

Full Length Research Paper

Induction of apoptosis in human breast adenocarcinoma MCF-7 cells by tannic acid and resveratrol

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Tannic acid (TA), a plant polyphenol, has been described as having anticarcinogenic, antioxidant, antimutagenic, antimicrobial, antiallergic, antiinflammatory and astringent properties. Resveratrol (RES), a phytoalexin phenolic compound, has been known as an antioxidant, anticarcinogenic, antiinflammatory, antimutagenic, antiproliferative, antiviral, antibacterial, estrogenic and vasodilator agent. Although molecular mechanisms of TA and RES responsible for anticancer activity are yet to be known, there are *in vitro* studies indicating that these substances contribute to anticancer activity by inducing apoptosis. In this study, the effects of TA and RES in MCF-7 breast cancer cells on apoptotic index, Fas-associated death domain (FADD) and Bak proteins were investigated. Terminal transferase-mediated dUTP nick end-labeling (TUNEL) method was applied for apoptosis and immunohistochemical staining for FADD and Bak proteins. Our study results show that TA and RES increased apoptotic index, percentage ratios of apoptotic proteins Bak and FADD in MCF-7 cell line.

Key words: Bak, Fas-associated death domain (FADD), MCF-7, resveratrol, tannic acid.

INTRODUCTION

Breast cancer is the most common type of malignant cancer among women worldwide. A recognized mechanism of breast cancer is an imbalance between cell proliferation and apoptosis, and tumor growth depending not only on the rate of proliferation but also on the rate of the apoptosis. It is generally believed that the balance between proliferation and apoptosis influences the response of tumors to treatments such as chemotherapy, radiotherapy and hormonal therapy. It has been suggested that, when these treatments fail, dysregulation of apoptosis may be the cause (Parton et al., 2001).

Apoptosis is a form of programmed cell death through death receptors in plasma membrane (extrinsic pathway)

or disruption of intracellular homeostasis (intrinsic pathway) (Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors, located on the cell membrane. The binding of the Fas ligands to Fas receptors, in association with adaptor molecules such as Fas-associated death domain (FADD), leads to cleavage and activation of caspases and culminating in apoptosis (Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). In contrast, the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell.

Permeabilization of mitochondrial membranes is controlled by a balance between the antagonistic actions of the proapoptotic and antiapoptotic Bcl-2 family members, leading to release of small molecules such as cytochrome c from intermembrane space to the cytosol

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(Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). Once released, cytochrome c forms a complex with Apaf-1 and procaspase-9, first resulting in activation of this initiator caspase and then of effector caspases. Proapoptotic protein Bak releases cytochrome c from mitochondria into the cytoplasm, and initiation of the caspase proteolytic cascade, eventually resulting in cell death (Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). Natural compounds and many chemotherapeutic agents with antitumor effects can trigger the apoptosis of cancer cells (Dong, 2000; Russo et al., 2005; Taraphdar et al., 2001). Phenolic phytochemicals such as tannins are natural constituents of tea, green tea, coffee, red wine, grapes, nuts and other plant products (Cowan, 1999; Taffetani et al., 2005). Plant-derived polyphenolic tannins (500 to 3000 Da) can be classified into two groups as hydrolysable and condensed tannins. The hydrolysable tannins, commonly called tannic acid (TA) contain either gallotannins or ellagitannins (Nam et al., 2001).

TA polyphenol has been described as having anticarcinogenic, antioxidant, antimutagenic, antimicrobial and astringent properties (Cowan, 1999; Nam et al., 2001; Khan and Hadi, 1998). TA and its structural monomer gallic acid are also capable of inducing apoptosis in animal cells (Khan and Hadi, 1998). Gallic acid acts as prooxidants in the induction of apoptotic cell death in human glioblastoma cells (Sakagami and Satoh, 1997). Inhibition of the proteasome by TA in Jurkat T-cells results in accumulation of two natural proteasome substrates, namely the cyclin-dependent kinase inhibitor p27 Kip1 and the proapoptotic protein Bax, followed by growth arrest in G1 and induction of apoptotic cell death (Nam et al., 2001).

