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Evaluation of Effects of Quercetin (3, 3′, 4′, 5, 7-pentohidroxyflavon) on Apoptosis and Telomerase Enzyme Activity in MCF-7 and NIH-3T3 Cell Lines Compared with Tamoxifen

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ABSTRACT

Objective: Quercetin has been shown to inhibit the proliferation of cancer cells. Tamoxifen is used for breast cancer. In this study, we have aimed to investigate the effects of quercetin and tamoxifen on telomerase enzyme activity and apoptosis in two cell lines.

Methods: In this study, 10, 50 and 100 μM of quercetin and tamoxifen were used to treat MCF-7 and NIH-3T3. Apoptosis was determined by the TUNEL method (Terminal Deoxynucleotide Transferase dUTP Nick End Label) and telomerase enzyme activity was determined by ELISA (Enzyme-Linked Immunosorbent Assay).

Results: In the NIH3T3 cell line, only 100 μM quercetin and tamoxifen induced significant apoptosis. In the MCF-7 cell line 10 μM and 100 μM quercetin in the 24th hour and 100 μM quercetin in the 72nd hour induce apoptosis. In 24th and 48th hours, 50 μM tamoxifen and 100 μM tamoxifen in the 24th and 48th hours induce apoptosis in the MCF-7 cell line. In MCF-7 and NIH-3T3 cell lines, all doses of quercetin and tamoxifen reduced telomerase enzyme activity compared to the control group.

Conclusion: In this study, it was shown that quercetin has similar effects to tamoxifen. However quercetin induces apoptosis more than decreasing telomerase enzyme activities, being different from tamoxifen. We hope that the findings will assist in developing new therapeutic pathways for preventing breast cancer. However, there should be many more studies in order to discover quercetin and other potential drugs.

Key Words: MCF-7, NIH-3T3, quercetin, tamoxifen, telomerase activity, apoptosis

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Introduction

Breast cancer is the most frequent cancer and the second leading cause of cancer mortality in women (1, 2).

The balance between proliferation and apoptosis is crucial in determining the overall growth or regression of the tumour in response to chemotherapy, radiotherapy, and more recently, hormonal treatments (3).

Apoptosis occurs in two pathways termed the mitochondrial pathways and death receptor mediated pathway. However, the mitochondrial pathway seems to dominate in breast cancer (3). In fact, defects in apoptotic pathways are now thought to contribute to a number of human disease, ranging from neurodegenerative disorders to malignancy (4).

Telomere dysfunction may also play a role in the genomic instability seen during carcinogenesis (5). In the majority of human cancers, telomerase activity is up-regulated or reactivated and a correlation between cancer and telomerase expression has been demonstrated (6).

Telomerase activity is suppressed during embriyonic differentiation in most somatic cells but remains active in some tissues, such as male germ cells, activated lymphocytes, and certain types of stem cell populations (7).

Quercetin is a member of a group of polyphenolic compounds known as flavonoids. Flavonoids, including quercetin, occur naturally in fruits, vegetables, nuts, seeds, flowers and bark (8).

Quercetin appears to have many benefical effects on human health, including cardiovascular protection, anti-cancer activity, anti-ulcer effects, anti-allergy activity, cataract prevention, anti-viral activity and anti- inflammatory effects (6, 9, 10).

Although the mechanisms by which quercetin exerts its activity remain unknown, there is evidence suggesting that the action of quercetin is probably mediated by interaction with the type II estrogen binding sites. Quercetin is a potent inhibitor of enzymes involved in signal transduction pathways including protein kinase C (PKC), tyrosine kinase, cdc25 phosphatase, PI-3 kinase, DNA topoisomerase II (11).

It has been shown that quercetin treatment causes cell cycle arrest of such as G1/S or G_2/M control points in differ-

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ent cell types. Moreover, quercetin-mediated apoptosis may result from the induction of stress proteins, distruption of microtubules and mitochondrial release of cytochrome c, and activation of caspases (10).

Tamoxifen is a non-steroidal anti-estrogen drug widely used in the treatment of patients with estrogen receptor (ER)-positive breast cancer (12).

