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RESEARCH ARTICLE

The Association Between Apoptotic Bak Protein And Quercetin in Breast and Colon Cancer Cell Lines

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Summary

Bak protein, which is one of the proteins stimulating apoptosis, leads to cell apoptosis by causing cytochrome c release from mitochondria membranes and followed by caspase activation. The aim of this study, the aim was to investigate the effect of quercetin, a flavonoid that has several biological activities especially the induction of antiproliferative effect and apoptosis in breast and colon cancer cell lines, on Bak protein. MCF-7 and CaCo-2 cell lines were treated with quercetin doses of 25, 50 and 100 µM. After the treatment, levels of Bak protein was investigated by staining with immunohistochemical method at 24th, 48 th and 72 nd hours. It was found that quercetin increased the Bak percentage at 24, 48, and 72 hours, with the most significant increase in the MCF-7 cell line was with 100 µM quercetin concentration at 48 hours and the most significant increase in the CaCo-2 cell line was with 25 µM quercetin concentration at all time points. In conclusion, it was found that quercetin is effective on levels of Bak protein and can also induce apoptosis via this mechanism and furthermore it was observed that this effect may appear in different cells at different concentrations and hours.

Key Words: *Apoptosis, Bak, CaCo-2, MCF-7, Quercetin.*

Meme ve Kolon Kanser Hücre Dizilerinde Apoptotik Bak Proteini ve Kersetin Arasındaki İlişki

Özet

Apoptozu uyaran proteinlerden Bak proteini, mitokondri zarlarından sitokrom-c'nin salınımını, devamında da kaspaz aktivasyonunu sağlayarak hücreyi apoptoza götürür. Çalışmamızda meme ve kolon adenokarsinoma hücre dizilerinde, özellikle antiproliferatif etki ve apoptoz indükleme olmak üzere çeşitli biyolojik aktiviteleri bulunan bir flavonoid olan kersetinin Bak proteini üzerine etkisinin incelenmesi amaçlanmıştır. Çalışmamızda, MCF-7 ve CaCo-2 hücre dizilerine, kersetinin 25, 50, 100 µM konsantrasyonlarda uygulandı. Uygulama sonrasında 24., 48. ve 72. saatlerde Bak proteini immunohistokimyasal yöntemle boyanarak incelendi. Kersetinin 24., 48. ve 72. saatlerde kontrol gruplarına göre % Bak oranını artırdığı, MCF-7 hücre dizisinde en anlamlı artışın 48. saat 100 µM, CaCo-2 hücre dizisinde tüm saatlerde en fazla 25 µM kersetin konsantrasyonunda olduğu belirlendi. Çalışmamızın sonucunda kersetinin Bak proteini üzerine etkili olduğu ve bu şekilde de apoptozu indükleyebileceği belirlenmiş olup, bu etkinin farklı hücrelerde farklı doz ve saatlerde olabileceği gözlendi. Ayrıca kersetinin CaCo-2 hücre dizisinde Bak proteini miktarını artırmada daha etkili olduğu göze çarpmaktadır.

Anahtar Kelimeler: *Apoptoz, Bak, CaCo-2, MCF-7, Kersetin.*

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INTRODUCTION

Apoptosis, also known as programmed cell death, is mediated by external and internal signals (1-3). Entrance of cells into apoptosis in cytoplasmic (extrinsic) pathway via extracellular signals occurs by binding of signal proteins such as Fas and TNF to the receptors on the target cell, which stimulates apoptotic pathway and stimulating the pathway to apoptosis (2,4). The caspases and Bcl-2 proteins are involved in the entrance of the cell into apoptosis via intracellular signals in the mitochondrial (intrinsic) pathway. The caspase gene family encodes the proteins that lead apoptosis of cells (2,3). The Bcl-2 gene family members encode for apoptosis stimulating (proapoptotic) and apoptosis inhibiting (anti-apoptotic) bcl proteins, which show these effects by acting antagonistically on each other. Of the members of this gene family, proteins such as Bcl-2, Bcl-X_L and Mcl-1 inhibit apoptosis, while proteins such as Bax, Bad, Bid, **Bak**, and Bcl-xs stimulate apoptosis (1,3,4).

The apoptosis stimulating proteins of Bcl-2 family, which also include the Bak protein, apoptosis of cells in the mitochondrial pathway by causing the release of cytochrome c (cyt-c) from mitochondrial membranes and then followed by caspase activation (1,4). All factors stimulating apoptosis such as DNA damage, growth factor (GF) deficiency, stimulation of the death receptors, radiation, and chemotherapy etc. may lead apoptosis of cells (1,4,5).