Resveratrol (3,5,4' trihydroxystilbene) (RES) is a phytoalexin phenolic compound (Corre et al., 2004). It has been found in many plants, including grapes, red wine, peanuts, berries and traditional oriental medicine plants and known as Kojikon the roots of *Polygonum cuspidatum* (Corre et al., 2004, 2005; Aziz et al., 2003). RES is an antioxidant, anticarcinogenic, antiinflammatory, anticoagulant, antimutagenic, antiproliferative, antimicrobial, estrogenic and vasodilator agent (Aziz et al., 2003; Corre et al., 2005; Filip et al., 2003).

RES suppresses the growth of breast cancer cell lines and inhibits the activity and the expression of several enzymes that play key functions in the regulation of the cell growth and apoptosis. RES increases the expression of apoptotic Bax and Bak and downregulates antiapoptotic Bcl-2 and Bcl-xL in MCF-7. This means that the ratio of Bcl-2 to Bax is important for the apoptosis induced by chemoprevention agent. The increased ratio of Bax to Bcl-2 might contribute to apoptosis induction in RES-treated MCF-7 cells (Corre et al., 2005). In this study, we aimed to investigate effects of TA and RES on apoptosis, FADD and Bak proteins in MCF-7 cell lines by comparing their effects.

MATERIALS AND METHODS

Cell culture

MCF-7 breast cancer cells were cultured in the chamber slides (Nunc™ Brand Lab-Tek NY, USA) using RPMI 1640 (Biological Industries Limited, Haemek, Israel) supplement with 10% fetal calf serum (Sigma-Aldrich Inc., St. Louis, USA) and penicillin-streptomycin (Sigma-Aldrich Inc., St. Louis, USA) and were grown as monolayers in a humidified atmosphere of 5% CO₂ at 37°C.

Cell treatment

TA (Acros Organics, New Jersey, USA) and RES (Sigma-Aldrich Inc., St. Louis, USA) were used to treat MCF-7 cells. MCF-7 cells were plated at a chamber slides and in a medium containing different concentrations of TA and RES [0, 25, 50 and 100 µM in dimethyl sulfoxide (DMSO)] for 24, 48 and 72 h.

Apoptotic index and immunohistochemical staining

Apoptotic index was determined by Apop Taq Plus Peroxidase *in situ* apoptosis detection kit (Chemicon International, Huissen, The Netherlands) using Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay. Immunohistochemical staining was done by the streptavidin-biotin-peroxidase staining method. An immunohistochemical detection kit (Lab Vision Corporation, Fremont, CA, USA) and its FADD and Bak antibodies (Neomarkers, Fremont, CA, USA) were used for immunohistochemical staining. Fresh medium containing 0, 25, 50 and 100 µM TA and RES concentrations were added to cells. Cells were incubated for 24, 48 and 72 h. Cells fixed on the chamber slides base were used to determine apoptotic index and percentage of FADD and Bak proteins. These fixed cells were first incubated with serum blocking solution (10 min) for determination of percentage of FADD and Bak proteins. Then they were incubated with FADD and Bak antibodies (1 h). After washing with PBS solution, they were incubated with biotin-labeled secondary antibody (10 min) and streptavidin-peroxidase conjugate (10 min). Thereafter, they were treated with substrate-chromogen solution (AEC) until staining was seen, after which cells were exposed to hematoxylin for 1 to 2 min. Preparations were mounted with mounting solution and evaluated.

Apoptotic nuclei and staining FADD and Bak proteins were counted at each chamber slides. 100 cells in the 30 different areas were evaluated in each slide. Evaluations of the stained preparations were performed using light microscopy by two of the authors and scored independently. Apoptotic index (APOi) = Number of apoptotic nuclei / total cell number × 100 (Garvin et al., 2006). Percentage of FADD and Bak proteins = (Staining cell / Total cell) × 100 (Liu et al., 1999).

Statistical analysis

Data were reported as means ± S.E. Statistical differences between control and TA- or RES- treated cells were calculated using the Student-t test for double comparing. A *p* value < 0.05 was considered to indicate a significant difference.

