Propolis from Turkey induces apoptosis through activating caspases in human breast carcinoma cell lines

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MCF-7 cell;
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Apoptosis;
Caspase

Summary
Propolis is a sticky substance that is collected from plants by honeybees that has anti-mutagenic and anti-carcinogenic properties with biological and therapeutic effects. The target of this study was to investigate the anti-apoptotic effect of propolis extracts (PE) on the caspase pathway in the human breast cell line MCF-7 in culture. Seven different propolis extracts, numbered PE 1–7, produced in their natural ecological environment, were collected from the Hacettepe University Beytepe Campus area in Ankara, Turkey. Individual extracts at 0.5, 0.25, 0.125 and 0.063 mg/ml were incubated with MCF-7 cells during 2 days culture. Cell growth and cytotoxicity were measured colorimetrically by MTT assay. Apoptotic cell death was determined by the TUNEL method (terminal deoxynucleotidyltransferase-biotin nick end-labelling) and caspase activity was investigated by immunocytochemistry using antibodies directed against caspase 6, caspase 8 and caspase 9. The results showed that the PE 5 and 6 extracts at 0.125 mg/ml dilution induced apoptosis in association with increased number of TUNEL positive cells. MTT results showed that cultures exposed to the same extracts and at the same dilution experienced better cell growth compared to those cultures exposed to the other extracts. Immunopositivity for all caspases was detected after treatment with all the extracts and at all dilutions, with stronger immunoreactivity for caspase 6 than caspases 8 and 9. Caspase 6 labelling was especially strong in PE 5 and PE 6. We conclude that propolis

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Introduction

Propolis, also known as ‘bee glue’, is a dark, sticky resinous substance, which is collected from leaf buds, twigs, trees and trunk wounds by bees such as *Castanea sativa*, *Populus* spp. and *Aesculus hippocastanum*. The bees attach the propolis with their hindlegs and carry it back to their colony, where it is combined with beeswax and used by worker hive bees to seal and sterilize the colony nest (Sorkun et al., 2001). Propolis, in general, contains a variety of chemical compounds including polyphenols (flavonoid aglycones, phenolic acids, and their esters, phenolic aldehydes, alcohols and ketones), terpenoids, steroids, amino acids and various inorganic compounds (Kartal et al., 2003) and has been used in folk medicine as a health aid since ancient times (Isla et al., 2001). Many biological properties, including antibacterial, antifungal, antiviral, local anaesthetic, anti-inflammatory, antioxidant, hepatoprotective, immunostimulating and cytostatic activities have been ascribed to propolis (Kujumgiev et al., 1999; Drago et al., 2000; Hegazi et al., 2000; Sforcin et al., 2000, Hegazi and El Hady, 2001; Isla et al., 2001; Kartal et al., 2003). Grange and Darvey (1990) reported that propolis showed antibacterial activity to Gram-positive bacteria, besides limited activity against Gram-negative bacteria. These findings were verified by Dobrowolski et al. (1991), who observed its fungicidal activity, mainly against dermatomycoses. Fungistatic and fungicidal effects of propolis, especially the susceptibility of yeasts to this product, were also observed (Dobrowolski et al., 1991; Kujumgiev et al., 1999; Ota et al., 2001). Propolis is marketed in different forms such as tablets, capsules, toothpaste, mouthwash preparations, face creams, ointments, lotions and solutions (Kartal et al., 2003). The medical applications of propolis led to an increased interest in its chemical composition as well as its origin (Bankova et al., 1989).

