Quercus pyrenaica Honeydew Honey With High Phenolic Contents Cause DNA Damage, Apoptosis, and Cell Death Through Generation of Reactive Oxygen Species in Gastric Adenocarcinoma Cells

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Abstract
Many studies have shown that honey with high phenolic contents prevents cancer formation. Furthermore, recent studies have demonstrated that honey can be used for the treatment of cancer as well as cancer prevention. Antineoplastic effects of honey are often associated with their antioxidant phenolic contents. However, very few studies have dealt with the association of phenolic contents of honeys in terms of antiproliferative effects. The aim of this study was, therefore, to elucidate the cytotoxic, genotoxic, apoptotic, and reactive oxygen species (ROS) generating effects of honey samples on the basis of their phenolic and flavonoid contents. Fourteen different honey varieties were collected from various parts of Turkey, and their characteristics regarding total phenols, flavonoids, and antioxidant contents were determined to test their effects on gastric cancer cells (AGS). For convenience, 2 honey varieties were selected, namely, Ida Mountains Quercus pyrenaica honeydew honey (QPHH-IM) having the highest phenolic and antioxidant content and Canakkale multifloral honey (MFH-C) with the lowest phenolic and antioxidant content. Levels of 11 different phenolic compounds in QPHH-IM and MFH-C samples were determined by LC-MS/MS. AGS cells were incubated with different concentrations of QPHH-IM and MFH-C for 24 hours, then the cell viability, DNA damage, apoptosis, and generation of ROS were determined. We found that QPHH-IM had more cytotoxic, genotoxic, and apoptotic effects than that of MFH-C. We think that these effects are probably related to pro-oxidant activities due to the high phenolic contents present. Therefore, further research on high-phenolic honey may contribute to the future development of cancer therapeutics.

Keywords
Quercus pyrenaica, honey, gastric carcinoma, apoptosis, DNA damage, reactive oxygen species, apitherapy

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Introduction
Gastric cancer (GC) is one of the most common cancer types and an important health problem as the second leading cause of cancer death worldwide.1 Adenocarcinoma is the most common type in approximately 90% of diagnosed GC cases. Given the current limitations in chemotherapy, radiotherapy, and surgical treatment, there is an increasing interest in complementary/alternative medicine approaches...
for gastric and other types of cancer.² The most important concern with anticancer drugs is their toxicity as side effects after treatments. However, natural compounds have been considered to be less toxic.

Honey is a natural product of honey bees, *Apis mellifera*. Honeydew honey (HH) is a type of honey obtained from the excretions of plant-sucking insects found on living parts of the plant or from their secretions from the living parts of plants.³ Polyphenolic compounds and phenolic acids found in honey vary according to geographical and climatic conditions. Some of these compounds have been reported as a specific marker for the botanical origin of honey.⁴ Due to its geographic location and ideal climatic conditions, Turkey is one of the most important producers of honey in the world.

Recently, honey has been tested and approved for its functional and biological properties such as antioxidant, anti-inflammatory, antibacterial, antiviral, anti-ulcer activities as well as antilipidemic and anticancer properties.⁵ In particular, the antioxidant properties of honey were shown to contribute to the prevention of various acute and chronic disorders such as diabetes, inflammatory disorders, and cancer.⁶ Phenolic acids and flavonoids are responsible for the antioxidant activity of honey.⁶ Flavonoids are well known to have antineoplastic effects due to their ability to scavenge free radicals.⁷ However, in recent years, researchers have focused on antiproliferative, genotoxic, and apoptotic effects as well as antioxidant and antiproliferative properties of honey. Antiproliferative effects have been demonstrated in a variety of cancer cell lines and tissues such as breast,⁸ colorectal, prostate, endometrial, and oral cancer.⁹ Furthermore, polyphenolic compounds in honey have also been considered to be one of the main factors responsible for the antiproliferative activity. However, the mechanisms of these opposite effects and their relation to the type and polyphenolic contents of honeys have not been elucidated in detail.

The aim of this study was to investigate the cytotoxic, genotoxic, apoptotic, and reactive oxygen species (ROS) generating effects of 2 different honey samples that were selected on the basis of their phenolic and flavonoid contents on GC cells.

**Materials and Methods**

**Honey Samples**

Fourteen different honey samples derived from chestnut, pine, cedar, oak, and multifloral were obtained from honey manufacturers from different regions of Turkey in 2018. The honey samples were stored at 4°C in the dark until analyses and dissolved with distilled water just before use for the biochemical and molecular analysis.

