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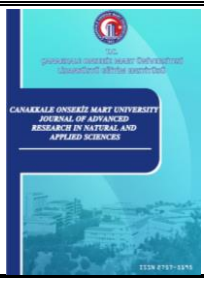

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Effects of Pervari Honey from Türkiye on Proliferation, Oxidative Stress, and Apoptosis of Human Breast Cancer Cells

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Abstract − Breast cancer is one of the most common causes of deaths worldwide. Major obstacles to treatment have contributed to the increasing popularity of complementary or alternative therapies. Although recent results support the mechanism that honey induces cell death, the full mechanisms are still unknown. This study investigates the potential use of Pervari honey (PH) as an in vitro therapeutic agent in breast cancer. Firstly, the antioxidant capacity and total phenolic content of PH were tested. In addition, MCF-7 and MDA-MB-231 breast cancer cells treated with PH were examined for cell viability, reactive oxygen species production, oxidative deoxyribonucleic acid (DNA) damage, and apoptosis. Our results show that PH treatment decreased cell viability dose-dependently and increased reactive oxygen species (ROS) levels, oxidative DNA damage, and apoptosis rate. The present study suggests that honey is a promising source to produce pharmaceuticals and nutraceuticals for breast cancer therapy.

Keywords – *Antioxidant, apoptosis, cancer, honey, oxidative* deoxyribonucleic acid (*DNA*) *damage, reactive oxygen species*

1. Introduction

Cancer is a disease that disrupts the natural mechanisms that control cell survival, proliferation, and differentiation. The process of cancer progression involves irreversible deoxyribonucleic acid (DNA) damage caused by the accumulation of mutant DNA, the excessive growth of mutant cells and the accumulation of abnormal cells in the form of tumors as a result of advanced genomic alterations, and finally, the spread of cancer cells during the metastatic process [1]. Although almost every organ in the body is susceptible to cancer, the most commonly affected are the liver, colon, and breast. Breast cancer is the most common cancer in women worldwide, with 2.3 million new cases in 2020 and 685,000 deaths [2]. However, most of the chemotherapeutic agents currently in use are not very effective. They may lose their efficacy due to the emergence of resistance and their numerous side effects [3]. Scientists have focused on the potential use of natural products to develop fewer toxic alternatives to chemotherapy and radiation for cancer.

There are numerous molecular subtypes of breast cancer cells, such as triple negative or positive [4]. The cells lacking the expression of both steroid receptors are the source of triple-negative breast cancer cells [5]. Human triple-negative breast cancer cells are modeled by the MDA-MB-231 cell line [6]. MCF-7 cells, on the other hand, are hormone-dependent triple-positive breast cancer cells that have estrogen receptors on the cell surface

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[7]. The proliferation of these cells is induced by estrogen [8]. The ER-positive subtype of breast cancer is the most common form of the disease. Due to its high estrogen receptor (ER) expression and extreme hormone sensitivity, MCF-7 is the most commonly used breast cancer cell line in research [9]. These two cell lines were used in our study to examine both subtypes of breast cancer.

Recently, many natural products containing a wide variety of molecular targets, including transcription factors, cytokines, chemokines, adhesion molecules, growth factor receptors, and inflammatory enzymes, and exhibiting novel structural features and mechanisms of action have been intensively studied in a variety of cancer cell lines because of their potential to be a good and cost-effective source of novel pharmacological structures [10-12]. In addition, combining natural products with established chemotherapeutic treatments has significantly improved patient survival as cancer cells respond better to chemotherapy and radiotherapy [13]. Honey, used as a natural ingredient in complementary and alternative medicine, stands out as a valuable bee product that contains many biologically active compounds with high antioxidant capacity and other health properties that make it a suitable source for exploring medicinal potential [14, 15]. In recent years, several benefits of honey have been reported, such as anti-inflammatory [16, 17], antimicrobial [18, 19], and antioxidant [20, 21] effects. In addition, the anticancer effects of honey have been demonstrated in various cancers, including renal [22], prostate [23], endometrial [24], cervical [25], and breast [26, 27] cancers.