RESULTS

Apoptosis

TA- and RES-induced apoptosis in MCF-7 cells

Apoptosis (programmed cell death) plays an important

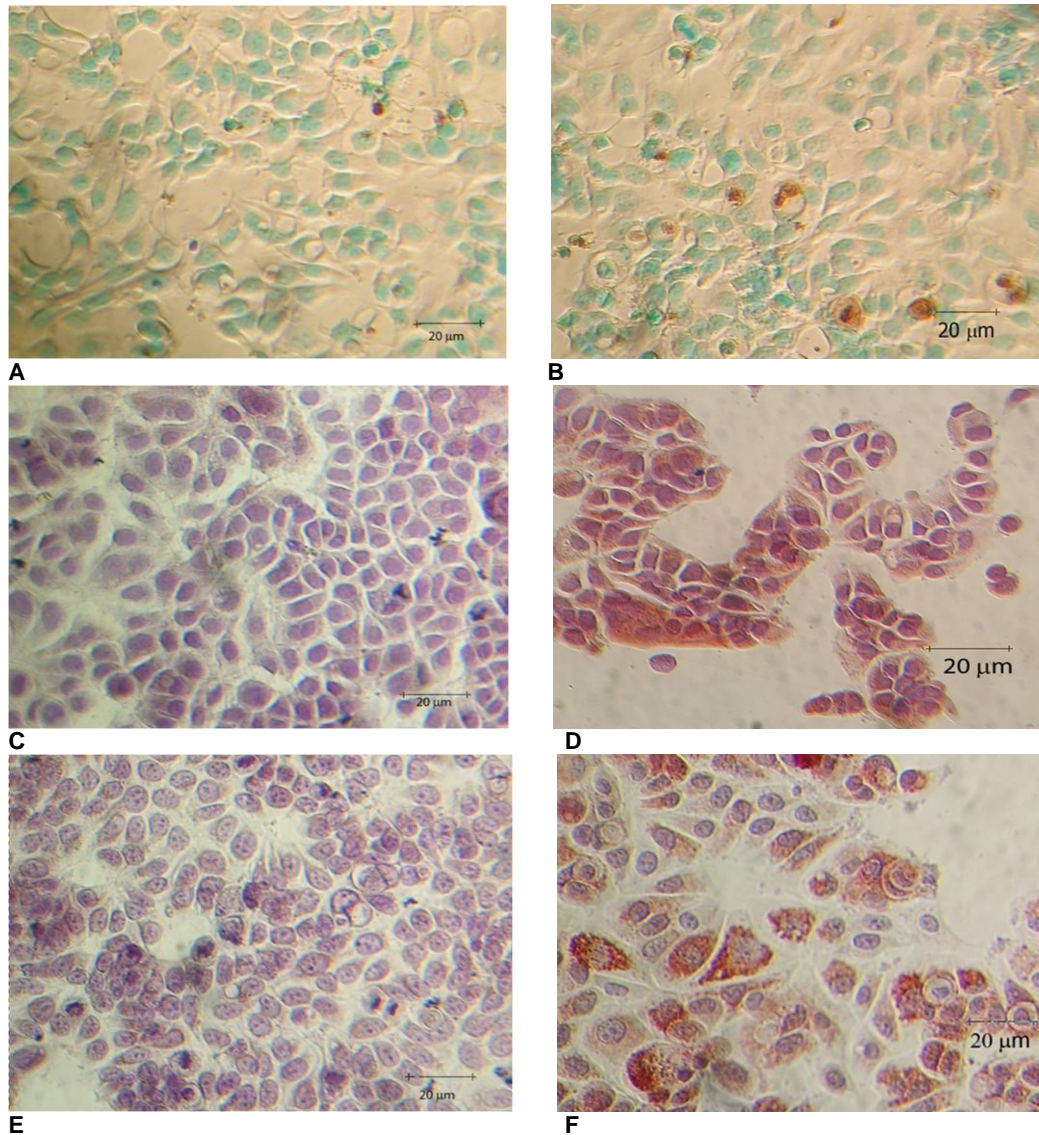


Figure 1. Apoptotic images of MCF-7 cell lines at 24th h 100 μM tannic acid ($p < 0.01$) (A), and 24th h control (B), the immunohistochemical images of FADD protein on MCF-7 cell line at 72nd h 100 μM resveratrol ($p < 0.01$) (C), and 72nd h control (D), the immunohistochemical images of Bak protein on MCF-7 cell line at 72nd h 100 μM tannic acid ($p < 0.001$) (E), and 72nd h control (F).

role in the multicellular organisms, and impairment of apoptotic function has been associated with several human diseases, including cancer (Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). Therefore, in the present study, we investigated whether or not TA and RES increased apoptosis in MCF-7 cells. The results illustrated in Figures 1A and B show that TA and RES induced apoptosis of MCF-7 cells. In particular, apoptotic index was increased at the concentration of 100 μM TA after 24 and 72 h and 50 μM TA after 72 h ($p < 0.01$), 50 μM TA after 24 and 48 h and 100 μM TA after 48 h and 25 μM TA after 72 h ($p < 0.05$) compared to control. On the other hand, apoptotic index was increased at the

concentration of 100 μM RES after 48 and 72 h ($p < 0.01$) compared to control (Table 1).

