Kafkas Univ Vet Fak Derg
16 (3): 397-404, 2010
DOI:10.9775/kvfd.2009.836

RESEARCH ARTICLE

Inhibitory Effects of Propolis on Human Osteogenic Sarcoma Cell Proliferation Mediated by Caspase Patway

Feyzan OZDAL KURT * H. Seda VATANSEVER ** Kadriye SORKUN ***
S. Ismet DELILOĞLU GURHAN **** Elgin TURKOZ ** Omur GENCAY *** Bekir SALIH *****

* Celal Bayar University, Faculty of Science, Department of Biology, 45140 Manisa - TURKEY
** Celal Bayar University, Faculty of Medicine, Department of Histology and Embryology, 45020 Manisa - TURKEY
*** Hacettepe University, Faculty of Science, Department of Biology, 06800 Ankara - TURKEY
**** Ege University, Department of Bioengineering, 35100 Izmir - TURKEY
***** Hacettepe University, Faculty of Science, Department of Chemistry, 06800 Ankara - TURKEY

Makale Kodu (Article Code): KVFD-2009-836

Summary

A natural product Propolis, is a resinous material gathered by honeybees from the buds and bark of certain trees and plants. Propolis contains various chemical components of biological activities, including antimutagenic, antioxidant, antibacterial, antiviral and anticarsinogenic. Therefore, the aim of this study is to investigate the antiapoptotic effect of propolis extracts (PE) using caspase pathway in the human osteogenic sarcoma cell line SAOS-2 in culture. The extracts which produced in ecologic environment were taken from the Hacettepe University, Beytepe Campus area-Ankara were used. Seven different PE at 0.5, 0.25, 0.125 and 0.063 mg/ml were added to SAOS-2 cell line for two days incubation. For cell proliferation and cytotoxicty analyses MTT, for apoptotic cell death determination TUNEL method, for distribution of caspase 6, caspase 8 and caspase 9 indirect immunocytochemistry analyses were used. After MTT analyses, the most effect was observed PE 7 at the 0.125 mg/ml dilution. The number of TUNEL positive cells was more detectable at PE 4 and 5 at the 0.063 mg/ml, and PE 7 at the 0.125 mg/ml dilutions. The immunoreactivity of caspase 6 was stronger than caspase 8 and 9. Moreover, density of caspase 6 staining was much better especially in PE 7 at the 0.125 mg/ml dilution. In conclusion, the mechanisms of apoptosis induction by PE may appear via caspase pathway because of its anticanserogenic effect. PE may be usefull in the cancer treatment protocol.

Keywords: *Propolis, SAOS-2, Cell line, MTT, Apoptosis, Caspase*

Kaspaz Yolağı ile Yönlendirilen İnsan Osteojenik Sarcoma Hücre Çoğalmasına Propolisin Baskılayıcı Etkisi

Özet

Doğal bir ürün olarak propolis belli ağaç ve bitkilerin kabuki ve tomurcuklarından arılar aracılığı ile elde edilen bir reçine materyalidir. Propolis antimutajenik, antioksidan, antibakteriyel, antiviral etkileride içeren biyolojik aktiviteleri olan çeşitli kimyasal bileşikleri içerir. Bu nedenle bu çalışmada insan osteojenik sarcoma hücre dizini SAOS-2 kültüründe kaspaz yolağı kullanılarak propolis ekstraktlarının (PE) antiapoptotik etkisi araştırılması amaçlandı. Ankara'da Hacettepe Üniversitesi Beytepe Kampüs alanında ekolojik çevrede üretilen ekstraktlar kullanıldı. 0.5, 0.25, 0.125 ve 0.063 mg/ml konsantrasyonlarda 7 farklı PE SAOS-2 hücre dizinine eklenerek 2 gün boyunca inkübe edildi. Hücre çoğalması ve toksisitesi için MTT testi ile apoptotic hücre ölümünün belirlenmesi için TUNEL metodu ve kaspaz dağılımı için kaspaz 6, 8 ve 9 immunohistokimyası analizi kullanıldı. Hücreler MTT analizi sonrasında en büyük etki PE 7 için 0.125 mg/ml dilüsyonunda görüldü. TUNEL pozitif hücre sayısı en fazla olan PE 4 ve 5 için 0.125 mg/ml dilüsyonu oldu. Kaspaz 6 immünoreaktivitesi kaspaz 8 ve 9'dan daha güçlü idi. Daha da önemlisi kaspaz 6 boyaması PE 7 için 0.125 mg/ml dilüsyonunda çok daha iyi idi. Sonuç olarak, PE ile indüklenen apoptosis mekanizmasının antikansorejenik etkisinden dolayı kaspaz yolağı ile oluşabilir. PE kullanımı kanser tedavi protokolünde yararlı olabilir.