Research into honey as a possible preventive and therapeutic supplement is gaining momentum. Numerous studies investigate the anticancer properties of different types of honey from different sources against various cancer cell lines and tissues [28]. The bioactive components of honey are generally consistent with its chemopreventive abilities. Certain studies have demonstrated the interference of bioactive chemicals in honey with pro-apoptotic, antioxidant, and anti-proliferative cell signaling pathways; however, the exact mechanism underlying this interference remains unclear [29].

The biochemical composition and pharmacological activity of honey varies from country to country and even from region to region within the same country due to geographic conditions, climate, and floral sources [30]. In Türkiye, which is the second largest honey producer in the world, with a production of 104.08 thousand tons in 2020 [31], there are different types of honey, such as chestnut, acacia, pine, and flower honey, due to the rich biodiversity of flora. Pervari honey (PH), produced by bees feeding on the rich flora consisting of hundreds of endemic plants such as clover, sage, and thyme and given at altitudes between 1600 meters and 2000 meters, is considered one of the best quality honey in Türkiye [32]. Although there are many studies in the literature on the anticancer properties of honey from different parts of the world [1], the number of studies on the anticancer and apoptotic effects of honey from Türkiye is limited [23]. This is the first study to investigate the cytotoxic effect of PH and its ability to induce apoptosis in human breast cancer cell lines. In this study, we hypothesize that PH has an apoptotic effect on breast cancer cells and can be used in the treatment plan for breast cancer.

2. Materials and Methods

2.1. Honey Sample

PH sample was kindly obtained from Erkan Bilen, Pervari Honey Cooperative, Siirt, Türkiye. The honey was stored in the dark at room temperature. PH was freshly dissolved in the complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin at a final concentration of 100 mg/mL and sterilized with a 0.2 μm syringe filter before being added to cells.

2.2. Total Phenolic Content

The total phenolic content of PH was measured using the Folin-Ciocalteu method described by Singleton and Rossi [33]. Briefly, 20 μL of the honey solution (100 mg/mL in distilled water) was combined with 100 μL of Folin-Ciocalteu reagent (1:10) and followed by 80 μL of 15% sodium carbonate. After mixing the ingredients,

the absorbance at 700 nm was measured. The results were reported as milligram gallic acid equivalents per kilogram honey (mg GAE⁄kg honey) following (2.1) using a standard gallic acid concentration (5-500 µg/mL) curve.

$$C = (C1 \times V) \div m \tag{2.1}$$

Here, C is total phenolic content (mg GAE ⁄ kg honey), $C1$ is gallic acid concentration obtained from the standard curve graph (mg⁄mL), V is honey solution volume (mL), and m is the honey weight (kg).

2.3. DPPH Assay

The antioxidant activity of PH was determined using the 2,2-diphenyl-1-picrylhydrazyl hydrate radical (DPPH) assay, according to Cheng et al. [34]. The honey sample was diluted in methanol at 1.5 to 100 mg/mL concentrations, and 0.1 mL from each dilution was mixed with 0.1 mL of DPPH (0.2 mM in methanol). The mixtures were left in the dark at room temperature for 30 minutes after gently shaking, and the absorbance was determined at 517 nm. The radical scavenging activity (RSA) of the honey (100 mg/mL) was calculated according to the following (2.2) and expressed as percent inhibition. Butyl hydroxytoluene (BHT) (100 mg/mL) was used as a positive control.

$$\%\ Radical\ scavenging\ activity = (1 - As/Ac) \times 100 \tag{2.2}$$

Here, As is the absorbance of the honey sample, and Ac is the absorbance of the control.

2.4. Cell Culture

Human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, and USA). They were propagated in DMEM/F12, supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. All cell passages used were between passages 20 and 30.

2.5. Cell Viability

Anti-proliferative activity of PH as a cytotoxic agent in MCF-7 and MDA-MB-231 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [35]. Briefly, cells were seeded in a culture medium at a density of 5×10^3 cells/well in 96 flat-bottomed well plates. After 24 hours of plating, honey samples were added at final concentrations ranging from 10-60 mg/mL in DMEM. After 48 hours of incubation, the medium was replaced with MTT dissolved in the medium at a final concentration of 0.5 mg/mL for a further 4 hours at 37°C. Then, the MTT-formazan was solubilized in DMSO, and the optical density was measured at 570 nm and a reference wavelength of 620 nm. Cytotoxic activity was calculated from the following (2.3):

$$\%\ Cell\ viability = (As \div Ac) \times 100 \tag{2.3}$$

Here, As is the absorbance of the PH-treated cells, and Ac is the absorbance of the untreated cells. The viability of the control cells was assumed to be 100%, and the viability of PH-treated cells was compared with that of the control, and the IC50 values of PH were determined for MCF-7 and MDA-MB-231 cells.