**Chemicals**

Human AGS cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Fetal bovine serum, penicillin-streptomycin (10 000 U/mL), 0.25% trypsin-EDTA, and phenol red were supplied by Life Technologies (Carlsbad, CA). Ham's F-12 K (Kaighn's) medium was obtained from Gibco/Invitrogen Corporation (Carlsbad, CA). Bax, Bel-2, caspase-3, and P-53 primer antibodies were provided by Santa Cruz Biotechnologies (Santa Cruz, CA), and ATP-Glo cell viability assay kit was provided by Promega (Madison, WI). Other chemicals such as 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH₂-DA), ethidium bromide (EB), acridine orange (AO), ninhydrin, acetic acid, aluminum chloride (AlCl₃), cadmium chloride hemi (pentahydrate), (+)-catechin, methanol sodium hydroxide (NaOH), gallic acid, L-leucine, 2,4,6-tripyridyl-S-triazine (TPTZ), sodium nitrite (NaNO₂), potassium persulfate (K₂SO₄), ferric chloride (FeCl₃), sodium chloride (NaCl), sodium carbonate (Na₂CO₃), ammonium ferrous sulfate, phosphoric acid (H₃PO₄), Coomassie Brilliant Blue, and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Chloroform, acetone, and methanol were purchased from Merck (Darmstadt, Germany). Standards in liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis were caffeic acid (98%, Sigma-Aldrich), p-coumaric acid (98% Sigma Aldrich), kaempferol (99%, Sigma-Aldrich), penduletin (95%, Supelco), apigenin (95%, Sigma-Aldrich), acacetin (95%, Sigma-Aldrich), luteolin (95%, Sigma-Aldrich), diosmetin (95%, Sigma-Aldrich), neptin (98%, Supelco), taxifolin (85% Sigma-Aldrich), and eupatilin (98%, Sigma-Aldrich).

**Determination of Total Polyphenol, Flavonoid, Antioxidant, Glucose, and Fructose Contents of Honey Samples**

The Folin-Ciocalteu method¹⁰ was used to determine total phenolic content of honey samples. One gram of honey sample was dissolved in 10 mL of distilled water and filtered through filter paper. Fifty microliters of filtered honey sample and 250 µL of 0.2 N Folin-Ciocalteu reagent was mixed with vortex and kept for 5 minutes at room temperature. Then, it was mixed with 200 µL of 0.7 mol L⁻¹ Na₂CO₃. After incubation at room temperature for 2 hours, the absorbance of the reaction mixture was measured at 760 nm against a blank using a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA). Gallic acid (0-300 mg L⁻¹) was used as standard to produce the calibration curve. The mean of 3 readings was used, and the total phenolic content was expressed in mg of gallic acid equivalents per100 g honey.

The total flavonoid content of the honey samples was determined according to colorimetric assay method developed by
Zhishen et al.11 Fifty microliters of filtered honey samples was mixed with 250 μL of distilled water and 15 μL of a 5% NaNO₂ solution. After 6 minutes, 30 μL of 10% AlCl₃ solution was added, then 100 μL 1 mol L⁻¹ NaOH was added, and the solution was incubated for a further 5 minutes at room temperature. The reaction mixture was mixed well, and the intensity of the red flavonoid-aluminum complex was measured at 510 nm using a Varioskan Flash Multimode Reader (Thermo Scientific). A standard curve of catechin was drawn within a concentration range of 5 to 50 mg/L. Total flavonoid content was expressed as mg of (+)-catechin equivalents per 100 g of honey.

The total antioxidant capacity was determined according to the photometric method developed by Erel.12 Briefly, 1 g of honey sample was dissolved by stirring in 1 mL of distilled water and then 5 μL of sample was added to 500 μL of ABTS⁺ reagent. The mixture was incubated at room temperature for 90 seconds, and the color inhibition of the ABTS⁺ radical was measured at 734 nm using a Varioskan Flash Multimode Reader (Thermo Scientific). Results were expressed in mmol trolox equivalents per 100 g of honey.

