Protective Effect of Bee Products Against Oxidative Damage in Erythrocytes

Eritrositlerde Oksidatif Hasara Karşı Arı Ürünlerinin Koruyucu Etkisi

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ABSTRACT

The positive effects of bee products on human health are due to their antioxidant composition. However, their possible protective efficacy against t-butylhydroxyperoxide (t-BHP)-induced oxidative damage on human erythrocytes has not been investigated. Our aim in this study is to investigate whether propolis, pollen and royal jelly have protective efficacy against t-BHP-induced oxidative damage in isolated erythrocytes. Propolis and pollen samples collected from various regions of Turkey were extracted by mixing with various solvents (water and ethanol). Commercially purchased royal jelly was also extracted with water. The erythrocytes collected from 15 volunteers were centrifuged and packaged by washing with isotonic saline. All RBC packets were pooled together. The final erythrocyte packet was divided into seven groups as control, water extract of propolis, ethanolic extract of propolis, water extract of pollen, water extract of royal jelly, positive control (quercetin) and t-BHP groups. Erythrocytes were first treated with extracts of bee products and then t-BHP was added. Protective activities of bee products were investigated by malondialdehyde (MDA), total oxidant capacity (TOS), total antioxidant capacity (TAS), superoxide dismutase (SOD) and catalase (CAT) activities. Statistically, one way ANOVA and post-hoc Tukey tests were applied. As a result of the study, it was found that all bee products contributed to keeping MDA levels close to the control group. ($p<0.05$). Again, while TOS levels of all bee products decreased compared to t-BHP, TAS levels increased ($p<0.05$). No significant effect of bee products on SOD and CAT enzyme activities was observed. ($p>0.05$).

Keywords: Antioxidants, Bee products, Erythrocyte, Oxidative stress

ÖZ

Arı ürünlerinin insan sağlığı üzerindeki olumlu etkileri, antioksidan bileşimlerinden kaynaklanmaktadır. Ancak insan eritrositleri üzerinde t-butilhidroksiperoksit (t-BHP) kaynaklı oksidatif hasara karşı olası koruyucu etkinlikleri araştırılmamıştır. Bu çalışmadaki amacımız propolis, polen ve arı sütünün, izole edilen eritrositlerde t-BHP indüklü oksidatif hasara karşı koruyucu etkinliğe sahip olup olmadığını araştırmaktır. Türkiye'nin çeşitli bölgelerinden toplanan propolis ve polen örnekleri çeşitli solventler (su ve etanol) ile karıştırılarak ekstrakte edildi. Ticari olarak satın alınan arı sütü de su ile ekstrakte edildi. 15 gönüllüden toplanılan eritrositler santrifüj edildi ve izotonik tuzla yıkanılarak paketlendi. Tüm RBC paketleri bir araya toplandı ve kontrol, sulu propolis ekstraktı, etanollü propolis ekstraktı, sulu polen ekstraktı, sulu arı sütü ekstraktı, pozitif kontrol (kuersetin) ve t-BHP ile olmak üzere yedi gruba ayrıldı. Eritrositler ilk olarak arı ürünlerinin ekstraktları ile muamele edilmiştir ve daha sonra t-BHP eklenmiştir. Arı ürünlerinin koruyucu etkinlikleri, malondialdehit (MDA), toplam oksidan kapasite (TOK), toplam antioksidan kapasite (TAK), süperoksit dismutaz (SOD) ve katalaz (KAT) aktiviteleri ile araştırıldı. İstatistiksel olarak, ANOVA ve post-hoc Tukey testleri uygulandı. Çalışma sonucunda tüm arı ürünlerinin MDA seviyelerini kontrol grubuna yakın seviyelerde kalmasına katkı sağladığı bulunmuştur. ($p<0.05$). Yine tüm arı ürünlerinin t-BHP grubuna göre TOK düzeylerini düşerken TAK düzeyleri ise yükselmiştir ($p<0.05$). Arı ürünlerinin SOD ve KAT seviyeleri üzerinde önemli bir etkisi gözlenmedi ($p>0.05$).