Anahtar sözcükler: *Propolis, SAOS-2, Hücre dizini, MTT, Apoptoz, Kaspaz*

İletişim (Correspondence)
☎ +90 533 415 8337
✉ feyzanozdalkurt@yahoo.com, feyzan.kurt@bayar.edu.tr

INTRODUCTION

Propolis or bee glue is a dark sticky resinous substance collected by bees from plant sources [1]. The chemical compounds of propolis are polyphenols (flavonoid aglycones, phenolic acids, and their esters, phenolic aldehydes, alcohols, and ketones), terpenoids, steroids, amino acids, and inorganic molecules [2]. Flavonoids are the main components that exert various biological activities and have been reported to inhibit the development of carcinogen induced experimental mammary cancer [3,4]. The anticancer activities of flavonoids may due to their apoptotic effect [4]. Many biological properties, including antibacterial, antifungal, antiviral, local anesthetic, anti-inflammatory, antioxidant, hepatoprotective, immuno-stimulating, and cytostatic activities have been also described for propolis [2]. The medical applications of propolis led to an increase interest to its chemical composition as well as its origin where they come from [5].

Apoptosis, the physiological mode of cell death, is related to the regulation of development and homeostasis in multicellular organism [6]. However, failure to undergo apoptosis is one of the mechanisms of oncogenesis and chemoresistance of transformed cells [7]. There are numerous stimuli that trigger apoptosis, including withdrawal of essential growth factors or hormones or engagement of various receptor/ligands including Fas/Fas ligand and tumour necrosis factor (TNF)/TNF receptor [8,9]. Furthermore, apoptosis is regulated by several different genes, which potentiate (p53; Bax; c-myc) or inhibit (Bcl-2; Bcl-xL; sentrin) programmed cell death [10,11]. In addition, the signaling pathway leading to apoptosis involves the sequential activation of cysteine proteases known as caspases. It has been well documented that caspase cascade involved in apoptosis includes both initiator caspases and effector caspases [12]. Caspases-2, -8, -10, -12 and caspases-3, -6, -7 were described as initiator and effector caspases, respectively.

Impairments in apoptotic signaling enable tumor cells to avoid apoptotic cell death and grow into tumor masses that are resistant to apoptosis [13]. Defects in regulation of apoptosis have been detected in both upstream and downstream of the apoptotic signal pathway in many types of human tumor cells [14,15]. Inducing apoptosis is one of the mechanisms for several therapeutic agents as shown in propolis by a number of studies [16,17]. Chen et al. has demonstrated that propolin A, B and C inhibited the proliferation of human melanoma cells through inducing a cytotoxic effect and triggering apoptosis [18,19] .

We investigated whether apoptotic cell death can triggered by the activation of caspase cascade in human osteocarcinoma cell lines (SAOS-2) after treatment with propolis, which is collected from different region in Turkey and one of the extracts from Brasil.

MATERIAL and METHODS

Preparation of Ethanol Extracts of Propolis (EEP)

Seven propolis samples named with number (PE 1-7) were collected from different region such as Tahtakopru, Sakarya, Saricicek yaylasi, Canakkale, Brasil, Van, Yalova, respectively. They were obtained from Hacettepe University, Faculty of Science, Department of Biology, Ankara, Turkey. They were hardened in a freezer and ground in a handy grinder. Then, 100 g of sample were dissolved in 300 ml ethanol (96%). This mixture was periodically stirred and incubated for four weeks at 30°C in a bottle closed tightly. After incubation, supernatant was filtered twice with Whatman No. 4 and No. 1 filter papers, respectively. The final filtered concentrated EEP solution was diluted in 1:10 ratio (w/v) with ethanol (96%). This diluted EEP solution was used for anti-microbial activity test. A portion of the same diluted solution was evaporated to dryness for GC-MS analysis. Then, about 5 mg of residue was mixed with 75 ml of dry pyridine and 50 ml bis(trimethylsilyl) trifluoroacetamide (BSTFA), heated at 80°C for 20 min and then the final supernatant was analyzed by GC-MS.

GC-MS Analysis

A GC 6890N from Hewlett-Packard (Palo Alto, CA, USA) coupled with mass detector (MS5973, Hewlett-Packard) was used for the analysis of the diluted EEP samples. Experimental conditions of GC-MS system were as follows: DB 5MS column (30 m×0.25 mm and 0.25 µm of film thickness) was used and flow rate of mobile phase (He) was set at 0.7 ml/min. In the gas chromatography part, temperature was kept for 1 min at 50°C and then increased to 150°C with 10°C/min heating ramp. After this period, temperature was kept at 150°C for 2 min. Finally, temperature was increased to 280 with 20°C/min heating ramp and then kept at 280°C for 30 min.

Cell Line and Cell Culture

The human osteocarcinoma cell line, SAOS-2, was purchased from the Animal Cell Culture Collection, HUKUK (Ankara, TURKEY). This adherent tumor cell line was maintained in RPMI 1640 (Sigma Chemical Co., St Louis, Missouri, USA) supplemented 10% heat inactivated fetal calf serum (FCS) (Sigma Chemical Co., St Louis, Missouri, USA), and 1% L-glutamine (Sigma Chemical

Co., St Louis Missouri, USA). Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2. When the cells were confluent, they were routinely subcultured using 0.25% trypsin-EDTA solution (Sigma Chemical Co., St Louis, Missouri, USA). Cells ($6x10^4$/ml/well) were cultured in twenty four-well plates for 24 h and then were incubated with propolis extracts of 0.5, 0.25, 0.125 and 0.063 mg/ml for 24 h. Untreated SAOS-2 cells were used as control group. All experiments were done in triplicate for each extract. After 24 h, the cells were either collected for viability and proliferation assays or fixed with 4% paraformaldehyde for immunohistochemical and TUNEL assays.