Apoptosis, programmed cell death, plays a critical role in the cyclic changes and maintenance of homeostasis in multicellular organisms (Schwartzman and Cidlowski, 1993). However, failure to undergo apoptosis is one of the mechanisms associated with oncogenesis and chemoresistance of transformed cells (Green and Reed, 1998). Apoptosis is characterized by DNA fragmentation and chromatin condensation and differs from necrosis, which is characterized by ruptured cell membranes and swollen nuclei. 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 tumor necrosis factor (TNF)/TNF receptor (Stellar, 1995; Nagata and Golstein, 1995). Furthermore, apoptosis is regulated by several additional genes, which potentiate (p53; Bax; c-myc) or inhibit (Bcl-2; Bcl-xL; sentrin) programmed cell death (Okura et al., 1996, Sattler et al., 1997). In addition, the signaling pathway leading to apoptosis involves the sequential activation of cysteine proteases known as caspases. It has been well documented that the caspase cascade involved in apoptosis includes both initiator caspases and effector caspases (Thornberry and Lazebnik, 1998). Caspases-2, -8, -10, -12 and caspases-3, -6, -7 have been described as initiator and effector caspases, respectively. Impairment of apoptotic signaling enables tumor cells to avoid apoptotic cell death and grow into tumor masses that are resistant to apoptosis (Wyllie et al., 1999). Defects in regulation of apoptosis have been detected both upstream and downstream of the apoptotic signal pathway in many types of human tumor cells (Reed, 1999; Igney and Krammer, 2002). Apoptosis is also an important phenomenon in chemotherapy-induced killing of tumor cells. Several recent reports have indicated that many anti-cancer drugs or cancer chemoprevention agents act though the induction of apoptosis to prevent tumor promotion and progression (Reed, 2002). In addition, many natural products used in cancer chemotherapy, including taxol (Bhalla et al., 1993), adriamycin (Friesen et al., 1996), VP16 and camptothecin (Kaufmann, 1989) have apoptosis-inducing activity. Inducing apoptosis is one of the mechanisms proposed for the therapeutic effects of propolis (Chen et al., 1996; Aso et al., 2004). Recent studies have demonstrated that propolin A, B and C inhibited the proliferation of human melanoma cells by inducing a cytotoxic effect and triggering apoptosis (Chen et al., 2004, 2007).
In the present study, we investigated the anti-tumor activity of naturally occurring propolis extracts (PE) and the association with apoptotic pathways via the caspase cascade when applied to human breast carcinoma cell lines.

Materials and methods

Preparation of ethanol extracts of propolis (EEP)

Seven propolis extracts were harvested from different places in Sakarya, Kemaliye, Çanakkale, Van, Yalova and Hacettepe University of Turkey (two extracts) in 2004. The propolis samples, numbered PE 1–7, were obtained from the Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey. They were hardened in a freezer then ground in a hand grinder. One hundred grams of each sample were dissolved in 300 ml of 96% ethanol. This mixture was periodically stirred and incubated for 4 weeks at 30 °C in a tightly closed bottle. After incubation, the supernatant was filtered twice with Whatman nos. 4 and 1 filter papers, respectively. The final filtered concentrated solution (concentrated EEP) was diluted in 1:10 ratio (w/v) with 96% ethanol to protect it from microbial attack. A portion of the same diluted solution was evaporated to dryness for gas chromatography–mass spectrometry (GC–MS) analysis. Five milligrams of residue was mixed with 75 μl of dry pyridine and 50 μl bis (trimethylsilyl) trifluoroacetamide (BSTFA), heated at 80 °C for 20 min and the final supernatant was analyzed by GC–MS.

GC–MS analysis

A gas chromatograph (Hewlett-Packard model 6890N, Palo Alto, CA, USA) coupled with a mass detector (MS5973, Hewlett-Packard) was used for the analysis of the diluted EEP samples. Experimental conditions of the GC–MS system were as follows: a 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 a 10 °C/min heating ramp. After this period, the temperature was kept at 150 °C for 2 min. Finally, the temperature was increased to 280 °C with a 20 °C/ min heating ramp and then kept at 280 °C for 30 min.

Cell line and cell culture

MCF-7, a human breast cancer cell line, was purchased from the Animal Cell Culture Collection, HUKUK (Ankara, Turkey). This adherent tumor cell line was maintained in RPMI 1640 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (FCS) (Sigma-Aldrich) and 1% L-glutamine (Sigma-Aldrich). Cells were cultured in a humidified atmosphere at 37 °C in 5% CO2. When the cells were confluent, they were routinely sub-cultured using 0.25% trypsin-EDTA solution (Sigma-Aldrich). Cells (6 × 103)/ml/well were cultured in 24-well plates and then were cultured in the presence of propolis extracts of 0.5, 0.25, 0.125 and 0.063 mg/ml for 24 h. Untreated MCF-7 cells were used as a control. After 24 h, the cells were either collected for viability and growth assays or fixed with 4% paraformaldehyde for immunocytochemical and TUNEL assays.

