Effects of Turkish propolis on expression of hOGG-1 and NEIL-1

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Background/aim: Propolis is a bee product with antioxidative, antimutagenic, and other beneficial properties, and it is used as a natural drug. It is rich in polyphenolic compounds. Its composition varies depending on the particular geographical region. Oxidative stress is caused by an imbalanced free radical production and antioxidant system. The effects of flavonoids on the expression of DNA repair enzymes have been examined previously; however, no study has investigated the effects of propolis. This study investigated the effects of ethanolic extracts of Turkish propolis (EEP) on the expression of DNA repair enzymes.

Materials and methods: The effects of EEP and tertiary-butyl-hydroperoxide (t-BHP) on cell viability were determined using MTT. DNA damage was determined using comet assay. mRNA expression of target enzymes was detected using RT-PCR.

Results: According to the cytotoxicity analysis, after a recovery time of 4 h, appropriate damage agent t-BHP and optimum EEP concentrations were 300 μM and 200 μg/mL, respectively. 8-Oxoguanine-glycosylase (hOGG-1) and endonuclease-VIII-like-1 (NEIL-1) expressions increased in the positive control group (t-BHP alone) and the study group (t-BHP+EEP). Maximum increase in NEIL-1 expression was at hour 12 in the positive control group and at hour 8 in the study group.

Conclusion: EEP can be considered as a potential source of functional food and pharmaceutical agents.

Key words: Propolis, DNA repair, NEIL-1, hOGG-1, comet assay, antigenotoxic effect

1. Introduction

Propolis, a bee product, is composed of pollens, oils, and special resinous and waxy substances collected by bees from the bark and cones of trees and plant sprouts (1). Propolis has been reported to possess antibacterial, anticarcinogenic, antiinflammatory, antioxidative, tumoricidal, and antimutagenic properties (2,3).

The biological effects of propolis are attributed to its polyphenol content. In addition to their antioxidant properties, due to their free radical scavenging effects, these polyphenols have also been reported to affect cell proliferation, inhibition of angiogenesis, and intercellular signal mechanism and stimulation of DNA repair enzymes (3,4).

Oxidative stress develops when an imbalance occurs between reactive oxygen species (ROS) production and the antioxidant system (5). ROS have adverse effects on the structure and functions of macromolecules in cells, such as DNA, carbohydrates, lipids, proteins, and enzymes (6,7).

Base excision repair (BER) can repair single-strand cleavage caused by ROS. BER enzymes include DNA glycosylases (hOGG-1, NEIL-1/2), endonuclease-III (NTH), apurinic apyrimidinic endonuclease (APE-1), DNA polymerases, and DNA ligases (8).

hOGG-1 is a functional enzyme with DNA glycosylase/AP lyase activity that breaks the N-glycoside bond between oxidized guanine (8-OHG) and deoxyribose. It thus eliminates 8-OHG and also forms a nonbasic region via cleavage of the phosphodiester bond with β-elimination reaction (9,10). NEIL endonucleases are homologues of the bacterial MutM/Nei family. NEIL-1 endonuclease has a wide substrate profile and can recognize and remove modified bases, including formamidopyrimidine A (FapyA), formamidopyrimidine G (FapyG), thymine glycol, and 5-hydroxy uracil, from DNA strands (11).

Although the effects of flavonoids on the expression of DNA repair enzymes have been studied previously, no studies have investigated propolis to date. The aim of this study was to investigate the previously reported mechanism of the antigenotoxic property of ethanolic extract of Turkish propolis (EEP) (12) and to determine

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whether or not this is due to induction of BER pathway enzymes.