Cell Viability and Proliferation Assays

MTT assay, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product was used to estimate cell viability and proliferation. Cells were incubated in humidified 5% CO_2 (in air) at 37°C with 0.5 mg/ml of MTT in the last 4 h of the culture period tested. SAOS-2 cells without any treatment were used as a positive control for all extracts. The medium was then decanted and 1 ml dimethylsulphoxide (DMSO, Sigma Chemical Co., St Louis, Missouri, USA) was added to each well to ensure dissolving of the formazan salts. The absorbance was immediately determined at 570 nm in an UV- visible spectrophotometer multiplate reader (VersaMax, Molecular Device, USA). All experiments were done in triplicate for each extract [20] .

In situ Cell Death Detection Assay

Immunohistochemical detection of cells undergoing DNA fragmentation was carried out using a terminal deoxynucleotidyltransferase-biotin nick end-labelling (TUNEL) method with a commercial in situ apoptosis detection kit (Dead End Colorimetric TUNEL system, Promega G7130). Cells, which were cultured in twenty four-well plates, fixed with 4% paraformaldehyde in PBS at 4°C for 30 min. After washing with in PBS twice for 3 min, they were incubated with 20 mg/ml proteinase K for 10 min. The cells were rinsed with PBS. Endogenous peroxidase activity was inhibited by 30 min incubation in 3% hydrogen peroxide in methanol at room temperature, and they were then washed several times in PBS. After that the samples were then incubated with equilibration buffer for 5 min and TdT-enzyme for 60 min at 37°C in a humidified atmosphere. The reactions were stopped by adding 2xSCC solution for 15 min at room temperature. They were then washed with PBS three times for 5 min. and incubated with streptavidin-peroxidase for 45 min. Each step was separated by careful washing in PBS and then incubated with a solution containing diaminobenzidine (DAB, Sigma Chemical Co., St Louis Missouri, USA) 50 µl for each samples for 5 min to visualize immunolabeling. Samples were then mounted with mounting medium (AML060, Scytek, Logan, Utah, USA) and viewed using an IX71 invert- fluorescence-phase microscope (Olympus, Tokyo, Japan). As negative staining control for TUNEL, TdT was omitted during the tailing of reactions. All experiments were done in triplicate for each extract. Staining was examined independently by two of the authors, who had no information about the samples. TUNEL positive cells were counted randomly chosen fields per case and data was given as a percentage. The percentage of apoptotic cells stained brown was determined. Cells in areas with necrosis or poor morphology were not analyzed [21].

Immunohistochemical Assay

Cultures were also assessed immunocytochemically for binding of antibodies against caspase-6, caspase-8 and caspase-9. Samples were fixed with 4% paraformaldehyde in PBS at 4°C for 30 min. After washing with 0.1% Triton X-100 in PBS (PBST) twice for 3 min, they were permeabilized with PBST at 4°C for 15 min. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 30 min at room temperature. Cells were then washed with PBS, and incubated with primary antibodies: anti-caspase-6 (Biocarta, USA), anti-caspase-8 (Neomarkers, Fremont, CA) and anti-caspase-9 (Neomarkers, Fremont, CA) in 1:100 dilutions in PBS, for 2 h. The cells were then incubated with biotinylated IgG (supplied ready to use by Zymed) for 30 min, followed by three washes in PBS and then with streptavidin-peroxidase conjugate (supplied ready to use by Zymed) for 30 min (Histostain-Plus Bulk Kits; Zymed, San Francisco, CA) and washed with PBS three times. They were incubated with a solution containing diaminobenzidine (DAB, supplied ready to use by Zymed) 50 µl for each samples (Histostain-Plus Bulk Kits; Zymed, San Francisco, CA) for 5 min to visualize immunolabeling. The negative immunohistochemistry controls received the same treatment as described above, but those were incubated with rabbit IgG or mouse IgG instead of the primary antibody. Samples were then mounted with mounting medium (AML060, Scytek, Logan, Utah, USA) then viewed using an IX71 invert-fluorescence-phase microscope (Olympus, Tokyo, Japan). All experiments were done in triplicate for each extract. Immunohisto-chemistry were evaluated semiquantitatively using an additive immunoreactive score that is negative (-), weak (±), mild (+), moderate (++) and strong (+++) reflecting signal intensity [21].

Statistical Analysis

Comparable data groups were evaluated using non-

parametric Kruskal-Wallis test. A p value lower than 0.05 was considered significant [22].

RESULTS

Cell Morphology

SAOS-2 cells were epithelial-like cells *(Fig. 1)* and were subcultured every 3 days. After treatments with propolis extracts, the morphology of SAOS-2 cells were found different compaired to that of control group. Their morphological features for apoptosis such as shrinkage of cells, condensation of chromatin were seen after treatment with all propolis extracts. However, the number of cells in some treated group was also less because of cell death *(Fig. 2)*.