By binding to ER, tamoxifen decreases the expression of estrogen- dependent gene stimulated by estrogen (12, 13).

Tamoxifen is known to arrest MCF-7 cell proliferation in the G_0 or G_1 phase of the cell cycle (14). It has been shown that tamoxifen induces apoptosis in both ER-positive and ER-negative human breast cancer cells by activating the caspase pathway (15). The dose response and time dependent apoptotic effect of tamoxifen was demonstrated in ER-positive MCF-7 and ER-negative MDA-MB-468 cells, but ER-positive MCF-7 cells were much more sensitive than ER-negative MDA-MB-468 cells (16).

The treatment of breast carcinoma cell lines MCF-7 and MDA-MB-231 with the anti-estrogen drug tamoxifen reduces telomerase enzyme activity in comparison with untreated cells (17). However the effect was longer lasting in the ER-positive cell line (MCF-7) (18).

It has been demostrated that the inhibition of telomerase by tamoxifen is due to an action of the hormone on the breast carcinoma cells (14).

We have aimed to investigate the effects of quercetin, which has not been used as a drug, on apoptosis and telomerase enzyme activity in MCF-7 and NIH-3T3 cell lines compared with tamoxifen, which is used in breast cancer.

Materials and Methods

Mouse fibroblast cell line (NIH-3T3) and human breast cancer cell line (MCF-7) were grown and cultured in the Department of Medical Biology, Eskişehir Osmangazi University, Eskişehir, Turkey in 2004.

NIH-3T3 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% foetal calf serum, 100 U/ml penicilin plus 100 µg/ml streptomycin, 4% sodium bicarbonate (9.2gr/ml) at 37^{0}C and 5% CO_2 in a humidified incubator. MCF-7 cell line were cultured in Miniumum Essential Medium (MEM) (Sigma) supplemented with 10% foetal calf serum, 100 mM sodium pyruvate, 2% sodium bicarbonate (7.5 gr/ml) and 1mg/ml Bovine insulin at 37^{0}C and 5% CO_2 in a humidified incubator.

Tested groups were identified as Dimethyl sulfoxide (DMSO) control, 10, 50, 100 µM doses of quercetin and tamoxifen. Quercetin and tamoxifen were dissolved in DMSO. Measurements were made at the24th, 48th, 72nd hours for apoptosis assay and at 48th, 72nd hours for telomerase enzyme activity in both cell lines.

The experiments were repeated three times in all doses. Apoptag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon) was used for apoptosis assay. To determine apoptotic index, apoptotic nuclei were counted at each chamber slide of cell lines culture. 500 cells of in the different 10 areas were evaluated in each slide.

Apoptotic index (APOi) was determined by formulation as below.

Apoptotic index (APOi): Number of apoptotic nuclei/ total cell number x 100.

To determine telomerase enzyme activity, TeloTAGGG ^{PLUS}-PCR ELISA (6) kit was used.

Data were analyzed using One Way ANOVA test for percentage apoptotic nuclei. For telomerase enzyme activity, data were analyzed using Students t test. A p value of <0.0.5 was considered to be statistically significant in the experiments. Changes according to dose and time were determined by regression and correlation analysis.

Results

In NIH-3T3 cell line as control, it was determined that 100 µM dose of quercetin induced significant apoptosis at the24th and 72nd hours according to 48th hour ($p<0.05$). However, no significant changes in other doses were detected. 100 µM dose of tamoxifen at the 72nd hour induced apoptosis as same as quercetin's effects in NIH-3T3 cell line ($p<0.05$) (Table 1, Figure 1).

