Effects of Turkish propolis extract on secretion of polymorphonuclear elastase following respiratory burst

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Abstract: Propolis is a natural, resinous, hive product with various bioactive properties. Polymorphonuclear leukocytes (PMNLs) use both oxygen-dependent (respiratory burst) and oxygen-independent mechanisms (releasing proteolytic enzymes such as elastase) to destroy infectious agents. The in vitro effects of dimethyl sulfoxide (DMSO) extract of Turkish propolis on the secretion of PMN elastase from PMNLs following respiratory burst were studied. Phorbol myristate acetate was used to stimulate respiratory burst. The Flow Cytometric CellProbe Oxidative Burst Test was used to determine if respiratory burst occurred or not and what its severity was after a 4 h incubation with propolis extracts (0 mg/mL, 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL). PMN elastase activity following respiratory burst was determined by Flow Cytometric CellProbe Elastase Test. At the 5 mg/mL concentration, the percentages of fluorescence positivities of DCFH, PMA Oxidative Burst Test were observed as 28 ± 0.316% and 27 ± 0.374% for untreated and PMA-stimulated PMNLs, respectively. At the concentration of 5 mg/mL, percentage of fluorescence positivities of RGES-Elastase Test were observed as 17.5 ± 0.412% and 5.6 ± 0.316% for untreated and PMA-stimulated PMNLs, respectively. It was concluded that DMSO extracts of Turkish propolis may exhibit anti-inflammatory activities by reducing respiratory burst and secreting PMN elastase.

Key words: Propolis, respiratory burst, polymorphonuclear elastase, polymorphonuclear leukocyte

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1. Introduction
Polymorphonuclear leukocytes (PMNLs) (neutrophils) are a class of white blood cells that play an important role in an organism’s defense against infectious agents such as microorganisms, virally infected cells, and tumor cells (Lorenzen et al., 2000; Laskay et al., 2008; Paula et al., 2009). Once an inflammatory response is initiated, neutrophils are activated and recruited to the sites of infection to ingest and destroy infectious agents by phagocytosis. Activated neutrophils use both oxygen-dependent and oxygen-independent mechanisms to kill microorganisms (Laskay et al., 2008).

The oxygen-dependent mechanism known as respiratory (oxidative) burst is characterized by the generation of excess amounts of reactive oxygen species (ROS) such as superoxide anion (·O2−), hydrogen peroxide (H2O2), hydroxyl radical (OH·), and hypochlorous acid (HOCl) (Ratajczak-Wrona et al., 2013). NADPH oxidase, superoxide dismutase (SOD), and myeloperoxidase (MPO) are the enzymes responsible for the generation of highly bactericidal ROS (Lorenzen et al., 2000).

Respiratory burst can be activated by chemotactants, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), and nonchemotactic stimuli, such as ionophore A23187 and phorbol myristate acetate (PMA), via different mechanisms (Lorenzen et al., 2000). PMA activates respiratory burst via protein kinase C (PKC) and 1,2-diacylglycerol (DAG) in a calcium-dependent manner (Radford et al., 1999).

Oxidative stress results from an increase in ROS concentration or a decrease in endogenous antioxidant capacities in organisms (Tabart et al., 2012). It is associated with tissue damage and contributes to the induction of chronic or acute inflammatory diseases such as cardiovascular disease, diabetes, arthritis, Parkinson’s, Alzheimer’s, and cancer development (Derochette et al., 2013).

The antioxidants have been shown to possess free radical scavenging and metal chelating properties (Morais et al., 2011). Thus, they serve as a defensive factor against free radicals (Aliyazıcıoğlu et al., 2005; Mohammadzadeh et al., 2007).