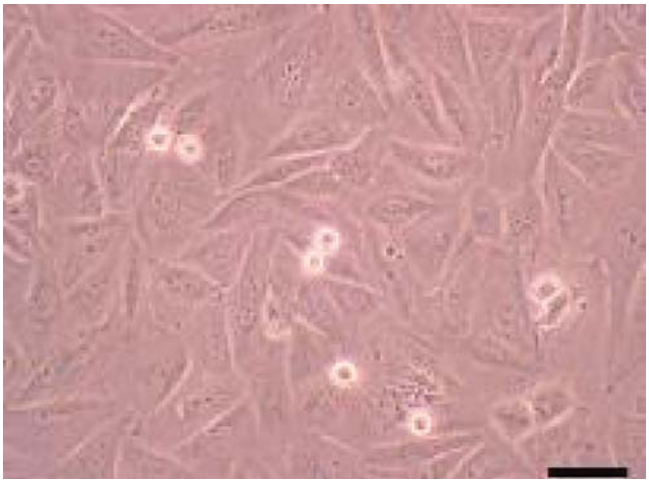

Fig 1. SAOS-2 cells after 24 h of culture period. They were growing as epithelial-like cells. Scale Bar: 25 µm

Şekil 1. 24 saatlik kültür periyodunda SAOS-2 hücreleri. Bu epitel benzeri hücrelerin çoğalması. Büyütme çizgisi 25 µm

Cell Proliferation

SAOS-2 cells were treated with PE at various concentrations for 24 h, and the cell viability was determined as described above by MTT assay. As shown in *Fig. 2*, all PE inhibited the growth of SAOS-2 cells in a dose-dependent manner. In addition, except PE 5 all PE in 0.5 mg/ml dilution were accelerated of proliferation of SAOS-2 cells. However this effect was gradually decreased in other dilutions *(Fig. 2)*. In contrast to that, the 0.125 mg/ml of PE 7 was more effective on inhibition of SAOS-2 cell proliferation when compared with other extracts and dilutions *(Fig. 2)*.

Effect of Propolis Extracts on Apoptosis Induction of SAOS-2 Cells

After treatment of SAOS-2 cells with seven types of propolis extracts for 24 h, the determination of apoptotic cells with TUNEL, the distributions of caspase-6, -8 and -9 with indirect immunohistochemistry were analyzed. The increased of numbers of TUNEL positive cells in all treated groups were significant when compared with control group *(Fig. 3)*. Propolis induced apoptosis in a dose-dependent manner and the effective dilutions of propolis extracts 4 and 5 at 0.0637 mg/ml, and 7 at 0.125 mg/ml were determined *(Table 1)*. According to the MTT assay, while proliferation was induced treated with 0.5 mg/ml dilution, cytotoxicity was observed in less dilutions of PE (except PE 5). After TUNEL assay, the number of apoptotic cells were increased in less dilution when compared with dense dilutions. This result were resembled with MTT assay results.

After immunohistochemical evaluation, the immunoreactivities of caspase-6, -8 and -9 were negative in SAOS-2 cells with treated 0.5 and 0.25 mg/ml dilutions from all type of extracts. While the immunoreactivities of caspase-6

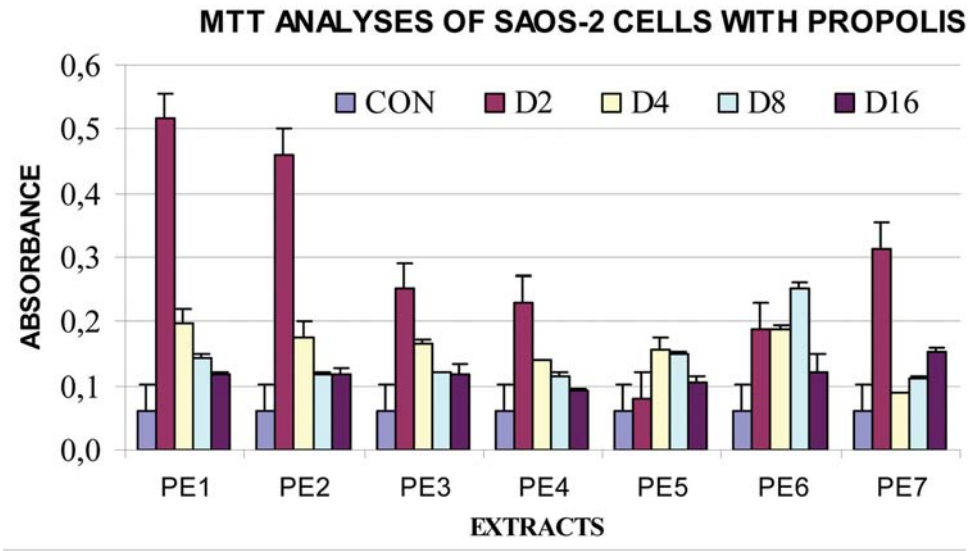

Fig 2. MTT analyses of SAOS-2 cells treated with various type and dilutions of PE

Şekil 2. PE'nın çeşitli dilüsyonları ve tipleri uygulanmış SAOS-2 hücrelerinin MTT analizleri