All doses of quercetin induced apoptosis when compared to controls in MCF-7 cell line ($p<0.05$). It was detected that a 10 µM dose of quercetin induced apoptosis significantly at the 24th

Table 1. Apoptotic index (APOi) in NIH-3T3 at 24th, 48th, 72nd hours

Groups	Doses (µM)	Apoptotic index (APOi) (%)									Statistically analysis (in hours)
		24th hour			48th hour			72nd hour			
Quercetin	10 µM	0.26±0.16	r=0.473**	R^2=0.223	0.14±0.13	r=0.154	R^2=0.024	0.27±0.19	r=0.378**	R^2=0.143	p>0.05
	50 µM	0.34±0.26			0.17±0.16			0.20±0.21			p>0.05
	100 µM	0.42±0.25			0.21±0.17			0.46±0.34			p<0.05 24.th-48th and 48th -72nd
Control (DMSO)	0	0.18±0.10			0.16±0.15			0.15±0.17			p>0.05
Tamoxifen	10 µM	0.24±0.15	r=0.328*	R^2=0.108	0.22±0.19	r=0.213	R^2=0.046	0.40±0.23	r=0.449**	R^2=0.202	p>0.05
	50 µM	0.22±0.14			0.29±0.20			0.38±0.17			p>0.05
	100 µM	0.27±0.17			0.27±0.19			0.50±0.19			p<0.05 24th-72th and 48th-72nd
Statistical analysis (Between groups)		p>0.05			p>0.05			p>0.05			

C: DMSO Control, Q: Quercetin, T: Tamoxifen, r: Correlation Coefficient, R^2: Coefficient of Determination, *p <0.05, **p <0.01

hour when compared to other hours ($p<0.05$). 100 µM dose of quercetin induced apoptosis at the 24th and 72nd hours ($p<0.05$). 50 µM and 100 µM doses of tamoxifen induced apoptosis at the 24th and 48th hours when compared to the 72nd hours and control at the 24th hour ($p<0.05$) (Table 2, Figure 2).

In NIH-3T3 cell line, it was found tha,t when compared with controls, quercetin and tamoxifen inhibit telomerase activity at the 48th and 72nd hours ($p<0.001$) (Table 3).

In MCF-7 cell line, telomerase enzyme activity was decreased by all doses of tamoxifen at the 48th and 72nd hours. The 100 µM dose of tamoxifen was more effective at the 48th and 72ndhours in MCF cell line ($p<0.001$). All doses of tamoxifen were significantly decreased when compared to the same doses of quercetin at both hours ($p<0.001$). However, only 10 µM tamoxifen was the same as 10 µM quercetin at the 72nd hour in MCF cell line. The telomerase enzyme activity was decreased by all doses of quercetin at the 48th and 72nd hours when compared to controls in MCF-7 cell line ($p<0.001$). However,100 µM tamoxifen was more effective at the 72nd hour. Additionally in this study, it was determined that quercetin and tamoxifen inhibit telomerase activity in a dose and time-dependent manner in MCF-7 cell line when compared with controls ($p<0.001$) (Table 4).

Discussion

Flavonoids have been found to inhibit the activity of protein tyrosine kinases, with the same potency as they inhibit growth of human gliosarcoma and squamous cell carcinoma, ovarian and NIH-3T3 cells (19).

In parallel with our findings, the activity of quercetin on the proliferation of endothelial cells, A549, BEL-7402, MKN-45 tumour cells and NIH-3T3 fibroblast cells is determined in a dose-dependent manner (20).

It was also known that quercetin has a wide range of biological activities, including inhibition of mutant p53 expression, the Na^+K^+ATPase, and androgen receptor expression and function in LNCap cells. It has antiproliferative activity in vitro against several types of cancer cells of human origin including ovarian, breast, leukaemic, v-H-ras NIH-3T3 transformed cells, and intestinal cells. It blocks the cell cycle at the G1/S transition in colon and gastric cancer as well as in leukaemic cells, but causes a G2/M block in breast and laryngeal cancer cells lines or in non-oncogenic fibroblasts (11).

These findings and our findings showed that quercetin and tamoxifen do not cause important side effects in NIH-3T3 cell line which is used as a control.

It has been suggested that the breast cancer antiproliferative effect of tamoxifen may be via a receptor-mediated cytostatic activity, non-specific activity, or a receptor-mediated cytotoxic activity. Previous reports have suggested that tamoxifen induced characteristic morphological changes consistent with apoptosis, including condensation of cytoplasm and convolution of nuclear and internucleosomal DNA fragmentation in breast cancer cells. This suggests that tamoxifen inhibits the growth of breast cancer by inducing apoptosis (21).