It is reported that dietary flavonols might play a role in the prevention of breast cancer, which is the leading cause of death in women and which develops due to several factors such as genetic characteristics, hormones, and environmental factors (6,7). It was shown that flavonols may reduce the risk of developing breast and other cancers and may increase the cytotoxicity in the cancer cells by stopping the cell cycle and inducing apoptosis (8).

The food constituents preventing the development of colorectal cancer are thought to increase apoptosis by leading to DNA damage and therefore believed to represent an important mechanism in the prevention of cancer (9). These food constituents include butyrate, flavonoids and glucosinates. Flavonoids are low molecular weight compounds that are present in plants. The average daily dietary intake of flavonoids is 1 gram or more (10).

Quercetin, a flavonoid, is present in vegetables and fruits like onion, tomato, green tea, apple and strawberry (6,11,12). Quercetin constitutes 25 milligrams of the total flavonoids taken by daily diets (13).

Quercetin has several biological activities such as antiproliferative effect and apoptosis induction (14, 17). In addition, it was reported that quercetin induces apoptosis via induction of growth inhibition, increasing of the number of cells in the sub-G1 phase, decreasing of the Bcl-2 protein level, and increasing of the Bax level, caspase-3 and PARP expression (8,15,16).

Studies regarding to the effect of quercetin in human colon cancer cell lines, was reported that quercetin inhibits survivin and cyclin D1 expression in Wnt/β signal pathway and inhibits the growth of cancer cells by regulating the genes related to cell cycle and apoptosis, and upregulating the tumor suppressor genes (18-21).

Natural compounds and many chemotherapeutic agents can have antitumor effects in cancer cells by triggering apoptosis (22-25). While it is believed that the intracellular targets of quercetin, which has significant antitumor activity, have different molecular pathways, the exact mechanisms of this activity are unclear (8, 26).

In order to contribute to the understanding of these pathways, it was aimed to investigate effects of quercetin on Bak protein, which stimulates apoptosis in breast and colon cancer cells.

MATERIALS AND METHODS

In this study, the human breast cancer cell line MCF-7 and the human colon cancer cell line CaCo-2 were used. The cells were seeded into 4-well chamber slides specifically designed for the staining procedure. MCF-7 cells were grown using RPMI 1640

(Roswell Park Memorial Institute 1640, Biological Industries Ltd., Haemek, Israel) supplement with 10% fetal calf serum (Sigma-Aldrich Inc., St. Louis, USA) and penicillin-streptomycin (Sigma-Aldrich Inc., St. Louis, USA). CaCo-2 cells were grown in a culture medium, called Eagle's minimal essential medium (Biowest, Nuaillé,France) containing 10% fetal calf serum and penicillin-streptomycin. Cells were maintained in a 5% CO_2 atmosphere at 37ºC.

The seeded cells were divided into four groups as follows: control (untreated) group group and 25, 50, and 100 µM quercetin (Sigma-Aldrich Inc., St. Louis, USA) groups. These groups were examined at three different time points (24th, 48 th and 72 nd hours), 24 hours after the seeding procedure, the medium over the cells were removed from the chamber slides using pipettes. Only fresh medium was applied to cells in the control group, while media containing quercetin concentrations were applied to cells in treatment groups. The media of the treatment groups were prepared so as to contain quercetin concentrations of 25, 50, and 100 µM per milliliter of medium by dissolving in dimethly sulfoxide [(DMSO) Sigma-Aldrich Inc., St. Louis, USA]. Cells were incubated for 24, 48 and 72 hours and then were fixed in flasks with fixation solution (Aseton:Methanol, 1:1). Immunohistochemical staining was performed by streptavidine-biotin-peroxidase staining method with the use of immunohistochemical detection kit (Lab vision Corporation, Fremont, CA, USA). Afterwards, the immunohistochemical staining steps were followed. Thereafter, they were treated with substrat-chromogen solution (AEC) until