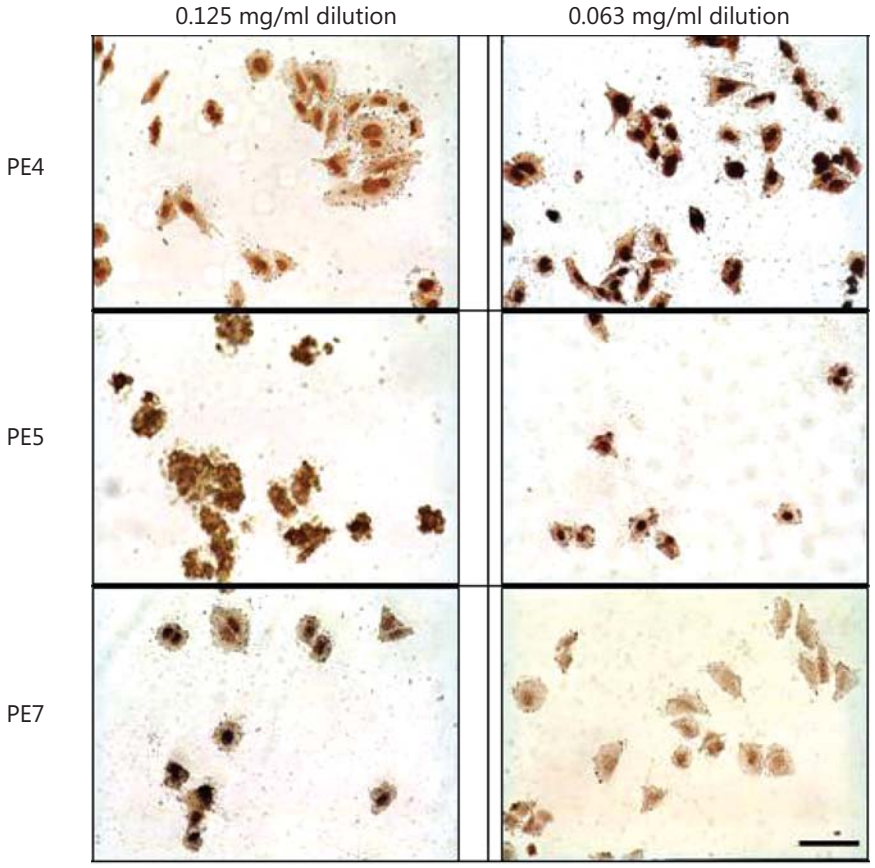

Fig 3. TUNEL staining of SAOS-2 cells after treated with different dilutions and types of PE in 0.125 mg/ml and 0.063 mg/ml dilutions. Scale Bar: 25 µm

Şekil 3. 0.125 mg/ml ve 0.063 mg/ml dilusyonlardaki PE'nın farklı dilusyon ve tipleri uygulanmış SAOS-2 hücrelerinin TUNEL boyaması. Büyütme çizgisi: 25 µm

Table 1. *The percentage of TUNEL positive SAOS-2 cells treated with various type and dilutions of propolis extracts (PE). The data were given as mean±SEM, * P<0.001*

Tablo 1. *Propolis ekstraktlarının çeşitli dilüsyonları ve tipleri uygulanmış SAOS-2 hücrelerinin TUNEL pozitifliği yüzdesi. Veriler ortalama±SEM ile gösterildi, * P<0.001*

mg/ml	PE 1 (%)	PE 2 (%)	PE 3 (%)	PE 4 (%)	PE 5 (%)	PE 6 (%)	PE 7 (%)
0.5	0	23±2.12	0	8.6±0.89	4.2±0.44	7.3±0.28	2.42±0.51
0.25	12±2.34	21±1.00	6.96±0.57	7.5±0.35	15.8±1.79	23±0.71	3.9±0.82
0.125	23±2.12	22.4±0.89	31±2.00	7.08±0.08	7.4±0.37	20±1.41	79.6±2.81*
0.063	20±1.41	19±1.73	8.8±1.79	82.4±2.51*	86.2±3.63*	26.06±0.69	27.58±1.52*

Table 2. *The staining intensity of anti-caspases-6, -8 and -9 in SAOS-2 cells treated with various types and PE dilutions*

Tablo 2. *PE'nın çeşitli dilüsyonları ve tipleri uygulanmış SAOS-2 hücrelerinde kaspaz-6, -8 ve 9'un boyama yoğunluğu*

SAOS-2	mg/ml	Caspase-6	Caspase-8	Caspase-9
PE 1	0.063	+/-	+	PE 7 +/-
	0.125	+/-	+/-	+/-
PE 2	0.063	+	+/-	+/-
	0.125	+	+/-	+/-
PE 3	0.063	+	+	-
	0.125	+/-	+/-	-
PE 4	0.063	++	+	-
	0.125	+	+	+
PE 5	0.063	++	+/-	-
	0.125	+	+/-	+
PE 6	0.063	+/-	+/-	-
	0.125	+	+/-	-
PE 7	0.063	++	+/-	+/-
	0.125	+	++	++

and -8 were detected in treated SAOS-2 cells with all type of extracts, the intensity of caspase-9 was less or negative (-), except at 0.125 mg/ml dilution of PE 7 *(Table 2)*. The moderate (++) expressions of caspase-6 were observed PE 4 and PE 5 at dilutions 0.0637 mg/ml, and PE 7 at 0.125 mg/ml dilution *(Fig. 4)*. In addition the moderate (++) immunoreactivities of caspase-8 and -9 were detected only in SAOS-2 cells treated with PE 7 at 0.125mg/ml dilution, these intensities were more detectable than other extracts and dilutions *(Table 2)*.