2. Materials and methods

2.1. Chemicals
Propolis samples were produced by honey bees (*Apis mellifera* L.) in Trabzon, Turkey. Foreskin fibroblast cells (CRL-2522) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Ethanol and NaCl were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Carlo Erba (Milan, Italy), penicillin-streptomycin and trypsin from GIBCO (Paisley, UK), Dulbecco's modified eagle medium (DMEM) from Lonza (Verviers, Belgium), fetal bovine serum (FBS) and polylysine from Biochrom (Berlin, Germany), phosphate buffer saline (PBS) tablet from Medicago (Uppsala, Sweden), sodium hydroxide from Riedal De Haen (Seelze, Germany), and RNA isolation and real-time PCR kits from QIAGEN (Hilden, Germany). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Extraction of propolis
First, 0.5 g of propolis was dissolved in 20 mL of ethanol. Following vortexing, this was incubated at 60 °C and 150 rpm for 24 h. Following incubation, the propolis was centrifuged at 4000 rpm for 10 min. Supernatants were filtered through filter paper and 0.22-μm filters, and 25×10^3 μg/mL stock EEP was used for experiments.

2.3. Cell culture
Fibroblast cells were cultured in DMEM containing L-glutamine, 10% FBS, and 1% penicillin and streptomycin in T-25 and T-75 flasks. This was accompanied by a 5% CO_2 supply at 37 °C in an incubator. Cells were passaged when they had attained 70%–80% growth in flasks.

2.4. Cell viability
 Fibroblast cells were cultured in 96-well plates at 5000 cells per well. After 24 h, the medium was removed and the cells were treated with different concentrations of tertiary-butyl-hydroperoxide (t-BHP; 100–600 μM) and EEP (50–250 μg/mL). Following a 24-h incubation, 190 μL of DMEM and 10 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye with a final concentration of 0.25 mg/mL were added to the wells. After a 2-h incubation, the well content was removed and 200 μL of DMSO was added to all wells, which were incubated for 30 min. Absorbance of the wells was read at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The results were calculated on the basis of control samples (cells without any test compound).

2.5. Determination of t-BHP concentration
A total of 2×10^5 fibroblast cells were cultured in a flask. After 24 h, cells were treated with 100–600 μM t-BHP for 1 h to determine the concentration resulting in DNA damage but not toxicity. After incubation, cells were washed with PBS and left to recover for 4 h. Cells were trypsinized and centrifuged for comet assay.

2.6. Determination of EEP concentration
EEP with final concentrations of 100, 150, and 200 μg/mL was added to the cells to determine the concentration that would prevent DNA damage without toxicity. Ethanol was used for a negative control (0 concentration). After 1 h, flasks were washed with PBS, and cells were treated with 300 μM t-BHP for another hour. Following incubation, flasks were washed and left for a recovery time of 4, 8, 12, or 24 h. Cells were trypsinized and centrifuged for comet protocol and RNA isolation.

2.7. Comet assay
A total of 40 μL of cell suspension was mixed with 80 μL of low melting point agarose in a polypropylene tube, spread on a slide coated with polylysine and normal melting point agarose, closed with a coverslip, and incubated at 4 °C for 5 min. Coverslips were removed and cells were incubated in lysis buffer for 1 h. Cells were then treated in an alkaline buffer (10 mM EDTA, 0.3 M NaOH, pH 13.1) for 30 min. Cells were held in 22 V (1 V/cm), 300 mA electrophoresis conditions for 20 min. After electrophoresis, cells were neutralized in buffer (Tris, pH 7.4) and incubated in ethidium bromide for 20 min. Cells were evaluated in an Eclipse E800 fluorescence microscope (Nikon, Tokyo, Japan) by filters (G-2A, excitation 510–565 nm, DM 575, BA 590) at 40× magnification. Visual analysis was used for scoring. Three slides were prepared for each group. One hundred cells from each of the slides were scored for DNA damage. Slide scoring was performed on a blind basis with the scorer blind to the treatment conditions of each slide. Selected cells were classified between 0 and 3, from nondamaged to most damaged, according to tail length. Excessively long tails and DNA spectra scored at 4 were not included (12).

All slides were scored with the following formula with a maximum damage possibility of 300:

Score = $(1 \times n_1) + (2 \times n_2) + (3 \times n_3)$ (n: cell number for every score) (13).