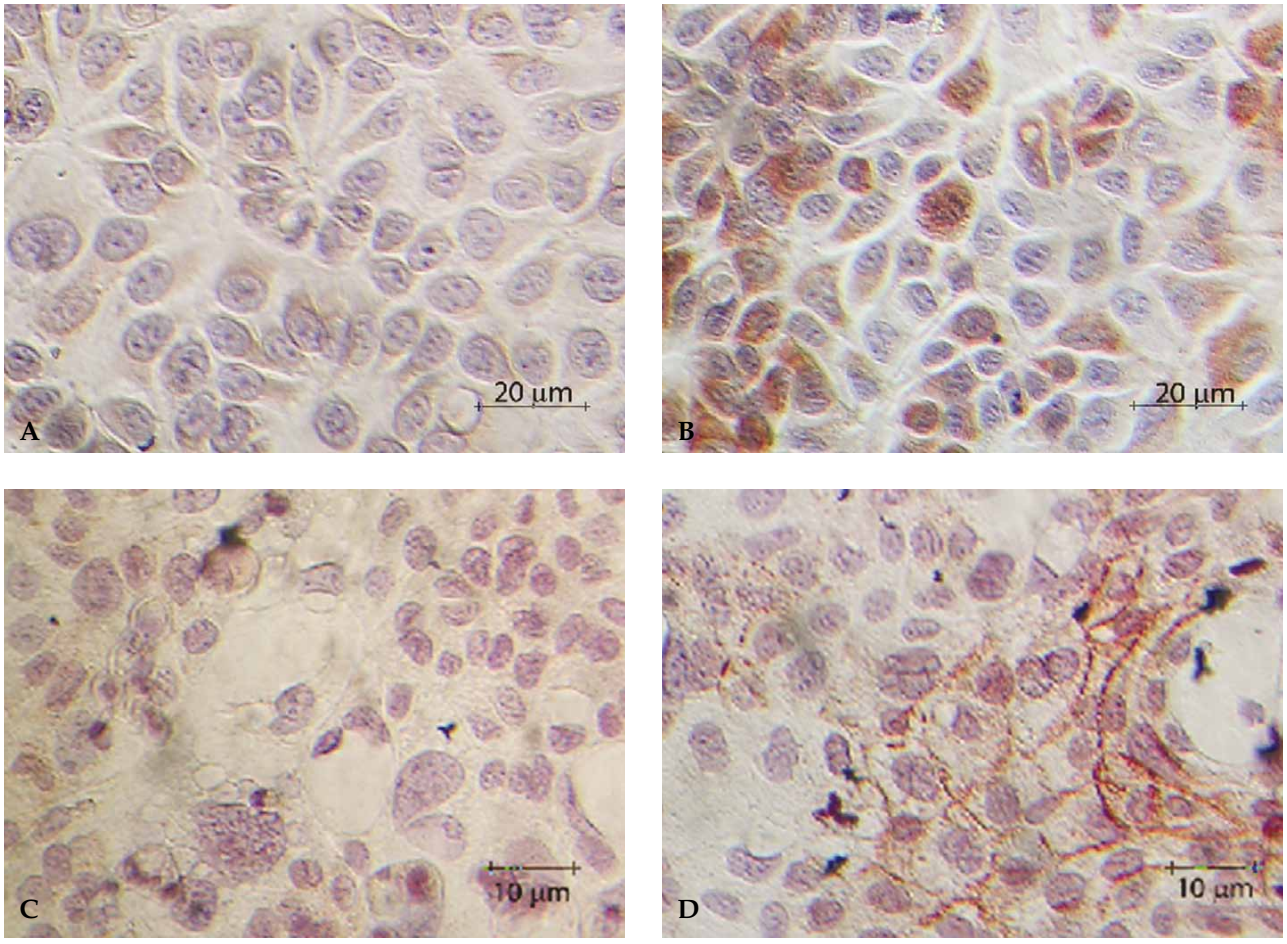

Figure 1. The immunohistochemical images of the Bak protein in the MCF-7 and CaCo-2 cell lines. MCF-7 cell line; (A) at 48th hours in the control group; (B) at 48 th hours with 100 µM quercetin. CaCo-2 cell line; at 48 th hours in the control group, (D) 25 µM quercetin-treated group at 48 th hours.

staining was seen, after which cells were exposed to hematoxylin. Preparations were mounted with mounting solution. The cells were examined under inverted microscope in a double-blind manner. For immunohistochemical index calculation, values that were obtained by counting the stained cells in five different randomly selected areas in each of the three different preparations (approximately 100 cells in each of the areas) were formulated as follows (15):

Immunohistochemical index: (Staining cells/Total cells) x 100

The cells were examined under inverted microscope following incubation with the primary antibody appropriate for the Bak protein.