In the oxygen independent killing mechanism, primary (azurophilic) granules of neutrophils release their proteolytic enzymes, such as elastase and cathepsins, and bactericidal proteins in phagolysosomes to digest...
Phagocytized microorganisms (Laskay et al., 2008). Proteolytic enzymes secreted by neutrophils contribute to the regulation of innate immunity, inflammation, and infection, and degrade the extracellular matrix components (Paula et al., 2009). Human PMN (neutrophil) elastase (HLE, HNE: EC 3.4.21.37) is a serine protease that cleaves neutral, nonaromatic dipeptides and has an important role in PMN-mediated proteolytic events and in the regulation of inflammation and infection (Korkmaz et al., 2008). PMN elastase is a positively charged 29 kDa molecule that is restricted to the extracellular space. It cleaves cell surface structures and molecules (Voynow et al., 2008). It was reported that PMN elastase participates in tissue destruction in a number of inflammatory disorders, including rheumatoid arthritis, cystic fibrosis, glomerulonephritis, emphysema, uveitis, Crohn’s disease, and depressive disorders (Değer et al., 1996; Paula et al., 2009).

Phytochemicals of fruits and vegetables such as polyphenols are considered to be of crucial nutritional importance in the prevention of chronic diseases such as cancer, and cardiovascular and neurodegenerative diseases. This may be related to their antioxidant activity as well their ability to regulate cellular activities of inflammation-related cells (such as mast cells, macrophages, lymphocytes, and neutrophils) (Tabart et al., 2012).

Propolis is a sticky, natural, resinous, hive product that honeybees produce by mixing their own waxes and salivated secretions with resins collected from the buds and barks of various plants. The chemical composition of propolis depends on the vegetation, climate, and season of the area from which it was collected (Banskota et al., 2001; Santos et al., 2003; Temiz et al., 2011). It is mainly composed of resin (50%), wax (30%), essential oils (10%), pollen (5%), and other organic compounds (5%) (Burdock, 1998). Organic compounds that are identified in different propolis samples are fatty acids, phenolic compounds such as flavonoids (pinocembrin, galangin, and pinobanksin), phenolic acids (caffeoylquinic acid derivatives), phenolic esters (caffeic acid phenethyl ester (CAPE)), aromatic acids (dihydrocinnamic acid, p-coumaric acid, ferulic acid, caffeic acid), aromatic aldehydes, terpenes, β-steroids, alcohols, sesquiterpenes, naphtalene, and stilbene derivatives (Marcucci et al., 2001; Castaldo and Capasso, 2002; Temiz et al., 2011).

Propolis has a long history of use in folk medicine and exhibits antioxidant, antimicrobial, antitumor, antiinflammatory, and immunomodulatory properties (Banskota et al., 2001; Santos et al., 2003; Arslan et al., 2012). These are generally attributed to the phenolic compounds in its content (Castaldo et al., 2002).

Fluorogenic substrates can be used to study hydrolytic and oxidative enzymes in living cells. CellProbe reagents consist of synthetic nonfluorescent enzymatic substrates that are characterized by their low molecular weight and high polarity. This allows them to diffuse through the cytoplasmic membrane of living cells without previous permeabilization (Prin-Mathieu et al., 2001).

The major aim of the present study was to determine whether respiratory burst status and the secretion of PMN elastase by neutrophils could be influenced by the incubation of Turkish propolis with DMSO extract.

2. Materials and methods

2.1 Propolis origin

Propolis samples (produced by honey bees (Apis mellifera L.) in various regions of Turkey) were provided by the Trabzon Agricultural Development Cooperative. They were mixed and named ‘Turkish propolis’.

2.2 Preparation of dimethylsulfoxide extract of propolis (DEP)

The propolis samples were ground (Retsch, ZM 200, Germany) and 5 g of ground propolis was dissolved in 5 mL of dimethyl sulfoxide (DMSO) [Sigma, Cat No: D4540-1L, ≥ 99.5% (GC)] (100% w/v) by continuous mixing for 5 h. It was then incubated at 37 °C in water bath (Nüve, Type: BM 402 Serial No: 03-1048, Turkey) overnight. After centrifuging at 800 × g for 15 min, it was filtered and 4 mL (1000 mg/mL) of extract was obtained. The extract volume was completed to 10 mL using deionized water. The filter procedure was repeated and 6 mL of extract was made up to 10 mL with deionized water to give a stock concentration of propolis extract of 240 mg/mL. The working extracts of propolis at concentrations of 0 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL were then prepared in phosphate buffered saline (PBS) [NaH2PO4·2H2O (Merck, K1305445), Na2HPO4·2H2O (Merck, K16290176)]. Extracts were filtered by 0.2-µm membranes (SCH Schleicher & Schuell FB 030/3 0.2 µm/7 bar mac).