Death receptors mediating apoptosis through the extrinsic pathway

TA- and RES treatment of MCF-7 cells results in an increase in apoptotic adaptor protein FADD

Recent studies have demonstrated that death receptors are important mediators of the apoptosis, particularly in chemotherapy-induced cancer cell apoptosis. FADD is an adaptor molecule that bridges the Fas-receptor and other

Table 1. Apoptotic index analyzed by TUNEL assay in MCF-7 cell line.

Group	Dose (μM)	24th h	48th h	72nd h
Apoptotic index				
Control	0	4.210 \pm 0.583	3.057 \pm 0.295	2.708 \pm 0.081
	25	4.040 \pm 0.346 ^{n.s}	3.850 \pm 0.278 ^{n.s}	4.255 \pm 0.330*
	50	6.778 \pm 0.300*	4.949 \pm 0.077*	5.032 \pm 0.302**
	100	7.905 \pm 0.578**	4.992 \pm 0.510*	4.521 \pm 0.286**
Tannic Acid	25	4.376 \pm 0.391 ^{n.s}	3.726 \pm 0.217 ^{n.s}	2.642 \pm 0.119 ^{n.s}
	50	5.567 \pm 0.172 ^{n.s}	4.156 \pm 0.192 ^{n.s}	3.210 \pm 0.138 ^{n.s}
	100	6.109 \pm 0.342 ^{n.s}	5.022 \pm 0.199**	3.791 \pm 0.160**
Resveratrol	25	4.376 \pm 0.391 ^{n.s}	3.726 \pm 0.217 ^{n.s}	2.642 \pm 0.119 ^{n.s}
	50	5.567 \pm 0.172 ^{n.s}	4.156 \pm 0.192 ^{n.s}	3.210 \pm 0.138 ^{n.s}
	100	6.109 \pm 0.342 ^{n.s}	5.022 \pm 0.199**	3.791 \pm 0.160**
FADD (%)				
Control	0	48.872 \pm 0.840	40.049 \pm 0.135	48.823 \pm 4.321
	25	54.717 \pm 1.980 ^{n.s}	59.016 \pm 1.384**	63.391 \pm 0.873 ^{n.s}
	50	58.805 \pm 2.970*	64.817 \pm 3.763***	61.197 \pm 1.618 ^{n.s}
	100	70.207 \pm 0.333***	68.567 \pm 1.044***	70.649 \pm 3.720**
Tannic Acid	25	51.677 \pm 1.673 ^{n.s}	53.052 \pm 3.361*	52.959 \pm 3.447 ^{n.s}
	50	53.142 \pm 2.680 ^{n.s}	65.346 \pm 0.921***	63.149 \pm 3.452 ^{n.s}
	100	66.695 \pm 0.663**	72.145 \pm 2.015***	74.583 \pm 3.327**
Resveratrol	25	51.677 \pm 1.673 ^{n.s}	53.052 \pm 3.361*	52.959 \pm 3.447 ^{n.s}
	50	53.142 \pm 2.680 ^{n.s}	65.346 \pm 0.921***	63.149 \pm 3.452 ^{n.s}
	100	66.695 \pm 0.663**	72.145 \pm 2.015***	74.583 \pm 3.327**
Bak (%)				
Control	0	54.012 \pm 0.503	52.984 \pm 0.551	56.580 \pm 2.248
	25	60.376 \pm 2.993 ^{n.s}	67.714 \pm 3.704*	62.414 \pm 0.770 ^{n.s}
	50	61.511 \pm 0.673 ^{n.s}	68.797 \pm 2.524*	68.913 \pm 1.079**
	100	74.084 \pm 0.615***	76.962 \pm 1.670**	74.922 \pm 0.688***
Tannic Acid	25	60.376 \pm 2.993 ^{n.s}	67.714 \pm 3.704*	62.414 \pm 0.770 ^{n.s}
	50	61.511 \pm 0.673 ^{n.s}	68.797 \pm 2.524*	68.913 \pm 1.079**
	100	74.084 \pm 0.615***	76.962 \pm 1.670**	74.922 \pm 0.688***
Resveratrol	25	43.366 \pm 4.706 ^{n.s}	58.231 \pm 0.64 ^{n.s}	71.350 \pm 4.065*
	50	51.468 \pm 0.076 ^{n.s}	61.629 \pm 1.105 ^{n.s}	80.452 \pm 3.268**
	100	63.798 \pm 3.309 ^{n.s}	75.909 \pm 3.130***	87.917 \pm 1.667***