Anahtar Kelimeler: Antioksidan, Arı ürünleri, Eritrosit, Oksidatif stres

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INTRODUCTION

In the recent years, side effects of synthetic drugs used in the treatment of diseases and resistance to these drugs have led people to the consumption of natural products. Natural products are promising sources for new pharmacological discoveries. One of the most widely used of these natural products is bee products. Bee products are used in many diseases, they are used to prevent the progression of the disease, reduce pain and treat the disease. The use of bee products for therapeutic purpose is called as apitherapy. Apitherapy is the use of bee products such as honey, pollen, royal jelly, propolis, bee venom and beeswax for medical purposes and is as old as beekeeping.[1-5] Propolis is collected from various resinous trees and plants by bees; due to content of flavonoids and phenolics, it has antibacterial, antiviral, antitumoral effects.[6] Pollen is a powder-like natural bee product with a content of phytochemicals, produced by bees from flowering plants mixing with nectar and bee secretions, and has different physiological and pharmacological activities.[7-10] Royal jelly is a special bee product made and secreted in the hypopharingeal and mandibular glands of honey bees. In scientific studies, pharmacological activities of royal jelly such as antioxidant, neurotrophic, hepatoprotective, hypotensive, antitumoral, antibiotic and anti-inflammatory have been reported.[11] In biological systems, atoms or molecules containing one or more unpaired electrons are called free radicals. Active oxygen derivatives of free radicals are also called oxidants.[12] These oxidants can be used in living organisms with antioxidant enzyme systems such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which are cytoplasmic, mitochondrial and extracellular forms. They are eliminated by antioxidants such as ceruloplasmin, transferrin, reduced glutathione (GSH), ascorbic acid and α-tocopherol.[13, 14] In the living system under normal conditions, oxidants and antioxidants are in a balance. However, the situation that occurs with the increase of oxidant substances in the living system endogenously or exogenously is called oxidative stress.[15] As a result of the reaction of increased free radicals with deoxyribonucleic acid (DNA), base and sugar modifications, base deletions and chain breaks have been reported to cause cell mutation, cellular dysfunction, and death.[16] As a result of the autoxidation of monosaccharides, hydrogen peroxide, peroxides and oxoaldehydes are formed. These are stated to bind to DNA and proteins and form cross-links.[17] Proteins are also sensitive to free radical damage. The effect of free radicals on proteins varies according to the amino acid content of the proteins. More damage occurs in sulfhydryl and amino groups on protein molecules with the effect of free radicals. However, proteins containing amino acids such as tryptophan, tyrosine, phenyl alanine, histidine, methionine, lysine and cysteine are more sensitive. It has been reported that proteins lose function as a result of their interaction with free radicals.[18, 19] Free radicals react, especially with membrane lipids, and cause the formation of MDA, the end product of lipid peroxidation, leading to membrane dysfunction and cell death.[20] t-BHP an organic hydroperoxide, is a compound frequently used in studies involving oxidative cell damage mechanisms. It has been demonstrated that organic hydroperoxides are formed by the addition of an oxygen to the alkyl radicals and / or the removal of a hydrogen atom from the peroxyl radicals. Alkoxyl or peroxyl radicals may be occured by t-BHP which accelerates chain reactions of lipid peroxidation. The reactions take place through metal ions and their complexes. The proposed mechanisms of action for the causes of t-BHP induced toxicity are as follows; changes in intracellular calcium balance followed by a decrease in glutathione and protein thiol levels, formation of DNA single strand breaks, initiation of lipid peroxidation or generation of tertiary butoxyl radicals, genotoxicity.[21] Erythrocytes prevent the

damage of reactive oxygen species or to reduce it by using its enzymatic and non-enzymatic effective antioxidant systems. When this enzymatic system could not scavenge the free radicals formed, imbalance of ROSs destroys lipids and proteins. The outside of the erythrocyte membranes is rich in phosphatides and proteins and is a very important target for free radicals. Increased MDA level results in changes in cell membrane polarity, charge sharing on the lipid phase surface and oligomer formation. The change between the enzymatic system and oxidative stress in the erythrocyte is associated with many special pathological conditions.[22] Erythrocyte cells are used due to easily obtaining method, therefore they may used for studies of protection mechanism of various natural products. Bee products are considered as a potential source of natural antioxidants that may improve the effects of oxidative stress underlying the pathogenesis of various diseases. Bee products show their capacity to eliminate free radicals with their phenolic compounds.[23] Our aims in the present study are (i)if bee products (propolis, pollen and royal jelly) may protect erythrocytes due to their antioxidant contents or not and (ii) which concentrations are effective for them.

MATERIALS AND METHODS

Ethical Approval

Ethical approval was obtained (Date: 01.12.2016, Decision No: 24237859-673) from Karadeniz Technical University (KTU) Faculty of Medicine Scientific Research Ethics Committee Presidency.

Preparation of Erythrocyte Packets and Extracts of Bee Products

Blood samples were taken from 15 subjects working in KTU Medical Biochemistry Research Laboratory (no smoking, alcohol use, drug use, chronic illness) into three EDTA tubes. The blood taken was centrifuged at 1690 g (Eppendorf - 5810) for 10 minutes and the plasma was removed. Isotonic saline solution up to three times of their volume was added to the blood cells and then centrifuged. After the leukocyte and platelet cells were removed, the erythrocyte package was isolated. Prepared erythrocyte packets were pooled and stored at +4 °C. The hematocrit (Hct) and hemoglobin (Hb) values of the erythrocyte packet were measured in the Biochemistry Laboratory of KTU, Faculty of Medicine. It was diluted with 0,9% NaCl so that the hematocrit ratio was 10%. Each propolis and pollen samples collected from various regions of Turkey were mixed, and mixing propolis sample was grinded to obtain a powder. 5 g of powdered propolis was added to 100 mL distilled water and to 100 mL 70% ethanol (sigma aldrich: 34852 Germany), 0,5 g pollen to 10 mL distilled water. They were subjected to incubate for at least 24 h at 60 °C with continuous shaking (nüve SL 350). At the end of the period, extracted bee products from the shaker incubator were filtered through a filter paper and then passed through 0.45 µm sterile filters (agilent technologies econofilter, USA), extracts of propolis and pollen at a concentration of 50 mg/mL were prepared. 4 g of commercial royal jelly (Fanus food company, Trabzon, Turkey-fo109) was weighed and added to 8 mL of distilled water. Royal jelly extract with a concentration of 50 mg/mL was prepared by taking the supernatant part by centrifuging at 10,000 g (Beckman coulter – allegra 64R) for 30 minutes.