2.3 Polymorphonuclear leukocyte (PMNL) isolation

Human blood with heparin was obtained from the Farabi Hospital’s Blood Bank, Karadeniz Technical University. PMNLs were isolated by centrifugation on a Ficoll density gradient and hypotonic lysis of red blood cells (Villagrasa et al., 1997). Briefly, 20 mL of whole blood in heparin was spread on 10 mL of Ficoll (BIOCHROM AG, Cat. No. 1 6113) and centrifuged at 800 × g for 25 min. The erythrocytes were lysed in hypotonic NaCl (Sigma-Aldrich, Cat No: S7653) solution and removed by centrifugation at 200 × g for 10 min. The lysis and centrifugation procedures were repeated twice. PMNLs were counted with an autoanalyzer (Coulter STKS, USA) and then resuspended in isotonic NaCl solution. The concentration of PMNLs was adjusted to 3.0 ± 0.5 × 10⁶ cells per mL. All experiments were performed 3 times.

2.4 Erythrocyte lysis

Human blood with heparin was collected from healthy volunteers and anticoagulated with sodium citrate (Sigma, Cat No: C-2630; 3.8%, v/v). Blood samples were centrifuged at 200 × g for 10 min. The erythrocytes were lysed in hypotonic NaCl (Sigma-Aldrich, Cat No: S7653) solution and removed by centrifugation at 200 × g for 10 min. The lysis and centrifugation procedures were repeated twice. The erythrocyte pellet was resuspended in a solution of 0.13 M NaCl, 1 mM CaCl2, and 1 mM MgCl2 (Sigma-Aldrich, Cat No: C-0375) and adjusted to 0.005% (v/v) of Triton X-100 (Sigma-Aldrich, Cat No: T8787). The final erythrocyte concentration was adjusted to 3.0 ± 0.5 × 10⁶ cells per mL. All experiments were performed 3 times.

2.5 Fluorescent substrate preparation

Fluorescent substrates were prepared by dilution with 1 M NaCl (Merck, K1003545) to achieve final concentrations of 100 μM (500 mg/mL) and 200 μM (1000 mg/mL) in a solution of DMSO (Sigma, Cat No: D4540-1L, ≥ 99.5% (GC)) and PBS (NaH2PO4·2H2O (Merck, K1305445), Na2HPO4·2H2O (Merck, K16290176)). Extracts were filtered by 0.2-µm membranes (SCH Schleicher & Schuell FB 030/3 0.2 µm/7 bar mac).

2.6 Hydrolysis assay

PMNLs were isolated by centrifugation on a Ficoll density gradient and hypotonic lysis of red blood cells (Villagrasa et al., 1997). Briefly, 20 mL of whole blood in heparin was spread on 10 mL of Ficoll (BIOCHROM AG, Cat. No. 1 6113) and centrifuged at 800 × g for 25 min. The erythrocytes were lysed in hypotonic NaCl (Sigma-Aldrich, Cat No: S7653) solution and removed by centrifugation at 200 × g for 10 min. The lysis and centrifugation procedures were repeated twice. PMNLs were counted with an autoanalyzer (Coulter STKS, USA) and then resuspended in isotonic NaCl solution. The concentration of PMNLs was adjusted to 3.0 ± 0.5 × 10⁶ cells per mL. All experiments were performed 3 times.