2.8. RNA isolation
Total RNA isolation kits (QIAGEN RNeasy) were used for RNA isolation from the cells. Purity of RNAs was determined with NanoDrop (Thermo Scientific, Waltham, MA, USA) by measuring the OD260/OD280 ratio.

2.9. mRNA expression analysis
β-Actin primers for forward 5'-TCA CCC AAC ACT GTG CCC ATC TAC GA-3' and for reverse 5'-TCG GTG AGG ATC TTC ATG AGG TA-3' were used as a reference, producing a 180-bp fragment (14). Specific primer pairs for NEIL-1 (forward: 5'-CGG CGG CTG CGT GGA GAA

GTC-3'; reverse: 5'-GTC CCA GCG GCC GAA CCG GCG-3') and hOGG-1 (forward: 5'-AAC AAC AAC ATC GCC CGC ATC ACT-3'; reverse: 5'-GCT AGC CCG CCC TGT TCT TCC-3') were previously described by Das et al. (15) and Collins et al. (16). Primers were manufactured by Metabion International AG (Martinsried, Germany).

Expression analysis was performed with a Roche Light Cycler 480-II (Rotkreuz, Switzerland) using the SYBR Green method. Complementary DNA was amplified in a 15-µL total volume containing SYBR Green master mix and 7.5 µM of specific primers. Real time-PCR was performed through amplification for 45 cycles of 94 °C (15 s), 50 °C (30 s), and 72 °C (30 s) after enzyme activation at 95 °C (15 min). Results were calculated with basic relative quantification according to β-actin expression of negative control samples (cells without any test compound).

2.10. Statistical analysis
All experiments were carried out in triplicate. All results are given as mean ± standard deviation (SD). Compatibility with normal distribution was determined using the Kolmogorov–Smirnov test. One-way ANOVA was used for comparing differences among the groups. P < 0.05 was regarded as significant.

3. Results
3.1. Cell viability
EEP was found to be nontoxic between concentrations of 50 and 200 µg/mL in cell viability analysis (Figure 1). t-BHP was found to be nontoxic between concentrations of 0 and 600 µM in cell viability analysis (Figure 2).

3.2. Comet analysis
t-BHP of 300 µM, which produces a 300 comet score in 4 h, was used as the damage concentration in all assays. Cells were pretreated with EEP (3 different concentrations) for 1 h. Comet scores were compared after damage with 300 µM t-BHP (Figure 3). There was no significant difference
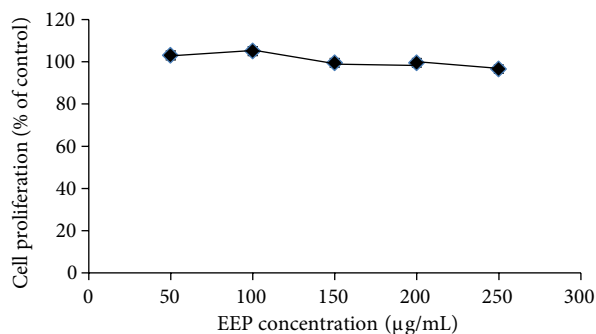
between hours in group A (P > 0.05), although there was a significant difference in group B (P < 0.0001). In groups C, D, and E there was a significant difference between hours 12 and 24 (P < 0.0001), but no significant difference was noted between other hours (P > 0.05). EEP concentration for expression analysis was determined at 200 µg/mL.

3.3. NEIL-1 and hOGG-1 mRNA expressions results
The two main glycosylase enzymes in the BER pathway (hOGG-1 and NEIL-1) were investigated. The mRNA levels for each enzyme were compared with control cells that were not exposed to oxidative stress with t-BHP. NEIL-1 mRNA expression slightly increased 1.5- and 2.7-fold after 8 h, and 1.2- and 5.5-fold after 12 h, with or without EEP treatment. After 24 h, it returned to levels below baseline in the EEP treatment group only (Figure 4).

hOGG-1 mRNA expression increased 1.18-, 1.43-, and 1.5-fold after 4, 8, and 12 h, respectively, with t-BHP treatment alone compared with the negative control. hOGG-1 mRNA expression only increased 1.3-fold after 12 h in the EEP and t-BHP treatment groups (Figure 5).

