-3′ (reverse)
GAPDH	5′-AGGGCTGCTTTTAACTCTGT-3′ (forward) 5′-CCCCACTTGATTTTGGAGGA-3′ (reverse)

5.5. Caspase-3 activity

Cells were then harvested and lysed with lysis buffer. Caspase-3 activity assay was performed with caspase-3 activity assay kit according to the manufacturer's instructions (APT131; Millipore).

5.6. Statistical analysis

In this study, data were collected from three different biological replicates and the results were plotted as mean ± SD. One-way ANOVA was used as statistical analysis, and Tukey's multiple comparison test was performed as a post hoc test using GraphPad Prism 7. p value < 0.05 was considered statistically significant.

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