2.7 Analysis of propolis extracts

Propolis extracts were prepared by mixing 5 g of propolis with 5 mL of DMSO (Sigma, Cat No: D4540-1L, ≥ 99.5% (GC)) and 5 g of ground propolis was dissolved in 5 mL of dimethyl sulfoxide (DMSO) [Sigma, Cat No: D4540-1L, ≥ 99.5% (GC)] (100% w/v) by continuous mixing for 5 h. It was then incubated at 37 °C in water bath (Nüve, Type: BM 402 Serial No: 03-1048, Turkey) overnight. After centrifuging at 800 × g for 15 min, it was filtered and 4 mL (1000 mg/mL) of extract was obtained. The extract volume was completed to 10 mL using deionized water. The filter procedure was repeated and 6 mL of extract was made up to 10 mL with deionized water to give a stock concentration of propolis extract of 240 mg/mL. The working extracts of propolis at concentrations of 0 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL were then prepared in phosphate buffered saline (PBS) [NaH2PO4·2H2O (Merck, K1305445), Na2HPO4·2H2O (Merck, K16290176)]. Extracts were filtered by 0.2-µm membranes (SCH Schleicher & Schuell FB 030/3 0.2 µm/7 bar mac).

2.8 Analysis of enzyme activities

PMNLs were isolated by centrifugation on a Ficoll density gradient and hypotonic lysis of red blood cells (Villagrasa et al., 1997). Briefly, 20 mL of whole blood in heparin was spread on 10 mL of Ficoll (BIOCHROM AG, Cat. No. 1 6113) and centrifuged at 800 × g for 25 min. The erythrocytes were lysed in hypotonic NaCl (Sigma-Aldrich, Cat No: S7653) solution and removed by centrifugation at 200 × g for 10 min. The lysis and centrifugation procedures were repeated twice. PMNLs were counted with an autoanalyzer (Coulter STKS, USA) and then resuspended in isotonic NaCl solution. The concentration of PMNLs was adjusted to 3.0 ± 0.5 × 10⁶ cells per mL. All experiments were performed 3 times.
2.4. Incubation of PMNLs with DEP

Isolated PMNLs were divided into 2 groups (Table). The 1st group was used for flow cytometric DCFH-PMA test and the 2nd one was used for RGES-Elastase test. Each group was divided into 2 subgroups. One subgroup was stimulated with PMA and the other was not. The PMA-stimulated group consisted of 100 µL of PMNLs, 100 µL of DEP at various concentrations (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL), 790 µL of RPMI-1640 cell culture medium, and 10 µL of phorbol 12-myristate 13-acetate (PMA) [Sigma, Cat No: P8139-10MG, ≥99% (TLC)]. PMA solution was prepared by diluting 1:100 in DMSO from stock solution of 1 mg/mL. A final concentration of 0.01% of DMSO solution was used as the 0 mg/mL of DEP. The untreated group consisted of 100 µL of PMNLs, 100 µL of DEP at various concentrations (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL), and 800 µL of RPMI-1640 cell culture medium (BIOCHROM AG, Cat. No.: F 1215). Final concentrations of DEP in each experimental group were 0.625 mg/mL, 1.25 mg/mL, 2.5 mg/mL, and 5.0 mg/mL. Each experimental group was incubated at 37 °C for 4 h in RPMI-1640 cell culture medium 6 flat-bottomed well plates at 37 °C and 5% CO₂ atmosphere (Steri-Cycle TermoForma Hepa Filter SN: 301896-251 model 381). The experiments were performed 3 times.