The amount of glucose present in honey samples was determined using the commercial kit working with the glucose oxidase method (Abbott Diagnostics, Lake Forest, IL). For measurement, 5 μL of sample or standard was mixed with 500 μL of reagent and incubated for 10 minutes at 37°C. The absorbance of the samples or standards was read against a blank within 60 minutes at 520 nm using a Multimode Reader (Varioskan Flash, Thermo Scientific). Results were analyzed using standard solutions (discussed later). A detailed methodology of uncertainty evaluations are available in the literature.15,16

**Measurement of Phenolic Contents of Honeys by High-Performance LC-MS/MS**

Ten grams of honey samples were extracted with 3 × 40 mL n-BuOH-water-CHCl₃. After the separation of phases, the organic phase was collected and evaporated until dryness. The residue was weighed to 10 mL in a volumetric flask and dissolved in 5 mL of MeOH in ultrasonic bath. Then, 100 μL of curcumin solution (from 100 ppm stock solution) was added as an internal standard and diluted to the volume with mobile phase, mixed and warmed to get a clear solution. The solution was filtered through a 0.45-μm Millipore Millex-HV filter, and the final solution (1 mL) was transferred into a capped auto sampler vial, from which 10 μL of sample was injected to LC for each run. The samples in the auto sampler were kept at 15°C during the experiment.14,15

LC-MS/MS experiments were performed on a Zivak Multitasker and Zivak Tandem Gold Triple quadrupole (Istanbul, Turkey) mass spectrometer equipped with a Fortis C18 column (150 × 3.0 mm id, 5 μm particle size). The mobile phase was composed of water (A, 0.1% formic acid) in water (B, 0.1% formic acid in methanol), the gradient program of which was 0 to 1.00 minute 70% A and 30% B, 1.01 to 20.00 minutes 100% B, and finally 20.01 to 25.00 minutes 70% A and 30% B. The flow rate of the mobile phase was 0.30 mL/min, and the column temperature was set to 30°C. The injection volume was 10 μL.15,16

The best mobile phase solution was determined to be a gradient of acidified methanol and water system. Such a mobile phase was found to be satisfactory for the ionization abundance and separation of the compounds. The best ionization of small and relatively polar antioxidants was obtained by electrospray ionization (ESI) source.16,17 The optimum ESI parameters were determined as 2.40 mTorr CID gas pressure, 5000.00 V ESI needle voltage, 600.00 V ESI shield voltage, 300.00°C drying gas temperature, 50.00°C API housing temperature, 55 psi nebulizer gas pressure, and 40.00 psi drying gas pressure.

During the validation experiments, curcumin was used as an internal standard. The validation parameters consisted of linearity, repeatability, recovery, limit of detection, and limit of quantification experiments. The linearity for each compound for the reported method was determined by analyzing standard solutions (discussed later). A detailed methodology of uncertainty evaluations are available in the literature.15,16

**Cell Culture Studies**

*Quercus pyrenaica* Honeydew Honey from Ida Mountains (QPHH-IM) and multifloral honey from Canakkale (MFH-C) possessing the highest and lowest phenolic, flavonoid, and antioxidant contents, respectively, were selected from 14 honey types, and cytotoxic, genotoxic, apoptotic, and ROS generating effects were tested on AGS cells via in vitro cell culture studies.

Human AGS cells are commonly used as a GC model for human stomach research. These cells were cultured in Ham’s F-12 (Kaighn’s) medium. In our study, the medium was supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. When the cells became almost confluent in 75 cm² plastic flasks, they were harvested weekly. For the experiments, the AGS cells were plated in a 96-well plate at a
density of $15 \times 10^3$ cells mL$^{-1}$ and a 6-well plate at a density of $18 \times 10^4$ cells mL$^{-1}$.

**Cell Viability Assay**

Cell Titer-Glo Luminescent Cell Viability Test Kit (Promega) was used to measure cell viability level. This method determines the degree of cell viability in proportion to the amount of ATP. For analysis, AGS cancer cells ($1.5 \times 10^5$ cells well$^{-1}$) were plated on 96-well plates. After 24 hours, the cells were incubated with different concentrations (range = 0.25% to 5% w/v) of QPH-H-IM and MFH-C. After incubation, the luciferin derivative and cell lysis solution were added as substrates. The luciferin derivative converts a light signal proportional to the current amount of ATP. Luminescence was measured using a Varioskan Flash Multimode Reader (Thermo Scientific) and normalized to control.

**Intracellular Reactive Oxygen Species Measurement**

The intracellular ROS production levels were measured by fluorometric method using a probe, 2',7'-dichlorofluorescein diacetate (H$_2$DCF-DA, Sigma, MO). Cells ($1.5 \times 10^5$ cells/well) were seeded in each well of 96-well plates. After 24 hours, they were treated with QPH-H-IM and MFH-C at different concentrations (0.25% to 5%) and incubated for another 24 hours. The cells were washed with phosphate-buffered saline (PBS) and incubated with 5 µM H$_2$DCF-DA for 30 minutes at 37°C in the dark. The cells were then washed, resuspended in PBS, and measured for the ROS contents using a fluorimeter (Varioskan Flash Multimode Reader, Thermo Scientific) and normalized to control.