caspase pathway after treated with less dilutions. The proliferation of SAOS-2 cells was suprisingly induced after treated with 0.5 mg/ml dilutions of PE (except PE 5). However, cytotoxicity was increased in less dilutions of PE (except PE 5). After both TUNEL and immunohistochemical results, while the number of effected cells after treated with PE were less in dense dilution, the number of TUNEL positive cells and immunoreactivities of caspase-6, -8 and -9 were strongly detected after tretaed with less dilutions. This results suggested that

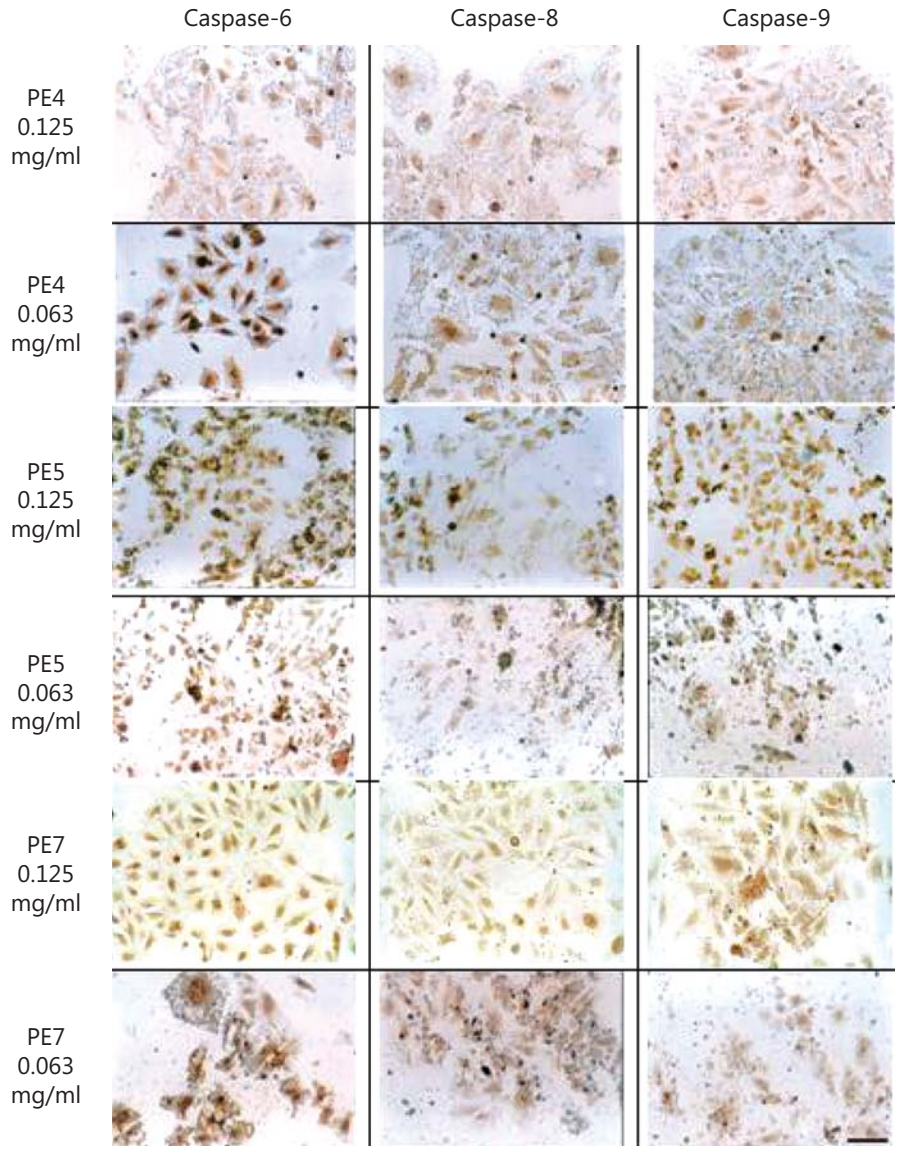

Fig 4. The distribution of caspase-6, caspase-8 and caspase-9 in PE 4, PE 5 and PE 7 with 0.125 mg/ml and 0.063 mg/ml dilutions of SAOS-2 cells. Scale Bar: 25 µm

Şekil 4. SAOS-2 hücrelerinin PE 4, PE 5 ve PE 7'nin 0.125 mg/ml and 0.063 mg/ml dilüsyon uygulamalarında caspase-6, caspase-8 ve caspase-9 dağılımı. Büyütme çizgisi: 25 µm

DISCUSSION

This research was performed to evaluate the effect of Propolis, which collected from different region of Turkey and Brasil, on the SAOS-2 osteosarcoma cell line via apoptotic pathway. Our data show that the propolis extracts were induced cell death and triggered of propolis may have both proliferation and cytotoxity on SAOS-2 cells, but these effects could depend on concentration of propolis extracts. In addition, the caspase activity was not triggered with dense dilution, therefore the proliferation of SAOS-2 cells were observed. But, the proliferation of SAOS-2 cells in control group were more or less similar with 0.5 mg/ml dilution of PE

5 treated cells. Therefore, different PE may have different effect on different cell type. In our results, PE 7 was more effective in both apoptosis and caspase activation.