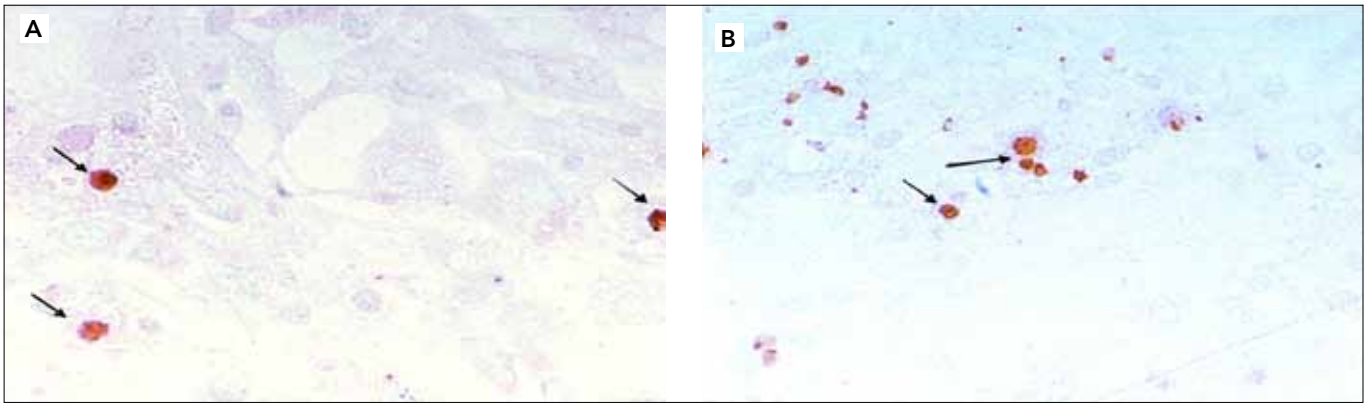

Figure 1. View of apoptotic nucleus in NIH-3T3 cell line to which are applied 100 µM quercetin (A) and 100 µM tamoxifen (B) at 72nd hour (counterstain in Methyl Green, x 400)

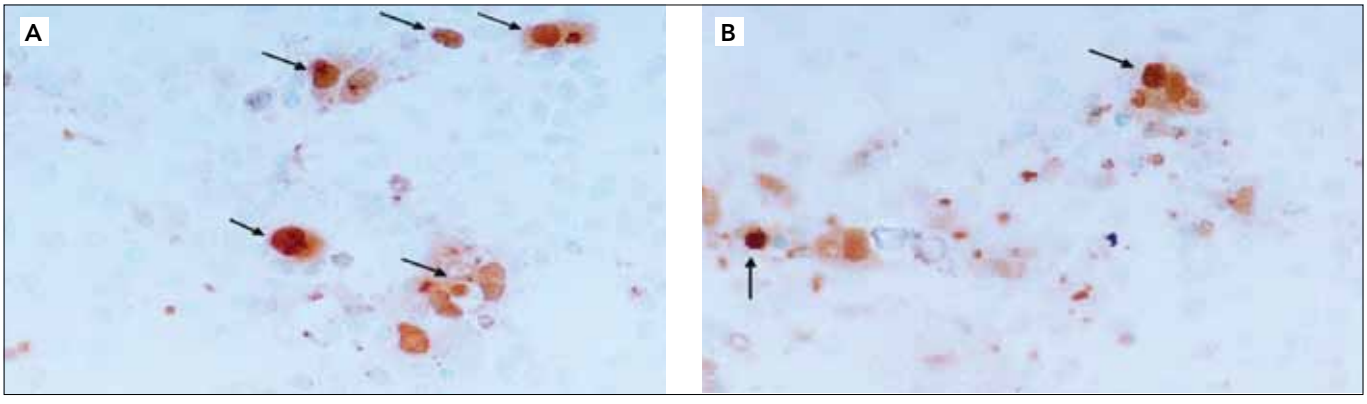

Figure 2. View of apoptotic nucleus in MCF-7 cell line to which are applied 100 µM quercetin at 72nd hour (A) and 100 µM tamoxifen at 24th hour (B) (counterstain in Methyl Green, x 400)