Cell viability and growth 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 growth. Cells were incubated in humidified 5% CO2 (in air) at 37 °C with 0.5 mg/ml of MTT in the last 4 h of the culture period tested. The medium was then decanted and 1 ml dimethylsulphoxide (DMSO, Sigma-Aldrich) 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, Sunnyvale, CA, USA). All experiments were performed in triplicate for each extract.

TUNEL assay for apoptosis

Immunocytochemical 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, Madison, WI, USA), according to kit instructions. Cells, which were cultured in 24-well plates, were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) at 4 °C for 30 min. After washing with 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 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 2 × SCC 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. They were then incubated with a solution containing 50 μl diaminobenzidine (DAB, Sigma-Aldrich) for each sample for 5 min to visualize immunolabeling. Samples were then mounted with mounting medium (AML060, Scytek, Logan, UT, USA) and viewed using an IX71 inverted-fluorescence-phase microscope (Olympus, Tokyo, Japan). As a negative control for TUNEL, TdT was omitted during the tailing of reactions. Staining was examined independently by two of the authors, who had no information about the samples. TUNEL-positive cells were counted in randomly chosen fields per case and data were given as percentages of total cells present. Cells in areas with necrosis or poor morphology were not analyzed.

**Immunocytochemistry to detect caspases**

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 (A4975, AppliChem, Darmstadt, Germany) 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% H2O2 for 30 min at room temperature. Cells were then washed with PBS, and incubated with primary antibodies: anti-caspase 6 (rabbit polyclonal, 12-01-16363, Biocarta, San Diego, CA, USA, dilution 1:100), anti-caspase 8 (rabbit polyclonal, RB-1200, Neomarkers, Fremont, CA, USA, dilution 1:100) and anti-caspase 9 (rabbit polyclonal, RB-1205, Neomarkers, Fremont, CA, USA, dilution 1:100) all for 2 h. The cells were then incubated with biotinylated IgG (both anti-mouse and anti-rabbit supplied ready to use by Zymed, San Francisco, CA, USA) 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) and washed with PBS three times. They were incubated with a solution containing 1:9 concentration of diaminobenzidine (supplied ready to use by Zymed) with 0.3% hydrogen peroxide (Histostain-Plus Bulk Kits; Zymed), 50 μl for each sample, for 5 min to visualize immunolabeling. Samples were then mounted with mounting medium (AML060, Scytek, Logan, UT, USA) and viewed using an IX71 inverted-fluorescence-phase microscope (Olympus, Tokyo, Japan). The negative controls received the same treatment as described above, but those were incubated with rabbit IgG in place of the primary antisera. Labelling in all negative control cases was negative. Immunolabeling was evaluated semi-quantitatively using an additive immunoreactive score reflecting signal intensity, as negative (−), mild (+), moderate (++), and strong (+++).

**Statistical analysis**

Comparable data groups were evaluated using ANOVA; p < 0.05 was considered significant. SPSS 10 software was used for analyses.

**Results**

**Cell morphology**

MCF-7 cells are epithelial-like cells that grow with typical epithelial colony morphology after 24 h in culture (Figure 1A). They were sub-cultured every 3 days. After treatment with propolis extracts, the shapes of MCF-7 cells were similar to those of the control group; however, the number of colonies was fewer because of increased cell death. In addition, the boundaries of cells were unclear and not visible and the chromatin condensation was also detected in the nucleus of the cells (Figure 1B). This altered morphology was seen in MCF-7 cells treated with all propolis extracts (Figure 1B).

**Cell viability and cytotoxicity**

MCF-7 cells were treated with propolis extracts at various concentrations for 48 h, and the cell viability was determined as described above by MTT assay. All propolis extracts at all concentrations inhibited the growth of MCF-7 cells in a dose- and time-dependent manner (Figure 2). Our results showed that PE 5 and PE 6 at 0.125 mg/ml were more effective in inhibiting MCF-7 cell growth when compared with other extracts and dilutions (Figure 2).