**Genotoxicity Assay**

Alkaline single cell gel electrophoresis assay (Comet Assay) was carried out with a slight modification of the method of Singh et al.$^{18}$ to assess the genotoxic effects of honey on AGS cells. AGS cells were plated on 6-well cell culture plates (approximately $2 \times 10^5$ cells per well) containing cell culture medium and incubated at 37°C in 5% CO$_2$ for 24 hours. Then, the honey samples below IC$_{50}$ concentrations were added and incubated for another 24 hours. The intracellular ROS production levels were measured by fluorometric method using a probe, 2',7'-dichlorofluorescein diacetate (H$_2$DCF-DA, Sigma, MO). Cells ($1.5 \times 10^5$ cells/well) were seeded in each well of 96-well plates. After 24 hours, they were treated with QPH-H-IM and MFH-C at different concentrations (0.25% to 5%) and incubated for another 24 hours. The cells were washed with phosphate-buffered saline (PBS) and incubated with 5 µM H$_2$DCF-DA for 30 minutes at 37°C in the dark. The cells were then washed, resuspended in PBS, and measured for the ROS contents using a fluorimeter (Varioskan Flash Multimode Reader, Thermo Scientific) and normalized to control.

**Immunoblotting Analysis**

AGS cancer cells were seeded on 6-well plates at $1.5 \times 10^5$ cells per well and incubated for 24 hours. They were then treated with honeys according to their IC$_{50}$ values. After 24 hours of incubation, the cells were harvested and prepared in NP-40 cell lysis buffer (2 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, and 0.2% NP-40 plus a protease inhibitor cocktail) for 30 minutes on ice and centrifuged at 14 000 × g (Beckman Coulter, Krefeld, Germany) for 10 minutes at 4°C. The final supernatant was then used as the cytosolic fraction. The protein concentration of the supernatant was determined using the Bradford protein assay method.$^{24}$ Proteins from cellular supernatants were separated on 8% to 10% polyacrylamide gel and transferred to a nitrocellulose membrane using the Trans-blot SD semipermeable electrophoretic transfer cell (Bio-Rad, Hercules, CA). Tris-HCl buffered saline with Tween 20 (TBST) with 5% nonfat milk were used for blocking the membrane. The primary antibodies, P-53, caspase-3, Bax, Bcl$_2$, and NF-κB (1/500 dilution),
were used after a night incubation (4°C). All samples were also blotted for β-actin to normalize protein amounts. TBST was used for washing the membrane and incubated with horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA) for another hour. Immunolabelled proteins were visualized with Pierce ECL Western staining substrate (Thermo Scientific) and captured with an imaging system (Vilber Lourmat Sté, Collégien, France).

**Statistical Data Treatment**

The experiments were repeated 3 times, and the results were expressed as mean value ± standard deviation (mean ± SD). Statistical evaluation was performed using analysis of variance (1-way ANOVA). Differences with a probability value of $P < .05$ were considered statistically significant. IC$_{50}$ values of honeys over the cell lines were calculated by nonlinear regression analysis. The statistical analysis was performed by using Statistical Package for Social Sciences (SPSS) version 21.

**Results**

**Total Phenol, Flavonoids, Antioxidant, and Carbohydrate Contents**

Total phenol and flavonoid contents of 14 different honey samples were compared in terms of phenol, flavonoid, and antioxidant content as well as glucose and fructose. From the different honey types, QPHH-IM showed the highest activity while MFH-C showed the lowest activity (Table 1). Hence, these 2 honey types were selected for further analysis.

Sugar is known to affect cell proliferation. Therefore, glucose and fructose contents of all honeys were also measured to exclude the possible effects of sugar on the cells. The results of the glucose and fructose contents and fructose/glucose ratios of the honey samples are presented in Table 2.

As shown in Table 2, there was no significant difference in terms of glucose and fructose levels between QPHH-IM and MFH-C; these honey types exhibited the highest and lowest phenolic, flavonoid and antioxidant contents demonstrated with boldface.