Table 2. Apoptotic index (APOi) in MCF-7 at 24th, 48th, 72nd hours

Groups	Doses (μM)	Apoptotic index (APOi) (%)						Statistically analysis (in hours)
		24th hour		48th hour		72nd hour		
Quercetin	10 μM	0.58±0.23	r=0.473** R²=0.223	0.36±0.32	r=0.154 R²=0.024	0.22±0.14	r=0.378** R²=0.143	p<0.05 24.th-48th and 24th -72nd
	50 μM	0.52±0.19		0.36±0.17		0.40±0.18		p>0.05
	100 μM	0.65±0.28		0.39±0.20		0.71±0.31		p<0.05 24.th-48th and 48 -72nd
Control (DMSO)	0	0.10±0.10		0.16±0.12		0.15±0.14		p>0.05
Tamoxifen	10 μM	0.22±0.14	r=0.328* R²=0.108	0.20±0.13	r=0.213 R²=0.046	0.15±0.14	r=0.449** R²=0.202	p>0.05
	50 μM	0.40±0.18		0.42±0.19		0.20±0.13		p<0.05 24th-72th and 48th-72nd
	100 μM	0.71±0.31		0.52±0.28		0.22±0.17		
Statistical analysis		p<0.05						p<0.05
(Between groups)		Q10-C						Q10-C
		Q50-C		p<0.05		p<0.05		Q50-C
		Q100-C		T100-C		Q100-C		Q100-C
		T50-C				Q100-T100		T50-C
		T100-C						T100-C

C: DMSO Control, Q: Quercetin, T: Tamoxifen, r: Correlation Coefficient, R²: Coefficient of Determination) (*p <0.05, **p <0.001

Table 3. Telomerase enzyme activities in NIH-3T3 cell line at 48th and 72nd hours

Groups	Doses (μM)	Telomerase enzyme activities			
		48th hour		72nd hour	
Quercetin	10	0.674±0.043	r=-0.574* R²=0.330	0.469±0.003	r=-0.550 R²=0.303
	50	0.544±0.076		0.516±0.054	
	100	0.673±0.041		0.521±0.018	
Control (DMSO)	0	5.765±1.035		4.433±1.293	
Tamoxifen	10	0.653±0.03	r=-0.588* R²=0.346	0.510±0.017	r=-0.579* R²=0.335
	50	0.633±0.025		0.65±0.017	
	100	0.557±0.021		0.402±0.046	
Statistical analysis		$C\text{-}Q_{10}$ (p<0.001)		$C\text{-}Q_{10}$ (p<0.001)	
(Between Groups)		$C\text{-}Q_{50}$ (p<0.001)		$C\text{-}Q_{50}$ (p<0.001)	
		$C\text{-}Q_{100}$ (p<0.001)		$C\text{-}Q_{100}$ (p<0.001)	
		$C\text{-}T_{10}$ (p<0.001)		$C\text{-}T_{10}$ (p<0.001)	
		$C\text{-}T_{50}$ (p<0.001)		$C\text{-}T_{50}$ (p<0.001)	
		$C\text{-}T_{100}$ (p<0.001)		$C\text{-}T_{100}$ (p<0.001)	
		$Q_{10}\text{-}T_{10}$ n.s		$Q_{10}\text{-}T_{10}$ n.s	
		$Q_{50}\text{-}T_{50}$ n.s		$Q_{50}\text{-}T_{50}$ n.s	
		$Q_{100}\text{-}T_{100}$ n.s		$Q_{100}\text{-}T_{100}$ n.s	

C: DMSO Control, Q: Quercetin, T: Tamoxifen, r: Correlation Coefficient, R²: Coefficient of Determination, *p<0.05, **p<0.01, n.s: not significant