4. Discussion
Molecular studies of Turkish propolis are limited in number. In a recent study, Aliyazicioglu et al. were the first to report the antigenotoxic activity of propolis by demonstrating that EEP reduced H_2O_2-induced DNA damage (12). The aim of the present study was to investigate the origin of the antigenotoxic activity of EEP. Was it induction of DNA repair enzyme expression, or not? We established that 100–200 µg/mL EEP significantly reduced 300 µM t-BHP-induced DNA damage. Similar studies have demonstrated that different propolis extracts from various countries also reduce DNA damage (2,12,13,17,18). hOGG-1 and NEIL-1 expression levels increased significantly according to the expression analysis with mRNA samples of EEP-treated cells.
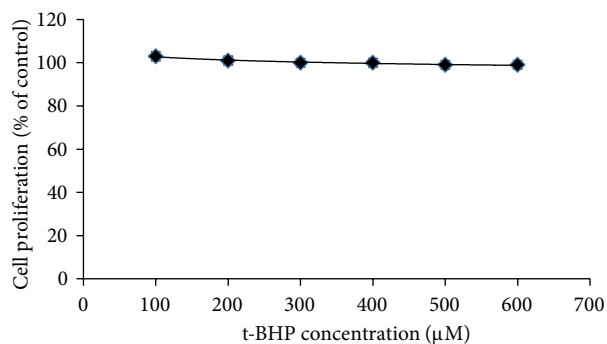

Figure 1. Effect of EEP on fibroblast cell viability shown by the MTT reduction test. Fibroblast cells were incubated for 24 h with increasing concentrations of EEP. Control cells were incubated in the presence of 1% ethanol. Values represent mean ± SD based on results obtained in at least 3 independent experiments.

Figure 2. Effect of t-BHP on fibroblast cell viability based on the MTT reduction test. Fibroblast cells were incubated for 24 h with increasing concentrations of t-BHP. Control cells were incubated in the presence of 1% PBS. Values represent mean ± SD based on the results obtained in at least 3 independent experiments.
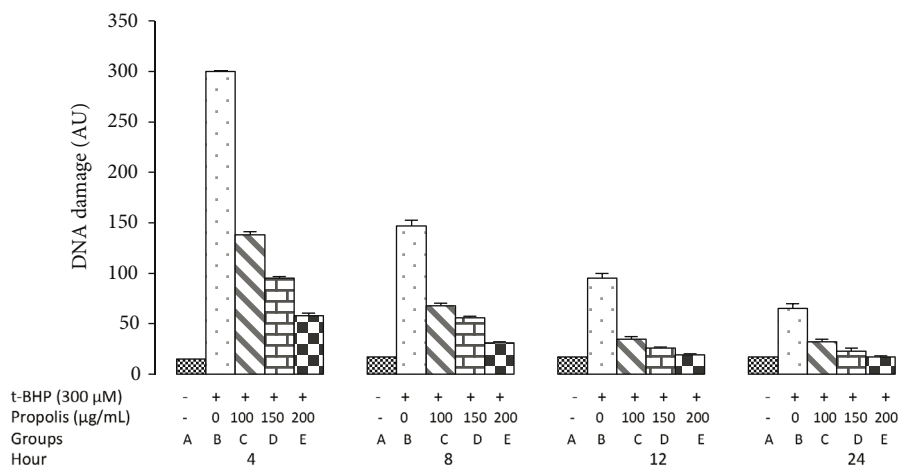
Figure 3. Effect of 1-h pretreatment of EEP (100, 150, and 200 μg/mL) on t-BHP (300 μM, 1 h)-induced DNA damage in fibroblast cells with 4, 8, 12, and 24-h recovery periods. DNA damage was assessed using comet assay. Comets were quantified by visual analysis, and values were calculated using the formula described in Section 2.7. Bars represent mean ± SD for at least 3 independent experiments.
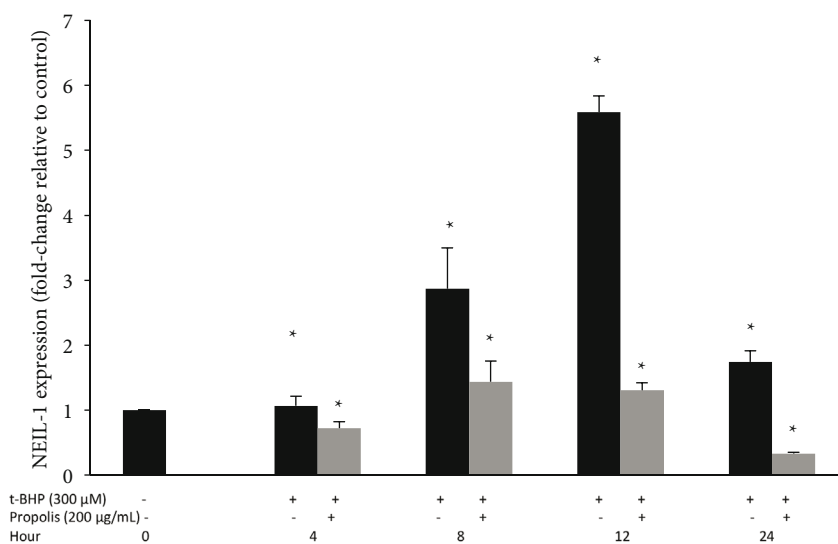