The comparisons between the groups were performed using one way variance (One way ANOVA) analysis, the Tukey and Holm-Sidak methods were used as the multiple comparison test and $p<0.05$ was considered significant. All analyses were performed using SPSS 15.0.

RESULTS

In this study, effects of the three different concentrations of quercetin on the Bak percentage at the specified hours in all groups are shown in **Table 1**.

When the effects of the quercetin concentrations on Bak percentage in the MCF-7 cell line were examined in comparison with the control group, it was observed that there was a statistically significant increase compared to control group, there was a significant increase in Bak percentage of MCF-7 cell line with the quercetin concentration of 100 µM at 48 hours.

When the effects of the quercetin concentrations on Bak percentage in the CaCo-2 cell line were examined in comparison with the control group, it was found that with the quercetin concentration of 25 µM there were significant increases at all three time points, with the greatest increase observed at 48th hours. Among quercetin concentrations, the concentration of 50 µM increased the Bak percentage at 72nd hours and the 100 µM concentration at 24th and 72nd hours. No differences were observed at the other concentrations and hours, compared to the control group.

According to these results, it was considered that quercetin increases the Bak protein amount and this polyphenol increases was found to be statistically more effective in CaCo-2 compared to MCF-7 cell lines suggesting that it may be more effective in colon cancer compared to breast cancer.

Table 1. The Bak percentages of the MCF-7 and CaCo-2 cell line groups by hours and concentrations.

Groups and Concentrations		**MCF-7**			**CaCo-2**		
Groups	**Concentrations (µM)**	**24th hour**	**48 th hour**	**72 nd hour**	**24 th hour**	**48 th hour**	**72 nd hour**
Control (Con)	0	55.62 ±2.10000	52.98 ±0.55104	56.58 ±2.24542	45.586 ±0,793	51.126 ±1.071	45.906 ±0.811
Quercetin (Q)	25	55.09 ±1.21000	61.85 ±2.74596	62.95 ±0.82281	59.640 ±1.132	63.856 ±0.392	61.520 ±1.211
	50	52.99 ±2.50272	59.44 ±3.48762	58.02 ±1.38597	49.070 ±0.655	53.840 ±0.672	53.943 ±1.563
	100	57.41 ±1.56321	66.76 ±3.69487	57.50 ±2.77162	54.006 ±1.583	55.966 ±1.572	56.233 ±0.534
Statistical Analysis		$Con\text{-}Q_{25}$ n.s	$Con\text{-}Q_{25}$ n.s	$Con\text{-}Q_{25}$ n.s	$Con\text{-}Q_{25}$ ***	$Con\text{-}Q_{25}$ ***	$Con\text{-}Q_{25}$ ***
		$Con\text{-}Q_{50}$ n.s	$Con\text{-}Q_{50}$ n.s	$Con\text{-}Q_{50}$ n.s	$Con\text{-}Q_{50}$ n.s	$Con\text{-}Q_{50}$ n.s	$Con\text{-}Q_{50}$ **
		$Con\text{-}Q_{100}$ n.s	$Con\text{-}Q_{100}$ *	$Con\text{-}Q_{100}$ n.s	$Con\text{-}Q_{100}$ **	$Con\text{-}Q_{100}$ n.s	$Con\text{-}Q_{100}$ **

Results are shown as mean ±S.E.values (n = 3)
not significant (ns), *p <0.05, **p <0.01, ***p <0.001)

DISCUSSION

While the dietary polyphenols were demonstrated to decrease the risk of chronic diseases such as cancer, the exact mechanism is not clear (27). Studies have shown that quercetin induces apoptosis in cancer cells. In a study of A549 and H1299 human lung cancer cells, it was reported that quercetin induced apoptosis in a dose-dependent manner (29).

The studies in MCF-7 cell line show that quercetin may lead to various morphological changes, which can cause apoptosis, and can induce the growth inhibition (14,16,28). A study of the human colon adenocarcinoma cell line SW480 also found that quercetin reduced the cell viability in a dose- and time-dependent manner and induced apoptosis dose-dependently (18).

Along with these studies showing the apoptosis inducing effects of quercetin, there are ongoing studies investigating the effects of quercetin on the different apoptotic pathways in order to determine its molecular targets. One of these studies, found that quercetin decreases the Bcl-2 protein level in the MDA-MB-231 breast cancer cell line, increases the Bax level, leads to an increase in the number of cells in the sub-G1 phase, and increases the caspase-3 and PARP expression (8,15).