FADD and Bak protein were analyzed by immunohistochemical staining assay in MCF-7 cell line. Apoptotic index, percentage of FADD and Bak protein were calculated from the control. Mean \pm S.E values are shown for three experiments (n = 3) (*p < 0.05, **p < 0.01, ***p < 0.001).

death receptors to caspase-8 through its death domain to form the death inducing signaling complex (DISC) during apoptosis (Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). Therefore, in the present study, we investigated whether or not TA and RES increased the levels of FADD protein in MCF-7 cells. As shown in Figures 1C and D, the percentage of FADD protein increased, following TA and RES treatment. In particular, FADD protein (%) ratio was increased at the concentration of 100 μM TA after 24 and 48 h and 50 μM TA after 48 h (p < 0.001), 25 μM TA after 48 h and 100 μM TA after 72 h (p < 0.01), 50 μM TA after 24 h (p < 0.05) compared to control. Otherwise, FADD protein (%) ratio was increased at the concentration of 50 and 100 μM RES after 48 h (p < 0.001), 100 μM RES after 24 and

72 h (p < 0.01), 25 μM RES after 48 h (p < 0.05) compared to control (Table 1).

Mitochondria mediating apoptosis through the intrinsic pathway

TA- and RES treatment of MCF-7 cells results in an increase in proapoptotic protein bak

Bcl-2 family proteins are important regulators in the apoptosis signaling pathway. These proteins include proapoptotic protein Bak (Bjelaković et al., 2005; Mousavi et al., 2008; Xu and Shi, 2007). Here, we examined the effects of TA and RES on the levels of Bak protein in MCF-cells. Figures 1E and F show the percentage of Bak

protein after exposure to TA and RES. In particular, % Bak protein ratio was increased at the concentration of 100 μM TA after 24 and 72 h ($p < 0.001$), 100 μM TA after 48 h and 50 μM TA after 72 h ($p < 0.01$), 25 and 50 μM TA after 48 h ($p < 0.05$) compared to control. Further, Bak protein (%) ratio was increased at the concentration of 100 μM RES after 48 and 72 h ($p < 0.001$), 50 μM RES after 72 h ($p < 0.01$), 25 μM RES after 72 h ($p < 0.05$) compared to control (Table 1).

DISCUSSION

Recent studies have suggested that TA and RES have a cancer preventative activity and they can induce apoptosis (Aziz et al., 2003; Corre et al., 2005; Kim et al., 2004; Nepka et al., 1999; Sakagami et al., 1997; Yang et al., 2000). Although there are studies that investigated the effects of RES on MCF-7 cell line, there are few studies about tannins (Bawadi et al., 2005; Cosan et al., 2009; 2010; 2011; Kim et al., 2004; Lanzilli et al., 2006; Pozo-Guisado et al., 2002).

In the current study, we demonstrated that TA can induce apoptotic activity in MCF-7 cell line (Table 1). In the studies about tannins, 2, 6, 12 and 24 μM condensed tannin were administrated to normal fibroblast lung (HEL 299), colon (CaCo-2), breast (MCF-7, Hs578T) and prostate (DU 145) cell lines. After 24 h, normal cells were alive but the cancer cell death was increased (Bawadi et al., 2005). In another study performed on prostate cancer cell line (LNCaP), TA 5 and 10 $\mu\text{mol/L}$ increased apoptotic index significantly at 72nd h as compared to control (Romero et al., 2002). In another study performed on human Jurkat T cells, TA 50 and 100 $\mu\text{g/ml}$ increased apoptotic cell death in a dose dependent manner at 24th h (Nam et al., 2001). The effect of TA on apoptosis is different at different doses and hours at different cell lines.

Our study also shows that RES can induce apoptotic activity in MCF-7 cell line (Table 1). In a study on MCF-7 cells, RES 12.5 μM has not affected apoptotic index at 48th h, but RES 50 μM has increased apoptotic index (Kim et al., 2004). In another study, RES 10, 50, 100 and 200 μM were administrated on MCF-7 and MDA-MB-231 cell lines and apoptosis was increased at MCF-7 in a dose proportional manner. The most effective dose was 200 μM at 36th h (Pozo-Guisado et al., 2002).