**Effect of propolis extracts on apoptosis induction of MCF-7 cells**

To determine the occurrence of apoptosis in MCF-7 cells treated with the seven types of propolis
Figure 1. MCF cells imaged under the inverted microscope: (A) MCF-7 cells after 24 h of culture. They consist of epithelial-like cells growing with typical epithelial colony morphology and (B) MCF-7 cells after treatment with different types of PE in 0.25, 0.125 and 0.063 mg/ml dilutions. Scale bars = 25 μm.
extracts, the cells were stained with TUNEL. After treatment with different types and various dilutions of propolis extracts for 48 h, the numbers of TUNEL positive cells in all treated groups (Figure 3B) were significantly greater when compared with the control group (Figure 3A). However, in MCF-7 cells treated with propolis extracts PE 5 and PE 6, the number of TUNEL positive cells was greater than in the other propolis extracts (PE 1–4, PE 7) (Table 1). The propolis induced apoptosis in a dose-dependent manner and the most effective dilutions of propolis extracts PE 5 and PE 6 were 0.125 mg/ml.

Caspase immunocytochemistry

Immunocytochemistry revealed positive immunoreactivities of caspase-6, -8 and -9 in MCF-7 cells treated with different types (PE 1–7) and various dilutions of propolis extracts (Table 2). Caspase immunoreactivity was not detected after treatment of the cells with propolis extracts at 0.5 mg/ml dilution. The immunolabelling of caspase-6 was mild (+) and moderate (+++) in treated MCF-7 cells treated with propolis extracts PE 1–4 and PE 7. This immunoreactivity was significantly increased in PE 5- and PE 6-treated MCF-7 cells (Table 2). Moderate (+++) and strong (++++) immunoreactivity of caspase-6 was detected, especially with 0.125 and 0.063 mg/ml dilutions of PE 5 and PE 6 (Figure 4). The immunoreactivity of caspase-8 was moderate (+++) and strong (++++) in MCF-7 cells treated with PE 1–6 and various dilutions, apart from propolis extract 7 (Table 2). Mild (+) and moderate (+++) labelling of caspase-8 was observed in MCF-7 cells treated with propolis extract 7 (Table 2). The labelling for caspase-9 was mild (+) and moderate (+++) in MCF-7 cells treated with propolis extracts PE 1–4 and PE 7. Similar to caspase-6 immunoreactivity, caspase-9 immunoreactivity was significantly increased in propolis extracts PE 5- and PE 6-treated MCF-7 cells (Table 2). Moderate (+++) and strong (++++) immunoreactivity of caspase-9, similar to caspase-6 immunoreactivity, was detected especially after treatment with 0.125 and 0.063 mg/ml dilutions of PE 5 and PE 6 (Figure 4). Caspase-8 immunoreactivities in the PE 5- and PE 6-treated MCF-7 cells were more than in the other groups. In addition, caspase-6 and caspase-9 immunoreactivities in all groups were similar, but these immunoreactivities were less than that of caspase-8. The control cells, which received no propolis treatment, were caspase immunonegative (Figure 4).

Discussion

Propolis has been used in folk medicine for the treatment of various ailments and has been demonstrated to have a broad spectrum of activities (Kartal et al., 2003; Grange and Darvey, 1990; Dobrowolski et al., 1991). However, its chemical composition and pharmacological activities vary according to the geographical and botanical origin. Chen et al. (2004, 2007) isolated two cytotoxic compounds from Taiwanese propolis, propolin A and propolin B, and have shown that they caused dose-dependent changes in five cancer cell lines at low dosages. Many reports have indicated that different types of propolis extracts significantly inhibit cell growth and reduce the differentiation or proliferation of tumor cells (Matsukawa et al., 1993; Marucci, 1995; Khalil, 2006; Padmavathi et al., 2006). However, the effect of propolis on cell death or growth is still questionable. Cytotoxic effects on tumor cells or interfering tumor suppressor proteins such as p53, p38 and MAPK are some of the suggested mechanisms to explain the anti-proliferative effect of propolis (Kaufmann, 1989). One of the mechanisms of the anti-tumor activity of propolis has been shown to be through the induction of apoptosis (Matsukawa et al., 1993). Chen et al. (2004) also demonstrated that while propolin C inhibited the proliferation of human melanoma cells through inducing a cytotoxic effect and triggering apoptosis, propolin A and propolin B efficiently induced apoptosis in the G2/M phase of the cell cycle. The other mechanism of propolis to induce apoptosis induction is its capability to inhibit the expression of telomerase by decreasing hTERT levels (Gunduz et al., 2005).