**Cell Viability Assessment**

The cell viability test was performed with AGS cells for 24 hours to evaluate the effect of QPHH-IM and MFH-C on cell growth. After incubation, the cytotoxic effect of honey was measured by ATP cell viability test. When cell viability of the control cells was accepted as 100%, the cell viability increased

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Table 1. Total Phenol and Flavonoid Contents and the Antioxidant Capacity for 14 Different Honey Samples. Ida Mountains Quercus pyrenaica Honeydew Honey (QPHH-IM), Chestnut Honeydew Honey (CNHH), Pine Honeydew Honey (PHH), Multifloral Honey (MFH).

| Honeys          | Total Phenolic Content (mg GAE/100 g) | Total Flavonoid Content (mg QE/100 g) | Total Antioxidant Capacity (Inhibition of ABTS %) |
|-----------------|---------------------------------------|---------------------------------------|--------------------------------------------------|
| CNHH (Düzce)   | 79.96 ± 6.02                          | 45.39 ± 4.80                          | 85.96 ± 0.35                                     |
| CNHH (Bursa)   | 89.52 ± 5.51                          | 48.38 ± 8.00                          | 86.75 ± 0.36                                     |
| CNHH (Rize)    | 84.50 ± 4.01                          | 46.52 ± 3.12                          | 85.81 ± 0.22                                     |
| CNHH (Balikesir) | 75.36 ± 5.10                        | 48.43 ± 5.95                          | 85.73 ± 0.19                                     |
| CNHH (Kastamonu) | 98.83 ± 10.15                      | 53.59 ± 3.02                          | 86.07 ± 0.15                                     |
| CNHH (Kocaeli) | 76.36 ± 6.15                          | 46.31 ± 4.85                          | 85.66 ± 0.29                                     |
| QPHH-IM        | **115.41 ± 9.95**                     | **77.36 ± 7.25**                      | **89.36 ± 0.16**                                 |
| MFH (Havran)   | 90.36 ± 4.15                          | 47.37 ± 4.95                          | 85.98 ± 0.05                                     |
| PHH (Muğla)    | 77.63 ± 7.51                          | 45.16 ± 5.01                          | 85.71 ± 0.18                                     |
| PHH (IM)       | 78.40 ± 6.95                          | 49.84 ± 4.65                          | 86.07 ± 0.24                                     |
| MFH (Çanakkale) | **67.66 ± 2.87**                      | **42.69 ± 2.71**                      | **84.09 ± 0.20**                                 |
| MFH (Balikesir) | 74.36 ± 3.95                          | 54.25 ± 4.15                          | 88.23 ± 0.17                                     |
| MFH (Bayburt)  | 88.36 ± 3.95                          | 52.84 ± 2.61                          | 88.68 ± 0.06                                     |
| MFH (Black Sea)| 73.56 ± 5.00                          | 48.13 ± 3.95                          | 88.39 ± 0.05                                     |

*The significant difference between total phenol, flavonoid and antioxidant contents of QPHH-IM and MFH. Honey samples with highest and lowest phenolic, flavonoid and antioxidant contents demonstrated with boldface.
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Table 2. Glucose, Fructose, Fructose + Glucose Levels in 100 g Honey Samples and Fructose/Glucose Ratio of the Samples.

| Honeys          | Glucose (g %) | Fructose (g %) | Glucose + Fructose (g %) | Fructose/Glucose Ratio |
|-----------------|---------------|----------------|-------------------------|------------------------|
| CNHH (Düzce)    | 23.76 ± 1.5   | 35.9 ± 0.8     | 59.70 ± 2.1             | 1.5 ± 0.1              |
| CNHH (Bursa)    | 23.83 ± 1.3   | 41.8 ± 0.7     | 65.67 ± 2.0             | 1.8 ± 0.2              |
| CNHH (Rize)     | 22.45 ± 0.9   | 23.5 ± 0.6     | 45.96 ± 1.8             | 1.0 ± 0.1              |
| CNHH (Balıkesir)| 26.49 ± 1.2   | 15.4 ± 0.5     | 41.85 ± 1.7             | 0.6 ± 0.1              |
| CNHH (Kastamonu)| 14.66 ± 0.8   | 22.3 ± 0.4     | 36.95 ± 1.3             | 1.5 ± 0.3              |
| CNHH (Kocaeli)  | 32.46 ± 1.4   | 26.8 ± 0.5     | 59.25 ± 1.8             | 0.8 ± 0.2              |
| QPHH-IM         | 33.98 ± 1.5   | 34.2 ± 0.7     | 68.16 ± 2.1             | 1.0 ± 0.3              |
| MFH (Havran)    | 28 ± 1.2      | 65.5 ± 1.1     | 93.47 ± 2.2             | 2.3 ± 0.2              |
| PHH (Muğla)     | 21.71 ± 1.3   | 28.1 ± 0.9     | 49.77 ± 1.9             | 1.3 ± 0.1              |
| PHH (IM)        | 44.75 ± 1.8   | 21.5 ± 0.7     | 66.27 ± 3.1             | 0.5 ± 0.2              |
| MFH (Çanakkale) | 32.9 ± 1.5    | 33.6 ± 0.8     | 66.50 ± 2.5             | 1.0 ± 0.3              |
| MFH (Balıkesir) | 25.72 ± 1.4   | 36.5 ± 1.1     | 62.24 ± 2.8             | 1.4 ± 0.2              |
| MFH (Bayburt)   | 19.81 ± 1.3   | 28.6 ± 1.2     | 48.41 ± 2.1             | 1.4 ± 0.1              |
| MFH (Black Sea) | 33.98 ± 1.2   | 33.1 ± 1.3     | 67.08 ± 2.0             | 1.0 ± 0.2              |