Table 4. Telomerase enzyme activities in MCF-7 cell line at 48th and 72nd hours

Groups	Doses (μM)	Telomerase enzyme activities			
		48th hour		72nd hour	
Quercetin	10	20.534±0.967	r=-0.615* R²=0.378	1.854±0.755	r=-0.570* R²=0.325
	50	16.580±1.280		6.707±0.563	
	100	12.712±1.073		11.150±2.355	
Control (DMSO)	0	194.476±5.917		381.36±11.561	
Tamoxifen	10	5.267±0.591	r=-0.592* R²=0.351	1.268±0.287	r=-0.588* R²=0.346
	50	4.724±0.688		0.830±0.142	
	100	3.512±0.456		0.251±0.090	
Statistical analysis		$C\text{-}Q_{10}$ p<0.001		$C\text{-}Q_{10}$ p<0.001	
		$C\text{-}Q_{50}$ p<0.001		$C\text{-}Q_{50}$ p<0.001	
		$C\text{-}Q_{100}$ p<0.001		$C\text{-}Q_{100}$ p<0.001	
		$C\text{-}T_{10}$ p<0.001		$C\text{-}T_{10}$ p<0.001	
		$C\text{-}T_{50}$ p<0.001		$C\text{-}T_{50}$ p<0.001	
		$C\text{-}T_{100}$ p<0.001		$C\text{-}T_{100}$ p<0.001	
		$Q_{10}\text{-}T_{10}$ p<0.001		$Q_{10}\text{-}T_{10}$ n.s	
		$Q_{50}\text{-}T_{50}$ p<0.001		$Q_{50}\text{-}T_{50}$ p<0.001	
		$Q_{100}\text{-}T_{100}$p<0.05		$Q_{100}\text{-}T_{100}$ p<0.001	

C: DMSO Control, Q: Quercetin, T: Tamoxifen, r: Correlation Coefficient, R²: Coefficient of Determination, *p<0.05, ***p<0.001, n.s: not significant

In one study, it was revealed that MCF-7 cells were somewhat more sensitive to stimulation at a low tamoxifen dose (1 μM), and cytotoxicity at higher tamoxifen doses (≥5 μM) compared with MDA-231 cells. 10 μM tamoxifen also induced time-dependent cytotoxicity in both cell lines, and MCF-7 cells were slightly more sensitive to tamoxifen. Morphologic changes in MCF-7 and MDA-231 cells induced by 10 μM tamoxifen at 24 hours were shown (22).

In parallel with our findings about quercetin, it is shown that 25 μM quercetin caused morphological changes which induce apoptosis in MCF-7 cells after 24 hour treatment (23).

It has been demonstrated that quercetin induces growth inhibition in the human breast carcinoma cell line MCF-7 through at least two different mechanisms; by inhibiting cell cycle progression and by inducing apoptosis (24).

It is shown that 20-80 μM doses of quercetin have cytotoxic effects and induce apoptosis in a dose-dependent manner in A549 and H1299 human lung cancer cell lines. It is also reported that higher doses of quercetin (60 and 80 μM) almost completely inhibited the cell proliferation (10).

It has been shown that quercetin and genistein have opposite effects on proliferation of human breast cancer cells at different concentration. Phytoestrogens stimulate the proliferation of MCF-7wt and MCF7SH cells at low and intermediate concentrations and are cytotoxic at high concentrations even for HeLa cells (25).

It has been shown that quercetin increases DNA fragmentation in a dose-dependent manner and induces apoptosis by caspase activation and arrests the G2/M phase in U937 cells (26).

We observed that, in MCF-7 cells, quercetin and tamoxifen significantly decrease telomerase enzyme activity in a dose- and time-dependent manner. However, quercetin was not as effective as tamoxifen.

In nasopharyngeal carcinoma cells, Ong et al. have showed that expression of p53 and the phosphorylated form of p53 were not increased by quercetin treatment. However, expression of the hypophosphorylated form of Rb was upregulated in a dose-dependent manner when cells were treated with 59.2 μM quercetin for 24 hour (27).

In another study, it has been demonstrated that quercetin inhibits the growth of A431 cells in a dose-dependent (10-50 μM) and a time dependent manner (12-72 hour). They also indicated that, among the flavonoids investigated, quercetin and luteolin were the most potent inhibitors of the proliferation of A431 and other cell lines, such as MiaPaCa-2, MCF-7 and Hep G2 (19).

The effects of quercetin on the proliferation of endometrial cancer cell line is shown by Kaneuchi et al. 10, 50,100 μM quercetin suppressed the cell growth to 49, 10 and 13% respectively (28).