The chemical composition and pharmacological activities of propolis may different according to the geographical and botanical origin [19]. In a recent study demonstrated that Taiwanese propolis, propolin A and propolin B, were inhibited five cancer cell lines growth in dose-dependent manner [4,19,23,24]. The mechanisms of the antitumor activity of propolis have been shown to be through the induction of cell cycle arrest, differentiation and initiation of apoptosis [18,25].

In the present study, the different propolis extracts were effected differently inhibition of growth and cell death. The PE 4, 5 and 7 in 0.063 mg/ml dilution were more effective than other types of PE on SAOS-2 cell death. The TUNEL positive cells were more detectable in PE 4 and 5 at 0.063 mg/ml dilutions, however these effect may originated from cytotoxicity. After immuno-histochemical evaluation, immunoreactivity of caspase-9 was weak or negative in all type of propolis extracts and dilutions. In addition, moderate immunoreactivity of caspase-6 was observed especially with 0.063 mg/ml dilutions of PE 4-5 and 0.125 mg/ml dilutions of PE 7 and treated groups. Therefore, the induction of apoptosis after propolis treatment could control programme cell death via extrinsic pathway using activation of caspase 8 and 6. In addition, moderate immunoreactivity of caspase-6, -8 and -9 were detected on SAOS-2 cells treated with PE 7 at 0.125 mg/ml dilution. Therefore, PE 7 may induced of cell death via both intrensec and extrensec pathways of apoptosis. This results supported that different type of PE could differ effect on cells after treated with different dilutions.

The caspase family plays an important role in both extrinsic and intrinsic pathway of apoptosis [26]. The caspase family includes 14 members, while the caspase-8 and -9 play a role in extrinsic and intrinsic pathway respectively, the caspase-6 express after activation of both apoptotic pathways with caspase-3. In our study suggested that the caspase pathway was activated after treatment with propolis extracts via caspase-8 expression and later on caspase-6 secretion. Apoptosis inducers are currently being used in cancer therapy [15]. In addition, different type of propolis extracts from different country was also induced caspase-dependent or independent apoptosis in different cancer cells [27-29]. Human tumor cells escape apoptotic cell that by avoiding the activation of upstream apoptotic signals and /or by upregulation of inhibitory factors in the apoptotic signal pathway [14,15,30,31]. Vatansever et al.[21] were demonstrated that different types of PE triggred the initiation of apoptosis on MCF-7 cells. While they were observed proliferation of MCF-7 cells after treated with dense dilutions of PE, induced of apoptosis in association with increased number of TUNEL positive cells were detected PE 5 and PE 6 extracts at 0.125 mg/ml dilutions [21]. In this study, PEs were triggered via caspase cascade increased expression of both caspase-8 and caspase-6 on SAOS-2 cells. In addition, on PE 4, 5 and 7 treated-SAOS-2 cells, expressions of both caspase-8 and caspase-6 were increased when compared with caspase-9 staining.

In conclusion, the mechanisms of apoptosis in propolis-treated tumor cell lines are still questionable. Propolis-induced apoptosis may be via the activation of caspase-8 and caspase-6, and the less activating of caspase-9, leading and causing of apoptosis on SAOS-2 cells. Propolis extracts were exhibited a dose-dependent inhibition of cellular proliferation and activation of apoptosis in the SAOS-2 cells. Additionally, extracts 4, 5 and especially 7 were induced more cytotoxic effect than other type of propolis extracts. Especially, PE 7 at 0.125 mg/ml dilution may suitable for treatment of SAOS-2 cells because of both induction of cell death and caspase activity. However, further in vivo studies are needed to establish the role of propolis extracts as a chemopreventive and/or therapeutic agent for cancer.

REFERENCES

1. Sorkun K, Suer B, Salih B: Determination of chemical composition of Turkish propolis. *Z Naturforsch,* 56, 666-668, 2001.

2. Kartal M, Yıldız S, Kaya S, Kurucu S, Topçu G: Antimicrobial activity of propolis samples from two different regions of Anatolia. *J Ethnopharmacol,* 86, 69-73, 2003.

3. Isla MI, Moreno Nieva MI, Sampietro AR, Vattuone MA: Antioxidant activity of Argentine propolis extracts. *J Ethnopharmacol,* 76, 165-170, 2001

4. Padmavathi R, Senthilnathan P, Sakthisekaran D: Therapeutic effect of propolis and paclitaxel on hepatic phase I and II enzymes and marker enzymes in dimethylbenz(a)anthracene-induced breast cancer in female rats. *Comp Biochem Physiol C Toxicol Pharmacol,* 143 (3): 349-354, 2006.

5. Bankova VS, Popov SS, Marekov NL: Isopentenyl cinnamates from poplar buds and propolis. *Phytochem,* 28, 871-873, 1989.