Abbreviations: CNHH, chestnut honeydew honey; QPHH-IM, Ida Mountains Quercus pyrenaica honeydew honey; MFH, multifloral honey; PHH, pine honeydew honey. Selected two honey samples according to the total phenol and flavonoid content showed with boldface.

Table 3. Validation and Uncertainty Parameters of LC-MS/MS Method.

| Compound      | Linear Regression | R²  | Recovery | LOD/LOQ (mg/kg) | U₉₅ (%) | QPHH-IM (mg/kg) | MFH-C (mg/kg) |
|---------------|-------------------|-----|----------|-----------------|---------|----------------|---------------|
| Salicylic acid| y = +0.2121x + 0.04| 0.99| 94.3     | 0.7/3.5          | 18.2    | 60.4           | 4.4           |
| Caffeic acid  | y = +0.2543x + 0.01| 0.96| 92.8     | 1/5.0            | 20.6    | 6.0            | <LOQ          |
| Kaempferol    | y = +0.0095x - 0.00| 0.96| 93.3     | 0.3/1.5          | 12.1    | 15.2           | <LOQ          |
| Penduelin     | y = +0.1385x - 0.00| 0.99| 100.1    | 0.6/3.1          | 7.8     | 1.2            | <LOQ          |
| Apigenin      | y = +0.1329x + 0.05| 0.98| 99.7     | 1.1/6.0          | 10.8    | 5.9            | <LOQ          |
| Acacetin      | y = +0.6369x + 0.07| 0.98| 95.3     | 1.2/6.0          | 5.7     | 7.0            | 2.5           |
| Luteolin      | y = +0.2217x + 0.03| 0.98| 99.8     | 0.7/3.5          | 4.2     | 3.7            | <LOQ          |
| Diosmetin     | y = +1.1820x + 0.32| 0.98| 100.2    | 0.6/3.0          | 3.8     | 0.7            | <LOQ          |
| Taxifolin     | y = +0.0735x + 0.00| 0.97| 91.8     | 3.1/15.0         | 10.1    | 4.2            | <LOQ          |
| Eupatilin     | y = +0.5231x + 0.07| 0.98| 96.2     | 0.9/4.0          | 15.7    | <LOQ           | <LOQ          |
| Nepetin       | y = +0.3282x - 0.06| 0.98| 100.1    | 2.2/11.0         | 10.6    | 0.6            | <LOQ          |

Abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; QPHH-IM, Ida Mountains Quercus pyrenaica honeydew honey; MFH-C, Canakkale multifloral honey.

to 108% at a concentration of 0.25% relative to the control 24 hours after addition of the honeys. At doses above this concentration, cytotoxic activity increased in a concentration-dependent manner (*P < .001). Higher doses of QPHH-IM resulted in greater cellular death than in MFH-C in AGS cells (*P < .05). The IC₅₀ concentrations were calculated as 17 and 45 mg/mL (1.7% and 2.5% w/v) for the QPHH-IM and for the MFH-C in AGS cells, respectively (Figure 1). These data indicate that both QPHH-IM and MFH-C have proliferative effects at lower concentrations; on the other hand, QPHH-IM with high phenolic content was more cytotoxic than MFH-C with low phenolic content at their higher concentration.

Reactive Oxygen Species Generation Assessment

We measured intracellular ROS formation by fluorometric method using the H₂DCF-Da probe. Low dose of honey samples (0.25%) decreased the intracellular ROS production in cancer cells (*P < .05). However, ROS production significantly increased at higher doses of the samples (1.5% for QPHH-IM and 5% for MFH-C [Figure 2]). There were close negative relationships between cell viability and ROS generating activity in both honeys (*r = −0.839, P < .001, for QPHH-IM and r = −0.853, P < .001, for MFH-C in AGS cells).