2.6. Flow Cytometric Analysis of Apoptosis

The FITC Annexin V Apoptosis Detection Kit with 7-AAD detected apoptotic cells (Biolegend, 640922). The cells were seeded in a 24-well plate with a density of 5×10^4 cells per well and treated with the IC50 doses of PH and Doxorubicin for 48 hours. Doxorubicin was used as a standard cytotoxic agent. Cells were harvested

and rinsed with 1× PBS after incubation. The cells were resuspended in 200 µL of Annexin V Binding Buffer and transferred to a new test tube with 100 µL of cell suspension. Cells were gently vortexed and kept for 15 minutes at room temperature in the dark after adding 5 µL of FITC Annexin V and 5 µL of 7-AAD viability staining solutions. Finally, 400 mL of Annexin V Binding Buffer was added to each tube, and a flow cytometer was used to analyze the cells (FACS Aria III, BD Biosciences, USA). The results were expressed as a percentage of early, late, and total apoptotic cells.

2.7. Intracellular ROS Production

The intracellular ROS generation levels were determined using dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent probe. The cells were seeded at a density of 1×10^4 cells per well in a 96-well plate. They were subsequently given the IC50 doses of PH and Doxorubicin for 48 hours. Doxorubicin was used as a standard cytotoxic agent. After incubation, the medium was removed, and cells were treated with 10 µM DCFH-DA for 30 minutes at 37 ^oC. The fluorescence intensity was measured using a microplate reader at 485 nm for excitation and 530 nm for emission wavelengths. The results are expressed as a percentage of the untreated control value, with the mean absorbance normalized. H_2O_2, a well-known ROS-inducing toxin, was used as a positive control to ensure the assay provided the predicted results.

2.8. Oxidative DNA Damage

The 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA kit (Fine Test, #EU2548, Wuhan Fine Biotech Co., Ltd., China) was used for quantitative detection of 8-OHdG in the supernatant of the cells. After the cells were treated with IC50 doses of PH and Doxorubicin for 48 hours, the ELISA assay was performed according to the manufacturer's protocol. Doxorubicin was used as a standard cytotoxic agent. The target concentration of the samples was interpolated from the standard curve obtained from the measurement of 450 nm absorbance.

2.9. Statistical Analysis

The cell viability (%) for each concentration of PH was calculated and expressed as a percentage relative to the control response. IC50 values of PH were also calculated. All values are expressed as the mean ± standard deviation of three (MTT assay) measurements. Statistical analysis was performed using a t-test two-tailed distribution, assuming two-sample unequal variance. $p < 0.05$ value was considered statistically significant (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). Data was analyzed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1. Total Phenolic Content

Phenolic compounds can inhibit cell proliferation and induce cell death in various cancer cell lines [36-39]. In this study, phenolic contents were expressed as total phenolic acid contents. The total phenolic content in PH was found to be 0.71 mg GAE/g honey by plotting the gallic acid standard calibration curve (R^2=0.9995). When comparing the contents with other studies, the content determined for PH is quite higher than those of other Turkish honey such as multifloral, chestnut, acacia, rhododendron (0.02-0.26 mg GAE/g honey; [20], clover, lavender, lime, astragalus (0.25-0.53 mg GAE/g honey; [40]), and lower than those of others such as chestnut, cedar, pine honey (31.6-98.6 mg GAE/g honey; [41]), chestnut, heather, oak (0.98-1.20 mg GAE/g honey; [40]), pine, thyme, citrus honey (0.88- 10.93 mg GAE/g honey; [42]). Considering these studies, it is clear that the phenolic content of honey samples varies in quality and quantity depending on the botanical origin and nectar source.