In the present study, the effects of differing geographical and botanical origin of propolis extracts on the growth, cytotoxicity and apoptosis of MCF-7 cells were investigated. The data demonstrated that propolis treatment was associated with a strong inhibition of growth and cell death via
Figure 3. Evaluation of TUNEL staining: (A) TUNEL staining of control untreated MCF-7 cells and (B) TUNEL staining of MCF-7 cells after treatment with different types of PE in 0.25, 0.125 and 0.063 mg/ml dilutions. Scale Bars = 25 μm.
apoptosis in a dose-dependent manner. The cells were treated with various types of propolis extract, and it was observed that all of propolis extract types had effects on both cell growth and apoptosis as demonstrated by the MTT and TUNEL assays. The propolis extracts PE 5 and PE 6 were more effective than other extract types on MCF-7 cell growth and also cell death. In addition, when different dilutions of extracts were used during treatment of cells, 0.125 and 0.063 mg/ml dilutions significantly increased the cell death and immunoreactivity of caspase-6, -8 and -9. The most effective propolis extracts and dilutions appeared to be PE 5 and PE 6 in 0.125 and 0.063 mg/ml concentrations. Compared to the other caspases investigated here, immunoreactivity of caspase-8 was greatest after treatment with all types of propolis extracts and dilutions. Especially strong immunoreactivity of caspase-8 was observed with 0.125 and 0.063 mg/ml dilutions of PE 5- and PE 6-treated groups. In addition, caspase-6 and caspase-9 immunolabelling were also detected on MCF-7 cells treated with propolis extracts. However, the labelling intensities of caspase-6 and -9 were lower than that of caspase-8. Strong caspase-8 and caspase-9 labelling were observed with 0.125 and 0.063 mg/ml dilutions of PE 5 and PE 6-treated MCF-7 cells. It is likely that more apoptotic cells were detected with lower concentration of propolis extracts because when the cells were treated with the higher concentration of propolis extracts, they die via the necrotic pathway before apoptotic pathway activation. The dosage of propolis extracts may therefore be important when planning optimum treatment.

Apoptosis inducers are currently being used in cancer therapy (Nagata and Golstein, 1995). In recent studies, attempts have been made to induce apoptosis directly by triggering core components of the cell death machinery, such as, Bcl-2 family protein, intracellular anti-apoptotic proteins (IAPs), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and caspases, and modulating apoptosis indirectly by targeting protein kinases, phosphatases, transcription factors, cell-surface receptors, proteasomes (Reed, 2002; Jäättelä, 2002). In addition, different types of propolis extracts from different countries also induced caspase-dependent or independent apoptosis in different cancer cells (Huang et al., 2007; Li et al., 2007; Weng et al., 2007). Examination of

Table 1. The percentage of TUNEL positive MCF-7 cells treated with various type and dilutions of propolis extracts (PE).

<table>
<thead>
<tr>
<th>Extracts</th>
<th>mg/ml</th>
<th>PE 1 (%)</th>
<th>PE 2 (%)</th>
<th>PE 3 (%)</th>
<th>PE 4 (%)</th>
<th>PE 5 (%)</th>
<th>PE 6 (%)</th>
<th>PE 7 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE 1</td>
<td>0.5</td>
<td>17.97 ± 7.71</td>
<td>5.75 ± 1.82</td>
<td>12.01 ± 2.19</td>
<td>5.11 ± 0.60</td>
<td>8.89 ± 2.94</td>
<td>8.47 ± 2.67</td>
<td>14.77 ± 5.84</td>
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<tr>
<td></td>
<td>0.25</td>
<td>12.44 ± 2.99</td>
<td>18.97 ± 6.32</td>
<td>8.80 ± 2.11</td>
<td>5.85 ± 2.10</td>
<td>13.06 ± 6.13</td>
<td>7.30 ± 2.11</td>
<td>4.70 ± 4.11</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>51.41 ± 0.12</td>
<td>26.38 ± 5.53</td>
<td>17.05 ± 7.47</td>
<td>63.30 ± 21.88</td>
<td>89.50 ± 1.26</td>
<td>98.00 ± 0.82</td>
<td>28.86 ± 7.85</td>
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<tr>
<td></td>
<td>0.063</td>
<td>100 ± 0.00</td>
<td>43.89 ± 11.92</td>
<td>28.18 ± 9.21</td>
<td>96.60 ± 2.27</td>
<td>98.75 ± 0.95</td>
<td>97.20 ± 1.49</td>
<td>63.17 ± 10.19</td>
</tr>
</tbody>
</table>