2.5. Determination of respiratory burst by flow cytometric DCFH-PMA test

DCFH, PMA oxidative burst test (Coulter, PN7547078) was used to determine whether or not respiratory burst occurred and what its strength was after a 4 h incubation of all experimental groups. This test includes 2',7'-dichlorofluorescein diacetate (DCFH-DA), which is a lipophilic, readily oxidizable substrate that easily crosses cell membranes. Inside the cell, cytosolic enzymes (esterases) deacetylate the DCFH-DA to form nonfluorescent 2',7'-dichlorofluorescein (DCFH). Then H₂O₂ in the cell oxidizes DCFH to 2',7'-dichlorofluorescein (DCF), whose green fluorescence at 525 nm is easily measurable on the flow cytometer. This measurement is utilisable for neutrophils and monocytes/macrophages. For each assay, 2 aliquots of 50 µL of the incubated cell suspension were added to tubes. They were incubated for 10 min in a water bath of 37 °C. At the end of incubation, 25 µL of CellProbe reagent was added to each warmed tube and gently mixed. A blank control tube was prepared by replacing CellProbe reagent by 25 µL of 0.01% DMSO solution in the tube containing the prewarmed second aliquot for each sample. All tubes were incubated for 10 min in a water bath of 37 °C and then placed on ice for 10 min to stop the enzymatic reaction. By using Flow-Set Fluorospheres (Beckman Coulter, PN 6607007), the fluorescence channel was adjusted to 22.7 ± 0.30 fluorescence (Coulter, Epics Elite) and the fluorescence intensity was determined at excitation of 468–509 nm wavelengths and emission of 504–541 nm wavelengths (Imrich and Kobzik, 1998).

2.6. Determination of PMN elastase activity by flow cytometric RGES-elastase test

The flow cytometric CellProbe RGES-elastase test (PN 7547104) was used to determine PMN elastase activity after a 4 h incubation of all experimental groups. This test consists of a synthetic, nonfluorescent enzyme substrate of 2 leaving groups conjugated to a dye molecule Rhodamine 110. RGES (Arginine-Glycine-Glutamicacid-Serine-Rho110) includes a part of fibronectin, which is a substrate of PMN elastase. When the bond between the leaving group and the dye is cleaved by the PMN elastase, fluorescence is released (Prin-Mathieu et al., 2001). The assay procedure was the same as DCFH, PMA oxidative burst test.

2.7. Statistical analysis

Data were analyzed by two-way ANOVA, using SPSS version 15.0. Then, to make multiple comparisons among

<table>
<thead>
<tr>
<th>DEP concentration (mg/mL)</th>
<th>DCFH-PMA test</th>
<th>RGES-Elastase test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With PMA</td>
<td>Without PMA</td>
</tr>
<tr>
<td>0</td>
<td>46.0 ± 0.469</td>
<td>32.1 ± 0.224</td>
</tr>
<tr>
<td>0.625</td>
<td>37.0 ± 0.509</td>
<td>29.0 ± 0.509</td>
</tr>
<tr>
<td>1.25</td>
<td>33.0 ± 0.40</td>
<td>31.0 ± 0.509</td>
</tr>
<tr>
<td>2.5</td>
<td>29.0 ± 0.316</td>
<td>29.0 ± 0.547</td>
</tr>
<tr>
<td>5</td>
<td>27.0 ± 0.374</td>
<td>28.0 ± 0.316</td>
</tr>
</tbody>
</table>

PMA: phorbol myristate acetate
groups, Tukey’s multiple comparison test was used and P < 0.001 level was considered as statistically significant.

3. Results

3.1. Respiratory burst by flow cytometric DCFH-PMA test

The effects of DEP at different concentrations on the percentage of fluorescence positivity of DCFH, PMA oxidative burst test with and without PMA are shown in the Table and Figure 1.

The obtained percentages of fluorescence positivities at 0 concentration for the untreated and PMA-stimulated PMNL cultures were 32.1 ± 0.223% and 46 ± 0.469% (mean ± SD), respectively. There was a significant difference at 0 concentration of DEP between the percentages of fluorescence positivity of PMA-stimulated PMNLs and of untreated PMNLs (P: 0.000). The percentage of fluorescence positivity decreased significantly as the concentration of DEP increased for PMA-stimulated PMNLs (P: 0.000). For untreated PMNLs, the decrease in percentage of fluorescence positivity was not significant between concentrations of 2.5 mg/mL and 5 mg/mL (P: 0.014). At the 5 mg/mL concentration, the percentages of fluorescence positivities were observed as 28 ± 0.316% and 27 ± 0.374% (mean ± SD) for untreated PMNLs and PMA-stimulated PMNLs, respectively.