In another study, RES 5, 10, 20 and 40 $\mu\text{g/ml}$ were administrated on MCF-7 cell lines and the effect on apoptosis was determined on 24th, 48th and 72nd h. The structural differences as chromatin concentration, nuclear fragmentation and collapse of the cell into membrane-bound apoptotic bodies were observed most in 10 and 20 $\mu\text{g/ml}$ RES treated cells. Consequently, apoptosis was increased in a dose proportional manner with the 10, 20 and 40 $\mu\text{g/ml}$ doses at 24th and 48th h. At 72nd h, it decreased to control levels as in the previous studies and

our study (Lanzilli et al., 2006). Investigating the studies performed, RES was observed to increase apoptosis in a dose and time dependent manner, but there are some differences at different doses and hours at different cell lines (Cao et al., 2005; Holian et al., 2002; Joe et al., 2002; Luzi et al., 2004). Our results show that TA and RES induced apoptosis in MCF-7 cell, in a time and dose independent manner in MCF-7 cell line (Table 1).

We also investigated the FADD proteins that are effective on extrinsic pathway and the Bak proteins that are effective on intrinsic pathway. In the study, TA-induced apoptosis in HL-60 cell line showed that expression of antiapoptotic Bcl-2 was dose-dependently decreased whereas the expression of proapoptotic Bax protein was increased. The expression of Fas and FasL was slightly increased (Chen et al., 2009). In a study on HepG2 liver cell line, epigallocatechin-3-gallate (EGCG), a tea polyphenol that is similar to TA increased Bak and Bax proteins at different doses and different hours in a dose dependent manner (Kuo and Lin, 2003).

There are a lot of studies on RES induced apoptotic pathways in human breast cancer. In a study that investigated the effects of RES, lower doses of RES ($\leq 4 \mu\text{M}$) increased cell proliferation at ER-positive human breast cancer cell lines for MCF-7 and higher doses of RES ($\geq 44 \mu\text{M}$) inhibited cell proliferation. The inhibition of cell proliferation was thought to be caused by sub G1 phase fraction, up-regulation of Bax and Bak proteins, down regulation of Bcl-XL protein and activation of caspase 3 and the induction of apoptosis (Nakagawa et al., 2001). RES 10 to 100 μM was reported to activate caspase at SW480 colon cancer cells. This activation was associated with the accumulation of Bak and proapoptotic proteins like Bax (Delmas et al., 2003).

In the studies performed, RES was proposed to increase the expression of Bax and Bak proteins responsible for intrinsic pathway (Kim et al., 2004; Nakagawa et al., 2001; Aziz et al., 2006; Fulda and Debatin, 2004; Shankar et al., 2007). In a study performed on colorectal cancer cell (CrCs), Bax and Bak proteins disappeared by clonal selection but the cells were reported to undergo apoptosis. Consequently, it was reported that RES may sensitize the cells to death receptor pathway mediated apoptosis induced by CD95, TNF- α and TRAIL, the ligands of death receptor (Fulda and Debatin, 2004; Shankar et al., 2007; Delmas et al., 2004). However, RES can function as a sensitizer for death receptor pathway-mediated apoptosis triggered by the death receptor ligands CD95, TNF- α and TRAIL (Fulda and Debatin, 2004; Shankar et al., 2007; Delmas et al., 2004).

In another study, it was reported that RES induced the recruitment and the redistribution of FADD, procaspase-8 and Fas in SW480 cells. The redistribution of Fas receptor on membrane by RES contributes to the induction of apoptosis on colon cancer cells (Delmas et al., 2003). Our results show that TA and RES increased

percentage of Bak and FADD in MCF-7 cell (Table 1).

Also in our study, no significant difference was observed between the percent ratios of Bak proteins that are effective on intrinsic pathway and the FADD proteins that are effective on extrinsic pathway. This result shows that the cells undergo apoptosis by two pathways in TA and RES-induced MCF-7 cells. Our results demonstrate that TA and RES are able to induce the apoptosis and apoptotic proteins Bak and FADD in MCF-7. TA and RES may be potentially used as a chemotherapeutic drug in the anti-breast carcinoma chemotherapy.

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