Genotoxic Assessment

For DNA damage analysis, AGS cells were treated with different doses of honey samples for 24 hours and the DNA damage was measured via the Comet Assay method. Damaged DNA appears in a bright head and comet, while undamaged DNA appears to be only round. After incubation, the % tail intensity significantly increased with the
increasing doses of honey samples and, when compared with MFH-C, DNA damage levels were significantly higher in QPHH-IM at higher concentrations (Figure 3).

These findings indicate that DNA damage level in cancer cells is related to the honey sample concentrations and their phenolic contents.

**Apoptosis Assessment**

Apoptosis is important in determining tumor formation and resistance to treatment. In our study, we performed AO/EB double staining and Western blot methods in order to evaluate apoptotic effects of different concentrations of honeys in cancer cells. AGS cells were incubated with both honey for 24 hours to demonstrate the morphological characteristics of apoptosis on cells. Cells were then stained with AO/EB double staining and examined under fluorescence microscopy (Figure 4). As shown in Figure 4, after 24 hours of incubation, as the administered dose of both honeys increased, the green-looking viable cell ratios decreased and the yellow-orange–looking apoptotic cell ratios increased. High doses of QPHH-IM caused more apoptosis in cancer cells than MFH-C (Figure 4).
Western Blotting Results

In order to investigate the relationship between the cyto-toxic effects of honeys and apoptosis signaling pathways, the expression levels of P-53, caspase-3, Bax, Nf-κB, and Bcl-2 proteins were analyzed by the Western blotting method. For this purpose, AGS cells were treated with honey at different concentrations for 24 hours. Cell extracts were used for Western blotting. The β-tubulin was used as control. The results showed that both MFH and QPH increased expression levels of P-53, caspase-3, and Bax protein in AGS cancer cells, while decreasing expression levels of Nf-κB and Bcl-2 (Figure 5).

However, expression levels were significantly higher in QPHH-IM than in MFH-C at increasing honey sample concentrations.

Discussion

The biological properties of honeys have been studied extensively. While most of the previous studies related to honey’s antioxidant and free radical scavenging properties,
studies on prooxidant properties have recently increased.\textsuperscript{22} In general, these adverse effects have been associated with phenolic contents of honey. However, the mechanism(s) of these opposite effects have not yet been fully understood. In addition, there is no study investigating the relationship between the therapeutic effects of honey on gastric adenocarcinoma cells and their phenolic contents. Some studies consider that dark honeys, including the HH, tend to have higher amounts of phenolic compounds,\textsuperscript{23,24} but studies on this issue are doubtful. Therefore, we selected these 2 types of honey (QPHH-IM and MFH-C) based on total phenol, flavonoid, and antioxidant contents from 14 different honeys. In addition, we also measured 11 different phenolic compounds in both QPHH-IM and MFH-C samples. These results demonstrated that total and separately measured phenolic compound levels supported each other and that QPHH-IM has a very rich content relative to MFH-C in terms of phenolic compounds.

Cancer cells have a different metabolism than normal cells, and glucose is the primary source of energy for the growth and proliferation of these cells.\textsuperscript{25} Diets that are high in sugar may potentially cause a metabolic switch from oxidative phosphorylation to glycolysis in tumor cells, which confers the ability to grow in hypoxic environments, fuels tumor growth and invasion, and prevents apoptosis.\textsuperscript{26} Carbohydrates are the main constituents, comprising about 95% of the honey dry weight, and the main sugars are the monosaccharides fructose and glucose.\textsuperscript{27} To rule out the possible effects of sugar on the cells, we measured the glucose and fructose content of honeys before cell culture analysis. There was no significant difference between QPHH-IM and MFH-C in terms of glucose and fructose content. Therefore, we think that biological effects of both honeys on AGS cells may be caused by other substances such as non-sugar phenolic compounds.