3.2. Antioxidant Activity

Antioxidants are important reducing substances that scavenge ROS. They have been reported to inhibit the growth of various cancer cells [43]. Honey samples from different regions have been shown to have antioxidant activity. Still, the number of antioxidants they contain is influenced by their botanical origin, variety, processing, handling, and storage [44, 45]. In this study, the antioxidant activity of PH was determined using the DPPH radical scavenging assay. PH showed higher activity (IC50=41.33 mg/mL) than the positive control BHT (IC50=58 mg/mL). In a study in which 16 different types of honey from Brazil were examined, the results of the DPPH test varied between 8.20 ±0.16 and 62.12 ±0.13 mg/mL IC50 value [21]. One recent study reported that the DPPH assay of chestnut honey samples from different regions in Türkiye was found between 6.32 ±0.35 and 17.06 ± 1.30 mg/mL IC50 value [46]. Therefore, it can be concluded that honey's antioxidant effect can vary depending on the phytochemical content, flower source, and geographical origin.

3.3. The Effect of Pervari Honey on the Proliferation of MCF-7 and MDA-MB-231 Cells

The effect of PH on the viability of MCF-7 and MDA-MB-231 cells was determined using the MTT assay, which indirectly measures the effect of a substance or extracts on cell survival and proliferation. The use of PH on MCF-7 and MDA-MB-231 cells at different concentrations over 48 hours caused decreased cell viability in a dose-dependent manner (Figure 1). PH treatment resulted in a significant decrease in cell viability at 50 and 55 mg/mL (IC50 doses) concentrations in MCF-7 and MDA-MB-231 cells, respectively, compared to the control group.

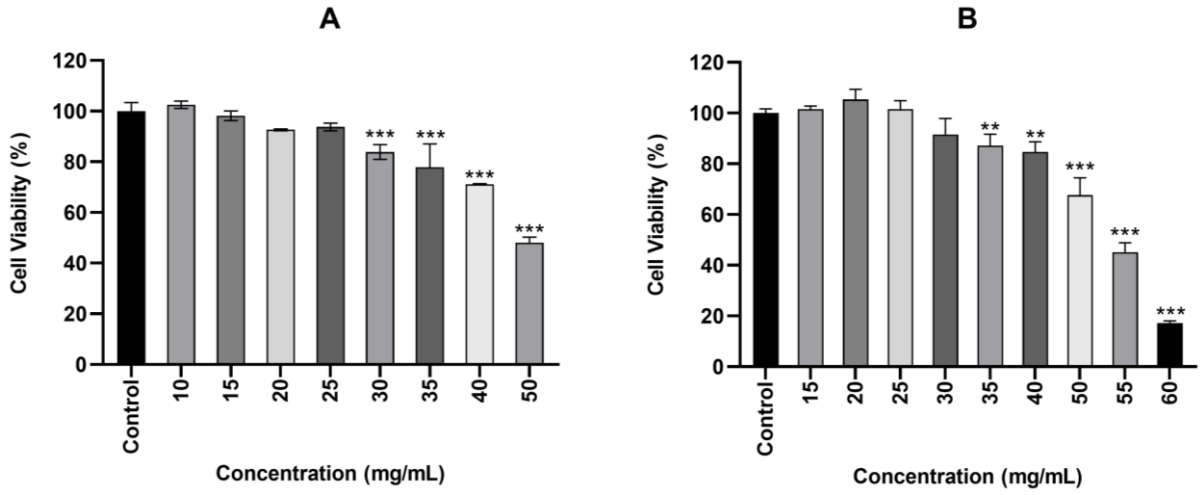

Figure 1. (A) Cytotoxicity evaluation of Pervari honey on MCF-7 cells using MTT assay. (B) Cytotoxicity evaluation of Pervari honey on MDA-MB-231cells using MTT assay. Untreated cells served as control. All the values are presented as mean ± SEM (n=3). The significance of difference is demonstrated as **p< 0.01 and ***p< 0.001 in comparison to the control group

Based on conclusive data from previous studies investigating the cytotoxic effects of different types of honey on breast cancer cell lines, we performed a PH treatment for 48 hours [41, 47]. In view of studies showing that polyphenols and phenolic acids inhibit cancer-related signaling pathways and processes [48, 49], the chemical composition of honey could be responsible for the different effects of various types of honey on viability/proliferation.