Rodgers et al. have showed that in the MCF-7 cell line, 50 μM quercetin treatment decreased DNA synthesis. RNA synthesis was significantly inhibited by 10 μM quercetin (23).

It has been shown that the most potent quercetin effect was found to be dose-dependent and chromatin condensation, an indication of apoptosis, was noticed on human colon cancer cells. Quercetin was found to distribute throughout the cell with higher amounts in the perinuclear and nucleoli areas (29).

Our results in MCF-7 cells were not consistent with several reports which showed that quercetin, H-89 or herbimycin A did not significantly inhibit telomerase enzyme activity (30).

Kudo et al. showed that, although 100 μM quercetin could not influence the HSP90 expression, which is essential for telomerase enzyme acitivity, quercetin might affect the interaction between HSP90 and actin filaments without influencing HSP90 expression itself. Thus quercetin delayed the reorganization of F-actin (31).

A study showed that 100 μM quercetin could not inhibit hTERT (human telomerase reverse transcriptase) mRNA in malignant melanoma cell line (32).

As is seen from the results of these studies, quercetin prevents cancer by decreasing telomerase activity more than by upregulating tumor suppressor genes (p53, Rb), suppressing DNA synthesis, decreasing cell proliferation and showing cytotoxic action.

Aldous et al. have found that telomerase activity in MCF-7 and MDA-MB 231 appears to be relatively unaffected by tamoxifen concentrations of 10^{-6}, 10^{-7} and 10^{-9} M.,but 10^{-8} M treated cells showed significantly lower activities than untreated control cells, although both cell lines demonstrated a rise in activity at 144 hours. The effects of the 10^{-8}M concentration on telomerase activity in both cell lines were significantly different from the other concentrations used (14).

Brandt et al. have shown that telomerase enzyme activities were significantly reduced following treatment with tamoxifen at 20 μM for 24 hour, showing a further reduction when the incubation was continued for 48 hours in the human hepatoblastoma cell line HepG2. In addition, while the rate of apoptotic nuclei was still low (about 5%) following 24 hour incubation of the cells, it increased up to 63% when the incubation time was prolonged to 72 hours (33). In another study which was carried out by the same researchers, it has shown that tamoxifen does not affect the transcription level olf hRT, hTERT, and TP1 in HepG2 cells. In contrast, the tamoxifen-induced down-regulation of telomerase activity in the breast cancer cell line MCF-7 was related to a decrease in HTERT mRNA expression. They also observed that protein kinase C activity was suppressed by tamoxifen. In conclusion, the tamoxifen induced down-regulation of telomerase activity in HepG2 cells may be due to the suppression of protein kinase C activity (34).

It has been shown that hTERT expression in cell lines showed the highest inhibition with tamoxifen (10 μM), but tamoxifen+quercetin (20 μM), showed agonistic effects and tamoxifen expression was recovered by the teatments (35).

In a study, researchers have found that type II EBS are expressed in non-small- cell lung cancer cell lines and primary tumors, and that both tamoxifen and quercetin have an antiproliferative effect on these tumor cells (36).

These findings together with our findings show that quercetin, like tamoxifen, does not affect the NIH-3T3 cell line with side effects. However, they positively affect with the MCF-7 cell line by decreasing telomerase enzmye activity and inducing apoptosis.

As expected in the MCF-7 cell line, tamoxifen has apoptotic effects. Tamoxifen prevents cancer by decreasing telomerase enzyme activity and increasing apoptosis in a time- and dose-dependent manner.

Quercetin's antiproliferative effects are shown by inducing apoptosis more than by decreasing telomerase enzyme activity in a time- and dose-dependent manner.

In conclusion, flavonoids such as quercetin which are being studied have benefical effects on cancer therapy. These effects, such as decreasing telomerase enzyme activity and inducing apoptosis, must be studied more extensively to help develop new therapeutic pathways. There should be many more studies in order to discover quercetin and other poten-

tial medicines. We hope that these findings may be helpful cancer prevention and therapy.

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Conflict of Interest

No conflict of interest was declared by the authors.

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