Figure 4. Relative mRNA levels of NEIL-1 under oxidant challenge. Fibroblast cells were pretreated with EEP (200 μg/mL, 1 h) after incubation with t-BHP (300 μM, 1 h)-induced DNA damage with 4, 8, 12, and 24-h recovery periods. Relative mRNA level of NEIL-1 versus β-actin was determined using RT-PCR. Each bar represents the mean ± SD for at least 3 independent experiments. *: P < 0.05 compared to negative control cells (with no treatment).

Antigenotoxic and antimutagenic activities of propolis have been reported in several in vivo and in vitro studies. Munari et al. demonstrated the protective effect of ethyl acetate extracts of *Baccharis dracunculifolia*, one of the major botanical sources of propolis, on methyl methanesulfonate (MMS)-induced DNA damage in V79 cell lines with simultaneous treatment and posttreatment.

They suggested that this action could be attributed to the free radical scavenging and inducing expression of the DNA repair enzyme activities of the polyphenolic content of *Baccharis dracunculifolia* (13).

In another study, Chen et al. investigated the effects of baicalin, a flavonoid isolated from the roots of *Scutellaria baicalensis*, on H$_2$O$_2$-induced DNA damage in NIH3T3
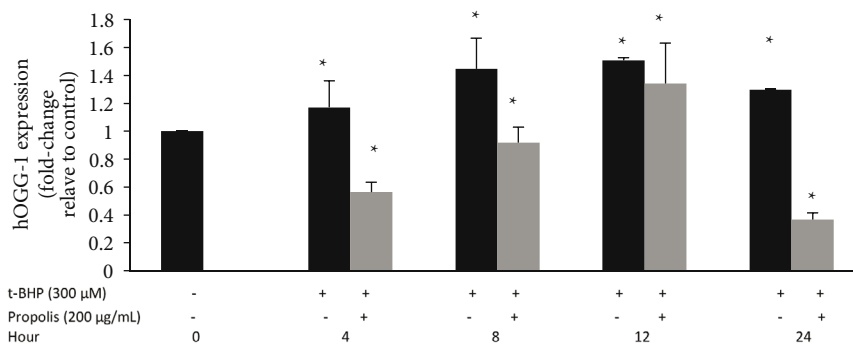
Figure 5. Relative mRNA levels of OGG-1 under oxidant challenge. Fibroblast cells were pretreated with EEP (200 µg/mL, 1 h) after incubation with t-BHP (300 µM, 1 h)-induced DNA damage with 4, 8, 12, and 24-h recovery periods. Relative mRNA level of OGG-1 versus β-actin was determined using RT-PCR. Each bar represents the mean ± SD for at least 3 independent experiments. *: P < 0.05 compared to negative control cells (with no treatment).

cell lines. They reported that DNA damage decreased significantly in a group of cells pretreated with baicalin for 24 h and damaged with H_2O_2 for 15 min compared to a damaged control group. They suggested that this might derive from stimulation of the DNA repair mechanism in addition to the antioxidant activity of baicalin (19).