A study of CaCo-2 human colon adenocarcinoma cell line reported that the 5-μM concentration of quercetin downregulates the expression of cell cycle genes, downregulates the cell proliferation and induces the stoppage of cell cycle. This study was also demonstrated that the cell proliferation decreased by 51.3%, however the percentage of cells in the G1 phase decreased and the percentage of cells in the subG1 phase increased with administration of quercetin (19).

Another study of colon carcinoma CO115 cell line shows that 100 μmol quercetin inhibits the cell growth in G1/S and G2/M phases by affecting the genes associated with cell cycle and apoptosis (21).

It is reported that quercetin induces apoptosis in the human prostate cancer cells by inhibiting Bcl-2 via the induction of Bax and regulating insulin-like growth factor binding protein-3; in the hepatoma HepG2 cells by inhibiting phosphoinositol 3- kinase/Akt and extracellular regulatory pathway; and in human lung cancer A549 cells via caspase-3 activation and mitochondria-dependent pathways (30,31).

In another study of quercetin, 24-hour administration of quercetin was reported to inhibit cell proliferation in the colon and breast adenocarcinoma cell lines in a dose-dependent manner (33).

Furthermore, it was reported that quercetin increases the DNA fragmentation in a dose-dependent manner and triggers apoptosis via caspase activation in the human leukemia U937 cells (32).

As suggested by above-mentioned studies, quercetin can show its effect via different pathways. In a study, the human liposarcoma SW 872 cell line was treated with 25, 50 and 100 μM concentrations for 24, 48 and 72 hours and it was observed that apoptosis increased in a time- and dose-dependent manner. In that study, where this increase was indicated to be associated with membrane potential loss, the growth inhibitory effect of quercetin was reported to be resulted from the stimulation of apoptosis that was demonstrated by mitochondrial membrane potential loss, degradation of PARP, downregulation of Bcl-2 and the activation of caspase-3, Bax, and Bak (34). That study determined that quercetin increased Bak protein level, hence apoptosis in a dose- and time-dependent manner. In conclusion, present study was found that the Bak protein levels increased compared to control group, but this increase was not dose- and time- dependent. Most importantly, it was concluded that quercetin may be more effective on the colon adenocarcinoma cell line than breast adenocarcinoma cell line.