3.2. PMN elastase activity by flow cytometric RGES-elastase test

The effects of DEP at different concentrations on the percentage of fluorescence positivity of flow cytometric CellProbe RGES-elastase test with and without PMA are shown in the Table and Figure 2.

The obtained percentages of fluorescence positivities at 0 concentration for the untreated PMNL and PMA-stimulated PMNL cultures were 27 ± 0.37% and 25.5 ± 0.36% (mean ± SD), respectively. There was a significant difference at 0 concentration of DEP between the percentages of fluorescence positivity of PMA-stimulated PMNLs and of untreated PMNLs (P: 0.000). The percentage of fluorescence positivity decreased significantly as the concentration of DEP increased for PMA-stimulated PMNLs (P: 0.000). For untreated PMNLs, the decrease in percentage of fluorescence positivity was not significant between concentrations of 2.5 mg/mL and 5 mg/mL (P: 0.576). At the 5 mg/mL concentration, the observed percentages of fluorescence positivities were 17.5 ± 0.412% and 5.6 ± 0.316% (mean ± SD), for untreated PMNLs and PMA-stimulated PMNLs, respectively.

4. Discussion

Following stimulation, PMNLs generate large amounts of ROS, called ‘respiratory burst’, and release their proteolytic activity.

![Figure 1. Percentage of fluorescence positivity of respiratory burst (DCFH, PMA oxidative burst test). The percentage of fluorescence positivity of DCFH, PMA oxidative burst test decreased significantly as the concentration of DEP increased for PMA-stimulated PMNLs (P: 0.000). (—): DEP added to PMA-induced PMNLs, (・・・): DEP added to untreated PMNLs (Bars represent mean ± SD) DEP: Dimethyl sulfoxide extract of propolis. PMA: phorbol myristate acetate. PMNLs: Polymorphonuclear leukocytes.](image1)

![Figure 2. Fluorescence positivity % of flow cytometric CellProbe RGES-elastase test (—): DEP added to PMA-induced PMNLs, (・・・): DEP added to untreated PMNLs (Bars represent mean ± SD) The percentage of fluorescence positivity of RGES-elastase test decreased significantly as the concentration of DEP increased for PMA-stimulated PMNLs (P: 0.000). DEP: Dimethyl sulfoxide extract of propolis. PMA: phorbol myristate acetate. PMNLs: Polymorphonuclear leukocytes.](image2)
enzymes (such as elastase) to destroy microorganisms (Laskay et al., 2008). ROS and elastase are involved in the pathogenesis of several diseases and tissue damage processes (Paula et al., 2009; Derochette et al., 2013). Phytochemicals of fruits and vegetables, such as polyphenols and flavonoids, have been shown to possess free radical-scavenging and metal chelating properties (Morais et al., 2011). With their antioxidant activities they serve as a defensive factor against free radicals (Derochette et al., 2013). This means that antioxidants may be effective in treating neutrophil- and ROS-dependent diseases.

Propolis has been shown to be capable of scavenging free radicals through their pharmacologically active constituents such as flavonoids (Mohammadzadeh et al., 2007). Thus, propolis is thought to improve health and prevent ROS-related diseases such as inflammation, heart disease, diabetes, and cancer by its antioxidant potential (Castaldo et al., 2002).

Total polyphenol content (Lotito et al., 2004), total flavonoid content, ferric reducing antioxidant potential (FRAP) (Mohammadzadeh et al., 2007), and total antioxidant capacity (TAC) (Erel, 2004) of DEP were analyzed in our laboratory. Total polyphenol content and total flavonoid content of DEP were determined as 48.7 ± 7.8 (mg gallic acid/g propolis) and 13.0 ± 1.0 (mg quercetin/g propolis), respectively. FRAP and TAC of DEP were determined as 59.5 ± 17.3 (mg Trolox/g propolis) and 8.8 ± 3.0 (mmol Trolox/100 g propolis), respectively (mean ± SD) (Barlak et al., 2011).