MMS, H_2O_2, ferrous sulfate, t-BHP, and doxorubicine have been reported to cause in vitro DNA damage in cells in antigenotoxicity studies (4,13,19,20). In this study, t-BHP was used to damage DNA in fibroblasts. t-BHP has been used to determine the cellular injury mechanism caused by acute oxidative stress (20). t-BHP is an organic hydroperoxide that forms alkali-labile regions and single-strand cleavage, causing oxidative DNA damage. DNA damage caused in this way can easily be determined using comet assay analysis (20,21). In this study, comet analysis was used to measure DNA damage due to its advantages over other methods, such as simplicity, economy, sensitivity, reliability, and speed (22,23).

In this study, comet scoring was performed using a scale of 0 (no damage) to 3 (most damage) in visual analysis in the comet assay between 100 and 600 µM t-BHP damaging concentrations. Maximum comet score would be 300 on this scale, and the t-BHP concentration was therefore selected as 300 µM. MTT analysis cell viability was compatible with the literature (20,24–27).

Comet analysis of cells carried out with 100, 150, and 200 µg/mL EEP and damaged with 300 µM t-BHP demonstrated that the highest antigenotoxic concentration was 200 µg/mL. This concentration also applied to cell viability and was therefore selected for use in expression analysis.

Cells are known to activate the BER pathway in oxidative base damage. BER mechanism enzyme NEIL-1 and hOGG-1 expressions were measured with relative quantitation at recovery times of 4, 8, 12, and 24 h. hOGG-1 mRNA expression increased in the positive control group as well as in the study group. Maximum expression levels in both groups were obtained at hour 12. At the end of hour 24, expression levels decreased below basal levels in the study group and remained at basal levels in the positive control group.

Gao et al. suggested that flavonoid naringenin prevents ferrous sulfate-induced DNA damage in LNCaP cell lines by inducing expression of 8-oxoguanine-DNA-glycosylase and DNA polymerase, enzymes involved in the BER mechanism. Those results differ from our study in that the highest expression level was determined at hour 8. At the end of hour 24, expressions were reported to have increased in the assay group and to have returned to basal levels in the positive control group (4). These differences may be due to the use of a different cell type, damage agent, and one type of flavonoid. The authors also reported that although LNCaP cell lines are resistant to oxidative stress, they have low repair capacity. In our study, total damage was eliminated in 24 h. This may be the result of a wide range of flavonoid contents in propolis, rather than a single flavonoid, and may derive from those flavonoids exhibiting a synergistic effect. Erdogan et al. investigated the content of Anatolian propolis, describing it as rich in phenolic compounds such as caffeic acid, gallic acid, *p*-coumaric acid, chlorogenic acid, myricetin, catechin, and luteolin (28). The solubility of Turkish propolis in different solvents was investigated by Çakıroğlu, who found that ethanolic extracts of Turkish propolis had high antioxidant capacity and were rich in quercetin (29).

Silva et al. observed the effects of luteolin, quercetin, and rosmarinic acid on t-BHP and light-sensitive compound Ro19-8022-induced DNA damage in PC12 cell lines. They observed that luteolin and quercetin

radical induced DNA repair enzyme hOGG-1 expression and prevented DNA damage through radical scavenging activity and rosmarinic acid via a direct effect. However, they reported that mRNA levels were unable to exceed negative control expression levels. Similarly, in our study, hOGG-1 expression at hour 4 was lower compared to the negative control group. This may be explained in terms of the dynamic equilibrium of oxidative DNA damage. This theory suggests that basic DNA damage forming in the presence of EEP may be less than in the absence of EEP. Oxidized base formation is an expected phenomenon, so DNA repair enzyme levels were not expressed in the negative control group (25).