3.4. Apoptosis Rate by Flow Cytometry Analysis

Apoptosis is a gene-controlled and programmed cell death characterized by physical changes such as the breakage of chromosomal DNA, swelling of the plasma membrane, and shrinkage of the cell. One of the most important mechanisms of cancer drugs is the induction of apoptosis. For this reason, researchers have focused

on the potential use of natural and synthetic chemicals for novel cancer treatments to increase the sensitivity and efficacy of drug-induced apoptosis. In flow cytometric analyses, propidium iodide, which binds to cellular DNA to detect apoptotic and necrotic cells at a very late stage, and the fluorescent antibody annexin V, which binds to phosphatidylserine, were used to detect apoptotic cells. In this study, after treatment with 50 and 55 mg/mL PH for 48 hours, PH mostly induced early apoptosis of 33.7% and 10.5% in MCF-7 and MDA-MB-231 cells, respectively.

In contrast, the percentage of apoptosis in the control group was 15.3% and 1.46% in MCF-7 and MDA-MB-231 cells, respectively, as shown in Figure 2. Doxorubicin treatment was more effective than PH in inducing apoptosis, with 59.6% and 29.5% early apoptosis rates for MCF-7 and MDA-MB-231 cells, respectively. Low necrotic cell rates (<4 %) were also observed in all cell groups (Figure 2).

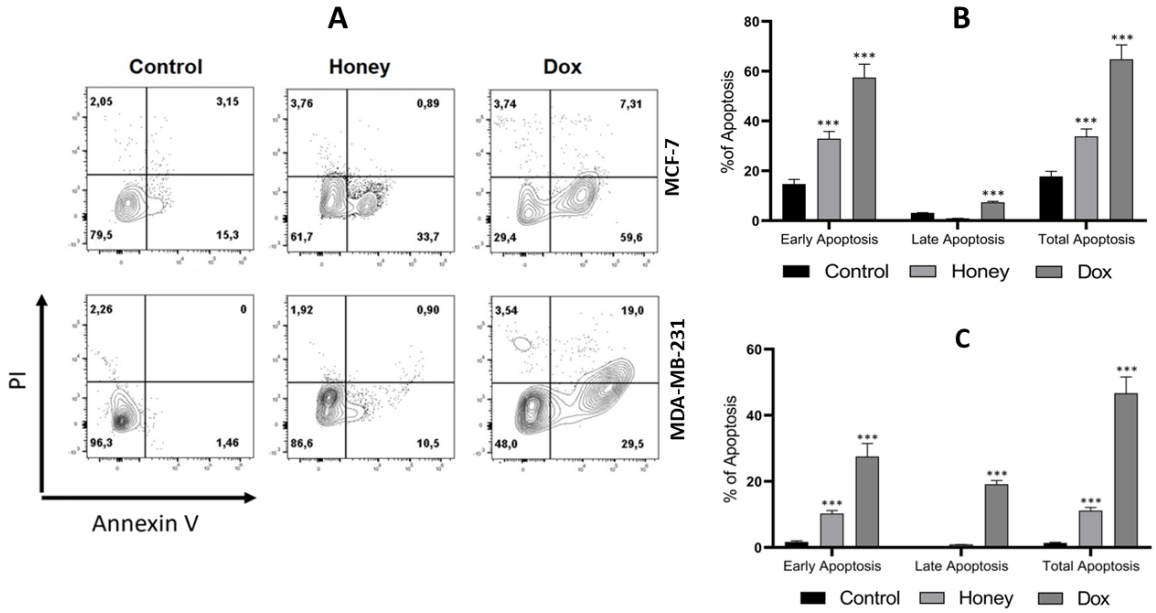

Figure 2. Apoptotic effect of Pervari honey treatment on breast cancer cells by flow cytometry. (A) The histogram represents the percentage of viable (left bottom), early apoptotic (right bottom), late apoptotic (right-top), and necrotic cells (left top). (B) The apoptotic ratio for MCF-7 cells after treatment with 50 mg/mL PH for 48 hours. (C) The apoptotic ratio for MDA-MB-231 cells after treatment with 55 mg/mL PH for 48 hours. The apoptotic ratio was determined by Annexin V FITC/PI assay. The bars represent mean values ± SEM of three independent experiments. The significance of the differences compared to the control group is indicated with $^{***}p<0.0001$