TUNEL positive cells were calculated 6.58 ± 0.43 in control group. This data was significant when compared groups PE 5 and PE 6 (p = 0.001). Values are mean ± SEM.

Table 2. The intensity of anti-caspases-6, -8 and -9 immunolabelling in MCF-7 cells treated with various types and propolis extracts (PE) dilutions.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>mg/ml</th>
<th>Caspase-6</th>
<th>Caspase-8</th>
<th>Caspase-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE 1</td>
<td>0.063</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PE 2</td>
<td>0.063</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td></td>
<td>0.25</td>
<td>–</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PE 3</td>
<td>0.063</td>
<td>+</td>
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<tr>
<td></td>
<td>0.125</td>
<td>+</td>
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<td>0.25</td>
<td>++</td>
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<tr>
<td></td>
<td>0.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PE 4</td>
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<td></td>
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<td></td>
<td>0.5</td>
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<tr>
<td>PE 5</td>
<td>0.063</td>
<td>++</td>
<td>++</td>
<td>+++</td>
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<td>0.125</td>
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<td></td>
<td>0.5</td>
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<td>PE 6</td>
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<td>0.125</td>
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<tr>
<td></td>
<td>0.5</td>
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<tr>
<td>PE 7</td>
<td>0.063</td>
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<td>++</td>
<td>+</td>
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the levels and activities of apoptotic effectors, inhibitors and regulators in human cancer cells and tissues has demonstrated that deregulation of apoptotic signal pathways is present in most human cancer cells. Human tumor cells escape apoptosis by avoiding the activation of upstream apoptotic signals and/or by up-regulation of inhibitory factors in the apoptotic signal pathway (Reed, 1999; Igney and Krammer, 2002). Activation of apoptotic signaling is achieved by either an extrinsic or an intrinsic pathway (Reed, 2000). Recently, extracts of propolis were shown to trigger various apoptotic pathway in different types of cancer cells (Chen et al., 2008; Izuta et al., 2008; Jin et al., 2008).

In this study the data obtained from immunocytochemistry supported the view that initiation of apoptosis was triggered via a caspase cascade with increased immunodetection of caspase-8 after propolis treatment of MCF-7 cells. Therefore, it is hypothesized that the apoptotic effects of propolis in MCF-7 cells are mediated by its ability to up-regulate the extrinsic pathway via caspase-8 signaling. In addition, in PE 5- and PE 6-treated MCF-7 cells, immunolabelling of both caspase-8 and caspase-9 was strongly increased, and the immunolabelling of caspase-6 was also increased. The present data demonstrated that apoptotic pathway induced both extrinsic and intrinsic pathways with increased levels of caspase-8 and caspase-9, respectively, especially after treatment with 0.125 and 0.063 mg/ml concentrations of PE 5 and PE 6.

In conclusion, it has still not been clearly demonstrated which mechanisms are responsible for apoptosis in propolis-treated tumor cell lines. Propolis-induced apoptosis may be via the activation of caspase-8 and caspase-9, and the activation of caspase-6, leading to DNA fragmentation, and

![Image: The distribution of caspase-6, caspase-8 and caspase-9 immunolabelling in control and treated cell cultures with either PE 5 or PE 6 in 0.125 and 0.063 mg/ml dilutions of MCF-7 cells. Scale Bar = 25 μm.]
finally apoptosis. Propolis extracts exhibited a dose-dependent inhibition of cellular growth and activation of apoptosis in the MCF-7 cell line. Moreover, extracts PE 5 and PE 6 induced greater cytotoxic effects than the other types of propolis extract at the same concentrations. Further in vivo studies are needed to establish the role of propolis extracts as chemopreventive and/or therapeutic agents for cancer.

References