Min et al. observed possible mechanisms of quercetin in protecting cells against H_2O_2-induced DNA damage in Caco-2 cell lines and reported that quercetin enhances DNA repair via radical scavenging and by inducing hOGG-1 expression (14). In contrast to our study, maximum hOGG-1 mRNA levels were seen at hour 4, dropped below basal levels at hour 8, and reached basal levels again at hour 12. The results of the 1-µM quercetin-treated group were similar to those of the positive control group, although the peak at hour 4 was not seen in this group, and this difference was not discussed. Maximum hOGG-1 levels in the 100-µM quercetin-treated group were obtained at hour 4 and gradually decreased to basal levels at the end of hour 12. These differences may be due to cell and damaging compound type and the use of a single flavonoid in the study.

NEIL-1 mRNA expressions were higher both in the study group and in the positive control group. Peaks in the positive control and study groups were seen at hours 8 and 12, respectively. In the study group, enzyme levels decreased below basal expression levels at the end of hour 24 and remained at basal levels in the positive control group. The effects of natural products on NEIL-1 mRNA expression levels have not been investigated before, and this is the first report in this field. Das et al. reported that hydrogen peroxide produced by glucose oxidase enhanced NEIL-1 mRNA and polypeptide levels 2- to 4-fold 6 h after treatment in HCT 116 cells. They interpreted this as a result of the transactivation of the NEIL-1 promoter region by ROS (15). The increases in NEIL-1 expression that started at hour 4 and persisted until hour 12 are therefore compatible with previous studies. EEP did not enhance expression in this group. This may be attributed to low DNA damage. High NEIL-1 expression compared to hOGG-1 in the t-BHP group may be due to the wide oxidized base substrate specificity of NEIL-1 or to the location of the damaged bases in the DNA strand (15,30,31).

Evaluation of comet scores indicated that EEP was quite effective in preventing DNA damage, and t-BHP-induced damage was mostly eliminated after 4 h of recovery. This antigenotoxic action may be the result of a positive effect of propolis on the antioxidant system at both activity and expression levels in a recovery time of 4 h, rather than the result of induction of the BER pathway enzymes, hOGG-1, and NEIL-1. It is also possible that pretreatment of EEP causes chelation of metal ions so that t-BHP application does not give rise to advanced Fenton reaction and DNA damage. At low concentrations, EEP protects cells from harmful chemical effects, but it is mutagenic at high concentrations. This may be ascribed to the Janus compound characteristics of propolis. This is provided by both enzyme induction in the antimutagenic system and saturation of enzyme systems in the DNA repair mechanism. Flavonoids in EEP are assessed as Janus compounds. Depending on their concentrations, they act as prooxidants or radical scavengers and exhibit mutagenic or antimutagenic activity (2,32,33). De Flora suggested that biopolyphenols exhibit antimutagenic or anticarcinogenic effects not only by scavenging radicals but also by chelating transition metals and inhibiting radical formation (34).

Additionally, increased NEIL-1 and hOGG-1 expressions in EEP-treated cells may be due to these vital DNA repair enzymes being tightly regulated. Small changes in the expression of these genes with toxic or nontoxic stimulants support this theory (25). Furthermore, if DNA damage has occurred in cells, the DNA damage checkpoint should be activated to arrest cell cycle progression until DNA is repaired (35). Additionally, the reactions of DNA repair systems are dependent on the use of ATP (36). In light of this information, EEP may play a pivotal role by decreasing ATP consumption in cells or preventing cell cycle arrest.

In conclusion, based on the comet assay results, the antigenotoxic effect of EEP may be attributed to preventing oxidative damage through radical scavenging, metal chelating, or modulation of antioxidant response, rather than increasing expression of DNA repair enzymes. EEP can be considered as a potential source of functional food and as a pharmaceutical agent.

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