It has previously been reported that a Malaysian honey, Tualang, significantly reduced the proliferation of human breast cancer cells MCF-7 (55.6%) and MDA-MB-231 (51.2%) and there was not much difference between the total apoptosis rate of the two cell lines [47]. However, in our study, the total apoptosis rate of MCF cells (34.6 %) was significantly higher than that of the MDA-MB-231 cell line (11.4 %). While the apoptotic effect of PH and Tualang honey was similar in MCF-7 cells, Tualang honey showed a significantly stronger apoptotic effect in MDA-MB-231 cells. Many research groups have studied the anticancer effect of honey from different regions of Türkiye on breast cancer cell lines [50, 51]. The study conducted by Seyhan et al. [41] showed that 85 % and 90 % of MCF-7 cells died after 48 hours of treatment with chestnut honey at a dose of 2.5 and 5 µg/mL, respectively, whereas 34.6% apoptotic cell death was observed in MCF-7 cells at PH dose of 50 mg/mL in our study. These studies show that honey's doses and effects vary depending on the source and geographical region from which it is obtained.

3.5. ROS Production

This study investigated the effect of PH on ROS production in breast cancer cells using the DCFH-DA assay. PH treatment with IC50 doses of PH and Doxorubicin for 48 hours significantly increased ROS production in

both cell lines (Figure 3). In normal cells, low levels of ROS contribute to cell proliferation [52]. Previous studies have shown that elevated ROS levels cause oxidative damage and trigger cancer cell death in many cancers, including breast cancer [53]. Therefore, the inhibitory effect of PH on cancer cell proliferation may be partly mediated by ROS-dependent mechanisms. Furthermore, PH caused higher ROS levels in MCF-7 cells than in MDA-MB-231 cells, and high ROS levels may also be associated with higher apoptosis rates in MCF-7 cells (Figure 2B). This result was similar to previous findings reported that cell apoptosis could be induced by stimulated ROS overexpression [54,55].

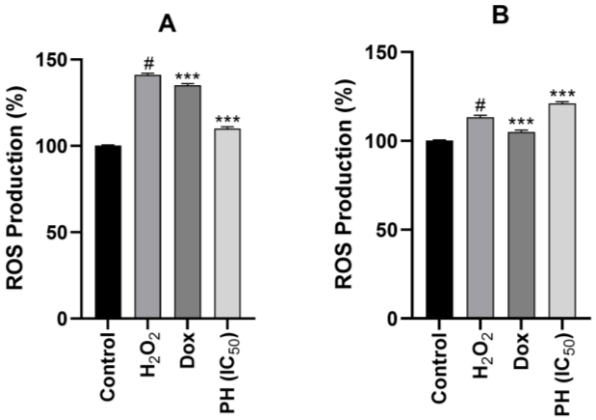

Figure 3. (A) Effect of treatment with Pervari honey on ROS production in MCF-7 cells. (B) Effect of treatment with Pervari honey on ROS production in MDA-MB-231 cells. Untreated cells served as control. All the values are presented as mean ± SEM (n=3). The significance of the difference is demonstrated as $^{\#}p<0.001$ compared to the control group and $^{***}p < 0.001$ compared to the H_2O_2 group

Reactive oxygen species (ROS) are by-products of mitochondrial metabolism and redox signaling [56]. If ROS homeostasis becomes unbalanced due to an excessive accumulation of ROS, this can lead to the development of various diseases, such as neurodegenerative diseases, cardiovascular diseases, and cancer. Depending on their concentration, ROS have a dual effect on cancer. Higher levels of ROS have been found to play a role in tumorigenesis and have a chemotherapeutic effect in suppressing cancer growth by promoting apoptosis and cell death [57].

Honey may also prevent cancer growth by regulating oxidative stress, i.e., by enhancing or inducing oxidative stress. The anticancer effects of honey, which it exerts via antioxidants or pro-oxidants, appear to depend entirely on the level of oxidative stress in cancer cells. If cancer cell survival depends on low levels of ROS and oxidative stress, honey acts as a pro-oxidant, thereby increasing ROS and oxidative stress [58]. In addition, few studies have addressed the effect of honey on intracellular ROS production to induce apoptotic cell death in cancer cell lines [59–61].