The compounds in DEP were analyzed by HPLC (Agilent 1100 Series) in our laboratory. A Hichrom LiChrospher RP18-5 (25 cm × 4.0 mm) column was used. In the mobile phase 30 mM NaH₂PO₄ and acetonitrile were used (Pietta et al., 2002). The compounds identified in the DMSO extract of Turkish propolis as having the largest peaks were quercetin (6.5%), pinocembrin (6%), chrysin (5%), kaempferol (2%), galangin (1.5%), and naringenin (1%) (Çakiroğlu et al., 2010).

4.1. Respiratory burst by flow cytometric DCFH-PMA test

Barlak et al. (2011) reported that DMSO (100%) extract of propolis was rich in polyphenols and flavonoids and the antioxidant potentials of the extracts were found to correlate with the amount of total phenolic compounds in them.

As shown in the Table and Figure 1, the percentage of fluorescence positivity of DCFH-oxidative burst test decreased significantly as the concentration of DEP increased for PMA-stimulated PMNLs (P: 0.000). The decrease in percentage of fluorescence positivity might be due to the antioxidant capacity of DEP.

In a previous study, the percentage of fluorescence positivities decreased (between 3.8% and 11.8%) as concentrations of DMSO extracts of both propolis and pollen increased for K-562 cell culture. They remained unchanged (between 20% and 83%) for mononuclear cell (MNC) culture, as measured by intracellular dichlorofluorescence (DCFH) test, using flow-cytometric fluorescence analysis (Alıyaziçioğlu et al., 2005).

It was reported that Broussochalcone A, a prenylated chalcone, inhibited O₂⁻ consumption in fMLP- and PMA-stimulated rat neutrophils in a concentration-dependent manner with IC₅₀ values of 70.3 ± 4.9 and 63.9 ± 7.1 mM and suppressed the enzyme activity of trypsin-treated rat brain protein kinase C in a concentration-dependent manner (Wang et al., 1997).

It was found that tea catechins inhibited intracellular ROS generation in a dose-dependent manner together with diphenyleiodonium chloride (DPI) in fMLP-stimulated human PMN leukocytes (Nishikawa et al., 2007).

It was reported that CAPE, an active component of propolis extract, completely blocks production of ROS in PMA-stimulated human neutrophils at a concentration of 10 µM through its antioxidative property (Sud’ina et al., 1993). It was found that artelastin, a prenylated flavone previously isolated from Artocarpus elasticus, inhibits ROS production in PMA-triggered human PMNs due to a strong superoxide radical scavenging activity (Cerqueira et al., 2008). It was reported that new synthetic pyrrolo-benzylisoquinoline derivatives inhibited fMLP-induced superoxide radical release in human neutrophils without superoxide radical scavenging activity (Hwang et al., 2005). Superoxide generation induced by FMLP and PMA was inhibited by phenolic acids and their derivatives in a concentration-dependent manner (Lee et al., 2005). It was reported that Baccharis grisebachii and Pluchea sagittalis dichloromethane extracts as well as Pluchea sagittalis aqueous extract reduced ROS production induced by the 3 stimulants used (H₂O₂, PMA, and fMLP) in human neutrophils by reacting directly with the free radicals (Pérez-García et al., 2001). It was suggested that incubation of PMNs with Achyrocline satureioides at the concentration of 0.0012–0.03 µg/mL quercetin equivalent reduced fMLP-induced ROS increase in a concentration-dependent manner (Cosentino et al., 2008). It was reported that stem bark aqueous extract of mango tree (Mangifera indica L.) (MSBE) and mangiferin exhibited a dose-dependent inhibitory effect on the chemiluminescence signal in PMA and zymosan-stimulated PMNs (Garrido et al., 2008). It was found that H₂O₂ generation by peritoneal macrophages of stressed mice was potentiated, and nitric oxide (NO) production by peritoneal macrophages of stressed mice was inhibited by propolis administration (Missima and Sforcin, 2007).