We investigated cytotoxic, genotoxic, and apoptotic activities and ROS production capacity in human gastric adenocarcinoma cells in order to understand the relationship between phenolic contents and antiproliferative effects. We have demonstrated that QPHH-IM inhibit cell proliferation significantly at concentrations as low as 0.4% (w/v) in AGS cells and antiproliferative activity increased in a dose-dependent manner. We found 50% inhibition after 24 hours incubation of AGS cells with the 1.7% final concentration for QPHH-IM and 2.5% for MFH-C using ATP-Glo cell viability assay kit. The well-known honeys that have antiproliferative activity on different cancer cells are manuka honey,\textsuperscript{28} tualang honey,\textsuperscript{29} and gelam honey.\textsuperscript{30} Cytotoxic doses of these honeys were quite different and vary according to the cell types. Fernandez-Cabezudo et al\textsuperscript{31} found inhibition of cell proliferation at final concentrations of 0.6% manuka honey. They found 40% inhibition after 24 hours incubation of MCF-7 cells with 5% final concentration of honey. Tualang honey was also shown to exhibit antiproliferative
levels of ROS increase cell proliferation. On the other hand, toxic effect on cancer cells. It has been shown that low doses of honeys cause DNA damage. The vast majority of the studies indicate that high doses of honeys in GC-MFH-C in AGS cells. As far as we know, there is no prior experimental evidence that high doses of honeys in GC-MFH-C in AGS cells. Generally, it has been known that honeys have prooxidant activity of honey. In fact, there is supportive evidence that ROS may have a proliferative or cytoxic effect on cancer cells. It has been shown that low levels of ROS increase cell proliferation. On the other hand, high levels of ROS increase DNA damage, apoptosis, and cell death. Normally, phenolic compounds are antioxidants and may inhibit oxidative damage as a consequence of their ability to inhibit ROS. Under certain conditions, however, such as low pH, high phenolic concentrations, and the presence of redox-active transition metals (Fe and Cu), phenolic compounds exhibit prooxidant activity. In particular, the Cu²⁺ concentration in cancer cells is higher than normal cells, making them more susceptible to the prooxidant activity of phenolic compounds. In the presence of Cu²⁺, the prooxidant activity of phenolic compounds is supposed to progress via generating OH⁻ radical in a Fenton-type reaction, which eventually leads to DNA damage and apoptosis in cancer cells.

DNA damage has been investigated in order to understand the mechanisms by which high doses of honeys in cancer cells cause cytotoxicity. We used the comet assay technique to measure the genotoxic effects of both honeys on AGS cells. This is one of the most important methods for the evaluation of DNA damage of different active substances in different cells. In this study, we found that QPHH-IM levels above 0.5% caused DNA damage and the same QPHH-IM doses resulted in higher DNA damage than MFH-C in AGS cells. As far as we know, there is no prior experimental evidence that high doses of honeys in GC cells cause DNA damage. The vast majority of the studies have been concerned with the protective effects of honey on DNA damage. However, it has been shown that gelam and tualang honeys induced DNA damage in different cancer cells in a dose-dependent manner. Our results are consistent with the results of these studies.

Morphological, biochemical, and molecular changes related to apoptosis in cells can be measured by different methods. In the study, apoptosis was analyzed by AO/EB double staining. Apoptotic, necrotic, and living cells can be distinguished by this method. The results of the present work revealed that while high doses of honeys increased apoptosis in AGS cancer cells, QPHH-IM with high phenolic content induced apoptosis more than that of MFH-C with low phenolic content. It has been reported that the anticancer drugs can kill the cancer cells by stimulating the apoptotic pathways. Phenolic compounds can affect the cellular redox status because of their prooxidant properties. This can lead to cell death as a result of DNA damage and apoptotic activity. Our results are consistent with the results of other recent studies. The vast majority of chemotherapists used in cancer treatment show their effects by inducing apoptosis. Although the mechanism has not yet been fully understood, studies have shown that honey has antiproliferative effects by inducing apoptosis in cancer cells as well as by multiple cell signaling pathways. A recent study to understand honey’s molecular mechanism of colon cancer cell growth inhibition has shown that honey-induced apoptosis upregulates P-53 and is accompanied by modulating the expression of proapoptotic and antiapoptotic proteins. Our results are similar to previous studies. We also found that both honey species cause a decrease in Bcl-2 signal expression and an increase in apoptotic P-53, Bax, and caspase-3 signal expression levels. These effects of QPHH-IM were more pronounced than MFH-C, especially at high doses.

NF-κB has an important role in the regulation of intracellular signal transduction and protein expression of various genes in the cell nucleus. Exceptional NF-κB activation is associated with the stimulation of proliferation and protection against apoptosis in malignant cells. Recent studies have focused on the inhibitory effect of honey on inflammatory-mediated NF-κB activation. We also showed that NF-κB expression levels decreased with the increasing concentrations of honey, and the degree of inhibition with QPHH-IM was significantly higher than MFH-C, especially at high doses. These results show that honey has not only inhibitory effect on inflammation but also anti-proliferative effect on cancer cells.

Conclusion

Data showed that low concentrations of honey samples had proliferative effects due to their antioxidant activity, whereas high concentrations had cytotoxic, genotoxic, and apoptotic effects due to their prooxidant activities in cancer cells. All these effects were higher with QPHH-IM application.
possessing the high phenolic content when compared with that of MFH-C, which possesses low phenolic content on AGS cancer cells. These preliminary results suggest that high-phenolic honey may contribute to the future development of cancer therapeutics.

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