6. Schwartzman RA, Cidlowski JA: Apoptosis: The biochemistry and molecular biology of programmed cell death. *Endocrinol Rev,* 14, 133-151, 1993.

7. Green DR, Reed JC: Mitochondria and apoptosis. *Sci,* 281, 1308-1312, 1998.

8. Stellar H: Mechanisms and genes of cellular suicide. *Sci,* 267, 1445-1449, 1995.

9. Nagata S, Golstein P: The Fas death factor. *Sci,* 267, 1449-1456, 1995.

10. Okura T, Gang L, Kamitami T, Wada T, Okura I, Wei CF, Chang HM, Yeh ET: Protection against Fas/Apo-1 and tumor

necrosis factor-mediated cell death by a novel protein, sentrin. *J Immunol,* 157, 4277-4281, 1996.

11. Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB, Fesik SW: Structure of Bcl-xL-Bak peptide complex: Recognition between regulators of apoptosis. *Sci,* 275, 983-986, 1997.

12. Thornberry NA, Lazebnik Y: Caspases: Enemies within. *Sci,* 281, 1312-1316, 1998.

13. Wyllie AH, Bellamy CO, Bubb VJ, Clarke AR, Corbet S, Curtis L, Harrison DJ, Hooper ML, Toft N, Webb S, Bird CC: Apoptosis and carcinogenesis. *Br J Cancer, 80 Supp l,* 34-37, 1999.

14. Reed JC: Mechanisms of apoptosis avoidance in cancer. *Curr Opin Oncol,* 11, 68-75, 1999.

15. Igney FH, Krammer PH: Death and anti-death: Tumour resistance to apoptosis. *Nat Rev Cancer,* 2, 277-288, 2002.

16. Chen JH, Shao Y, Huang MT, Chin CK, Ho CT: Inhibitory effect of caffeic acid phenethyl ester on human leukemia HL-60 cells. *Cancer Lett,* 108, 211-214, 1996.

17. Aso K, Kanno S, Tadano T, Satoh S, Ishikawa M: Inhibitory effect of propolis on the growth of human leukemia U937. *Biol Pharm Bull,* 27, 727-730, 2004.

18. Chen CN, Wu CL, Lin JK: Propolin C from propolis induces apoptosis through activating caspases, Bid and cytochrome c release in human melanoma cells. *Biochem Pharmacol,* 67, 53-66, 2004.

19. Chen CN, Wu CL, Lin JK: Apoptosis of human melanoma cells induced by the novel compounds propolin A and propolin B from Taiwenese propolis. *Cancer Lett,* 245, 218-231, 2007.

20. Deliloglu-Gürhan SI, Vatansever HS, Özdal-Kurt F, Tuğlu I: Characterization analysis of osteoblast derived from bone marrow in modified culture system. *Acta Histochem,* 108, 49-57, 2006.

21. Vatansever HS, Sorkun K, Deliloglu-Gürhan SI, Özdal-Kurt F, Turkoz E, Gencay O, Salih B: Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines. *Acta Histochem,* 3, (Epub ahead of print), 2009.

22. Ergün G, Aktaş S: ANOVA modellerinde kareler toplamı yöntemlerinin karşılaştırılması. *Kafkas Univ Vet Fak Derg,* 15 (3): 481-484, 2009.

23. Marucci MC: Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie,* 26, 83-99, 1995.

24. Khalil ML: Biological activity of bee propolis in health and disease. *Asian Pac J Cancer Prev,* 7 (1): 22-31, 2006.

25. Matsukawa Y, Marui N, Sakai T, Satomi Y, Yoshida M, Matsumoto K: Genistein arrests cell cycle progression at G2-M. *Cancer Res,* 53, 1328-1331, 1993.

26. Reed JC: Mechanisms of apoptosis. *Am J Pathol,* 157, 1415-1430, 2000.

27. Huang WJ, Huang CH, Wu CL, Lin JK, Chen YW, Lin CL, Chuang SE, Huang CY, Chen CN: Propolin G, a prenylflavanone, isolated from Taiwanese propolis, induces caspase-dependent apoptosis in brain cancer cells. *J Agric Food Chem,* 55 (18): 7366-7376, 2007.

28. Li H, Kapur A, Yang JX, Srivastava S, McLeod DG, Paredes-Guzman JF, Daugsch A, Park YK, Rhim JS: Anti-proliferation of human prostate cancer cells by ethanolic extracts of Brazilian propolis and its botanical origin. *Int J Oncol,* 31 (3): 601-606, 2007.

29. Weng MS, Liao CH, Chen CN, Wu CL, Lin JK: Propolin H from Taiwanese propolis induces G1 arrest in human lung carcinoma cells. *J Agric Food Chem,* 55 (13): 5289-5298, 2007.

30. Özcan A, Atakişi E: Apoptosisin biyokimyasal önemi. *Kafkas Univ Vet Fak Derg,* 9 (1): 95-100, 2003.

31. Balcan E, Arslan Ö, Gümüş A, Şahin E: Tunikamisinin embriyonik ve yenidoğan fare dalak dokularına etkisi. *Kafkas Univ Vet Fak Derg,* 15 (5): 651-660, 2009.