Paula et al. (2009) reported that tamarind (Tamarindus indica L.) fruit pulp extract (ExT) inhibited fMLP or PMA triggered neutrophil reactive oxygen species generation in a concentration-dependent manner.
Derochette et al. (2013) found that NDS27, a highly water-soluble form of the polyphenolic molecule curcumin, significantly and dose-dependently inhibited ROS production in PMA- or fMLP- stimulated equine PMNs and human promyelocytic leukemia cells (HL-60). They also found that the activity of myeloperoxidase released by PMNs and HL-60 cells was decreased by NDS27.

Tabart et al. (2012) showed that blackcurrant extracts (leaves, berries, and buds) significantly and dose-dependently inhibited ROS production of PMA-activated neutrophils. They also observed dose-dependent inhibition of MPO activity with all the explants tested.

Castro et al. (2008) reported that resveratrol (RESV; trans-3,5,4′-trihydroxystilbene) exerted a dose-dependent and significant inhibitory effect on the production of reactive oxygen species in kidney leucocytes of turbot (Psetta maxima (L.)) after stimulation of respiratory burst activity with PMA. They also reported that RESV strongly inhibited intracellular and extracellular MPO activity and induced a decrease in MPO mRNA levels in turbot neutrophils.

Çakıroğlu et al. (2010) found that the compounds identified in the DMSO extract of Turkish propolis as having the largest peaks are quercetin, pinocembrin, chrysin, kaempferol, galangin, and naringenin. As mentioned above, phenolic acids and their derivatives, flavonoids such as prenylated chalcone, catechins, curcumin, resveratrol, and propolis inhibit respiratory burst stimulated by different stimuli through different mechanisms.

In the current study it was observed that DEP significantly and concentration-dependently inhibited PMA-stimulated respiratory burst in human PMNLs. The inhibition effect of DEP on respiratory burst might be mediated by its antioxidant potential due to its polyphenolic content. It was suggested that propolis may be used as a therapeutic agent in the treatment of ROS-related diseases.

4.2. PMN elastase activity by flow cytometric RGES-elastase test

As shown in the Table and Figure 2, the percentage of fluorescence positivity of RGES-elastase test decreased significantly as the concentration of DEP increased for PMA-stimulated PMNLs (P: 0.000). The decrease in percentage of fluorescence positivity might be due to the antioxidant capacity of DEP.

We observed that DEP significantly and concentration-dependently inhibited PMA-stimulated PMN elastase secretion in human PMNLs. The inhibition effect of DEP on PMN elastase secretion might be mediated by its antioxidant potential due to its polyphenolic content.

Paula et al. (2009) reported that the tamarind (Tamarindus indica L.) fruit pulp extract (ExT) inhibited neutrophil NADPH oxidase activity and elastase activity.

Dalli et al. (2008) reported that fMLP-induced superoxide anion generation, elastase release, and chemotactic migration were inhibited by dry extract of leaves and flowers of Crataegus laevigata in human neutrophils at the concentration of 0.75–250 µg/mL. Kanashiro et al. (2007) demonstrated that quercetin, myricetin, kaempferol, and galangin, all found in propolis, inhibited elastase release from neutrophils.

As mentioned above, fruit pulp extract leaves and flowers of Crataegus laevigata and quercetin, myricetin, kaempferol, and galangin, all found in propolis, inhibit secretion of elastase. The results obtained in the current study suggest that the effect of DEP on secretion of PMN elastase might be mediated by its antioxidant potential due to its polyphenolic content.

We observed that DEP, which is rich in flavonoids, inhibited respiratory burst and PMN elastase secretion within PMNLs probably by its free radical scavenging activity due to its polyphenolic and flavonoid content.

In conclusion, DMSO extracts of propolis may have anti-inflammatory properties, probably due to their antioxidant potentials. Propolis may also be used as a therapeutic agent in the treatment of neutrophil-dependent diseases. Further studies are needed to investigate the mechanisms through which these effects of DMSO extract of Turkish propolis occur.

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