3.6. Oxidative DNA Damage

The increased level of 8-hydroxyguanosine (8-OHdG) indicates oxidative DNA damage [62]. Oxidative stress was triggered either by the production of ROS or by the depletion of intracellular antioxidant defense mechanisms. Antioxidants protect against oxidative stress and can effectively scavenge free radicals [63]. In the present study, after treatment with IC50 doses of PH and Doxorubicin for 48 hours, PH was found to increase 8-OHdG levels by causing oxidative DNA damage in MCF-7 and MDA-MB-231 cells. In both cell lines, 8-OHdG levels were close to each other in the PH-treated group, 49 and 51.5 ng/mL for MCF-7 and MDA-MB-231 cells, respectively.

Furthermore, compared to the control group, the increase in the MDA-MB-231 cells ($p<0.001$) was significantly higher than in the MCF-7 cells ($p < 0.05$) (Figure 4). Contrary to our results, Musarrat et al. [64] found that the ER-positive cell line MCF-7 showed a 9.3-fold higher 8-OHdG level than the ER-negative cell line MDA-MB 231. The changes in the genome of breast cells are probably caused by the oxidative attack of

ROS resulting from estrogen-induced oxidative stress in combination with the receptor-mediated proliferation of the damaged cells [65]. However, the higher amount of oxidative DNA damage in MDA-MB-231 cells suggests that the stimulation of proliferation in triple-negative breast cancer with poor prognosis may be associated with non-estrogen factors, although estrogens stimulated proliferation and DNA damage in breast cancer [66]. Moreover, PH-induced elevated 8-OHdG levels in MCF-7 and MDA-MB-231 cells could contribute to increased apoptotic rates in these cells, and PH may have potential in the treatment of breast cancer.

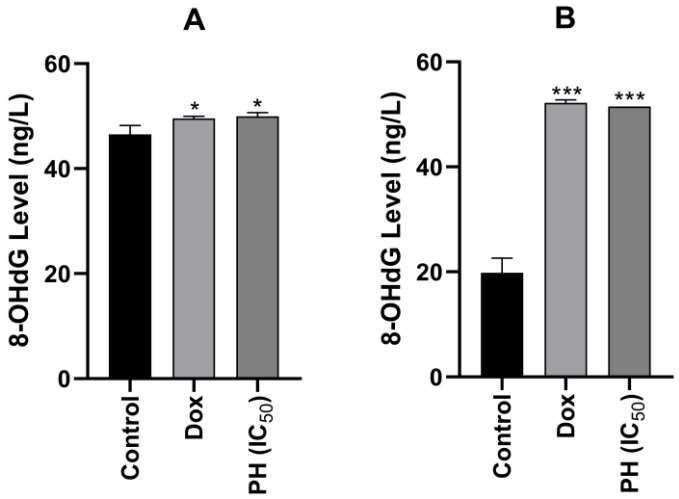

Figure 4. (A) Effect of Pervari honey on oxidative DNA damage in MCF-7 cells. (B) Effect of Pervari honey on oxidative DNA damage in MDA-MB-231 cells. 8-OHdG ELISA assay was performed to measure oxidative DNA damage. Untreated cells served as control. All the values are presented as mean ± SEM (n=3). The significance of the difference is demonstrated as $^{*}p< 0.05$ and $^{***}p< 0.001$ in comparison to the control group

4. Conclusion

PH is the best-selling honey in Türkiye. However, the effect of PH on breast cancer is still unknown, and there is no information about the phenolic profiles of the honey. This study investigated the anticancer effects and phenolic profiles of PH for the first time. PH may be a promising candidate for studies to develop targeted therapies and reduce certain excessive adverse effects of current approaches. These results suggest that PH has great potential for preventive and rehabilitative use for treatment and further experiments are needed to explore the signaling pathways.

Author Contributions

The first and second authors devised the main conceptual ideas and developed the theoretical framework. The first, second, and third authors performed the experiment and statistical analyses. The fourth author supported the study for methodology and software usage. The fifth author directed and supervised this study. The first author wrote the manuscript with support from the second and fifth authors. The fifth author reviewed and edited the paper. All authors read and approved the final version of the paper.

Conflicts of Interest

All the authors declare no conflict of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

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