REFERENCES

1. Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. *J Cell Mol Med.* 2003; 7(3):249-257.
2. Sprick MR, Walczak H. The interplay between the Bcl-2 family and death receptor-mediated apoptosis. *Biochimica et Biophysica Acta*. 2004; 1644:125-132.
3. Twomey C, McCarthy JV. Pathways of apoptosis and importance in development. *J.Cell. Mol. Med*. 2005; 9(2):345-359.
4. Pagliari LJ, Pinkoski MJ, Gren DR. Apoptosis signaling: a means to an end. *Handbook of Cell Signaling*. 2003; 3(331):431-439.
5. Gulbins E, Dreschers S, Bock J. Role of mitochondria in apoptosis. *Experimental Physiology.* 2003; 88(1):85-90.
6. Adebamowo CA, Cho E, Sampson L, et al. Dietary flavonols and flavonol-rich foods intake and the risk of breast cancer. *Int J Cancer*. 2005; 114:628–633.
7. Corre LL, Chalabi N, Delort L, et al. Differential expression of genes ınduced by resveratrol in human breast cancer cell lines. *Nutrition and Cancer*. 2006; 56(2):193–203.
8. Chien SY, Wu YC, Chung JG, et al. Quercetin-induced apoptosis acts through mitochondrial- and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells. *Hum Exp Toxicol.* 2009; 28:493-503.
9. Watson AJM. Apoptosis and colorectal cancer. *Gut.* 2004; 53:1701-1709.
10. Wei YQ, Zhao X, Kariya Y, et al. Induction of apoptosis by quercetin: ınvolvement of heat shock protein. *Cancer Research*. 1994; 54:4952-4957.
11. Aherne SA, O'Brien NM. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition.* 2002; 18:75–81.
12. Mıddleton E, Kandaswamı C, Theoharıdes TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev.* 2000; 52:673–751.
13. Yang K, Lamprecht SA, Liu Y, et al. Chemoprevention studies of the flavonoids quercetin and rutin in normal and azoxymethane-treated mouse colon. *Carcinogenesis*. 2000; 21(9):1655-1660.
14. Choi JA, Kim JY, Lee JY, et al. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *Int J Oncol.* 2001; 19:837–844.
15. Choi EJ, Bae SM, Ahn WS. Antiproliferative effects of quercetin through cell cycle arrest and apoptosis in human breast cancer MDA-MB-453 cells. *Arch Pharm Res.* 2008; 31(10):1281-1285.
16. Nichenametla SN, Taruscio TG, Barney DL, et al. A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition.* 2006; 46(2):161-183.
17. Hatipoglu A, Basaran A, Dikmen M, et al. 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 to compared with tamoxifen *Medical Journal of Trakya University*. *Date Accepted: May 27, 2010* Doı: 10.5174/Tutfd.2010.03372.3
18. Shan BE, Wang MX, Li RQ. Quercetin inhibit human SW480 colon cancer growth in association with inhibition of cyclin D1 and survivin expression through wnt/β-catenin signaling pathway. *Cancer Investigation*. 2009; 27(6):604–612.
19. Van Erk MJ, Roepman P, Van der Lende TR, et al. Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancerrelated mechanisms in colon cancer cells in vitro. *Eur J Nutr.* 2005; 44:143–156.
20. Richter M, Ebermann R, Marian B. Quercetin induced apoptosis in colorectal tumor cells: possible role of EGF receptor signaling. *Nutr Cancer*. 1999; 34:88–99.
21. Murtaza I, Marra G, Schlapbach R, et al. A preliminary investigation demonstrating the effect of quercetin on the expression of genes related to cellcycle arrest, apoptosis and xenobiotic metabolism in human CO115 colon-adenocarcinoma cells using DNA microarray. *Biotechnol Appl Biochem*. 2006; 45(Pt 1):29–36.
22. Dong Z. Effects of food factors on signal transduction pathways. *BioFactors*, 2000; 12:17–28.
23. Russo M, Tedesco I, Iacomino G, et al. Dietary phytochemicals in chemoprevention of cancer. *Curr Med Chem Immun, Endoc & Metab Agents*. 2005; 5:61-72.
24. Taraphdar AK, Roy M, Bhattacharya RK. Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. *Current*

Science. 2001; 80:1387-1396.

25. Cosan D, Soyocak A, Basaran A, et al. The effects of resveratrol and tannic acid on apoptosis in colon adenocarcinoma cell line. *Saudi Med J*. 2009; 30(2):191-195.
26. Park CH, Chang JY, Hahm ER, et al. Quercetin, a potent inhibitor against β-catenin/Tcf signaling in SW480 colon cancer cells. *Biochemical and Biophysical Research Communications.* 2005; 328(1):227-234.
27. Granado-Serrano AB, Martín MA, Bravo L, et al. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-Kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). *J Nutr.* 2006; 136:2715–2721.
28. Rodgers EH, Grant MH. The effect of the flavonoids, quercetin, myricetin and epicatechin on the growth and enzyme activities of MCF7 human breast cancer cells. *Chemico-Biological Interactions*. 1998; 116:213-228.
29. Kuo PC, Lıu HF, Chao JI. Survivin and p53 Modulate quercetin-induced cell growth inhibition and apoptosis in human lung carcinoma cells. *The Journal of Biological Chemistry*. 2004; 279:55875-55885.
30. Vijayababu MR, Kanagaraj P, Arunkumar A, et al. Quercetin-induced growth inhibition and cell death in prostatic carcinoma cells (PC-3) are associated with increase in p21 and hypophosphorylated retinoblastoma proteins expression. *J Cancer Res Clin Oncol.* 2005; 131:765-771.
31. Chang J, Hsu Y, Kuo P, et al. Increase of Bax/Bcl-XL ratio and arrest of cell cycle by luteolin in immortalized human hepatoma cell line. *Life Sci*. 2005; 76:1883-1893.
32. Kim OH, Kim YH, Lim JH, et al. Quercetin arrests G2/M phase and induces caspase-dependent cell death in U937 cells. *Cancer Letters*. 2006; 240(2):234-242.
33. Van der Woude H, Gliszczynska-Swiglo A, Struijs K, et al. Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. *Cancer Letters.* 2003; 200:41–7.
34. Huang SL, Hsu CL, Yen GC. Growth inhibitory effect of quercetin on SW 872 human liposarcoma cells. *Life Sciences*. 2006;79:203-209.