Determination of Optimum t-BHP Concentration

In order to find the t-BHP concentration to be used in the experiment, different concentrations (0,50,100,200,400,750,1500 µM) of t-BHP (sigma-aldrich-41665) (dissolved in phosphate buffered solution (PBS), 0,5 M, pH=7,4) were added to erythrocyte packets and left to incubate at 37 °C for 1 h. Later, MDA measurement was

performed [24] (Versamax Molecular Devices, Sunnyvale, CA, USA) in the erythrocyte packets and the optimum t-BHP concentration was selected as 750 µM, which caused a significantly higher damage compared to as 0 concentration (p <0,05).

Determination of Optimum Concentrations of Bee Products

Various concentrations bee products (5-400 µg/mL for water extract of propolis, 50-500 µg/mL for ethanolic extract of propolis, 1-10 mg/mL for water extract of pollen, and 10-40 mg/mL for water extract of royal jelly), and 1-10 µg/mL quercetin (sigma-aldrich-Q4951) [(dissolved with dimethyl sulfoxide (DMSO) (Merck-472301)] as a positive control were treated to the erythrocyte packets and left to incubate for 2 h at 37 °C. Afterwards, 750 µM t-BHP was treated to all bee products and quercetin solutions, and incubated at 37 °C for 1 h. MDA measurement was performed for all treatments. Statistically significant (p <0,05) concentrations according to the lowest MDA results were selected (200 µg/mL for water extract of propolis, 100 µg/mL for ethanolic extract of propolis, 5 mg/mL for water extract of pollen, 30 mg/mL for water extract of royal jelly, 2 µg/mL for quercetin).

Experimental Groups

The final erythrocyte package was divided into seven groups as control (only was treated with PBS) , water extract of propolis, ethanolic extract of propolis, water extract of pollen, water extract of royal jelly, positive control (quercetin) and t-BHP groups.

Biochemical Analyses

Except for the control and the t-BHP groups, extracts of bee products and quercetin in determined optimum concentrations were firstly added to erythrocyte packets and incubated at 37 °C for 2 h. Afterwards, the experimental protocol was completed by adding t-BHP at the determined concentration (750 µM) to all groups except the control group and incubating again at 37 °C for 1 h. Then, MDA[24], TOS[25] and TAS[26] assays (Rel Assay Diagnostics, Gaziantep, Turkey) were performed. SOD and CAT enzyme activities were measured from the remaining supernatant after the removal of erythrocyte membranes. [27, 28] Finally, Oxidative stress index (OSI) levels were calculated with the TOS/TAS ratio.

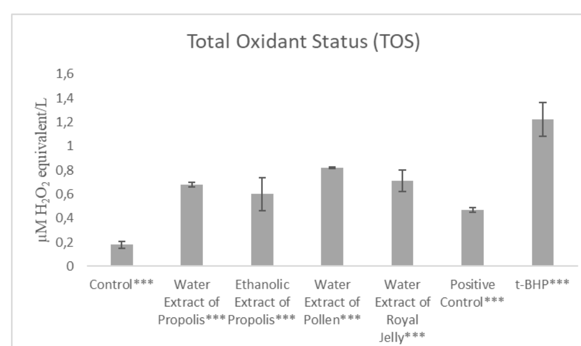
Statistical Analysis

The data were transferred to SPSS 23 (Statistical Package for the Social Sciences) computer package program and evaluated statistically. One way ANOVA and post-hoc Tukey tests were used for the evaluation of more than two independent groups that fit the normal distribution.

The values obtained were expressed as mean ± standard deviation (x ± SD) and p<0,05 was considered as statistically significant.

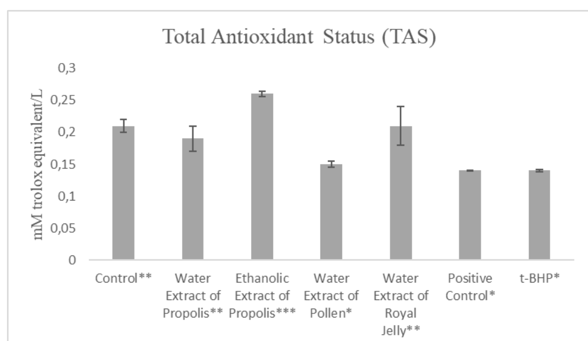
RESULTS AND DISCUSSION

The effects of bee products on antioxidant parameters in human erythrocytes damaged by t-BHP at the concentrations determined in the preliminary study (data not shown) are shown in for TOS (Figure 1), for TAS (Figure 2), for MDA (Figure 3), for SOD (Figure 4) and for CAT (Figure 5). Besides, the Oxidative Stress Index (OSI) was calculated and the results are given in Figure 6.



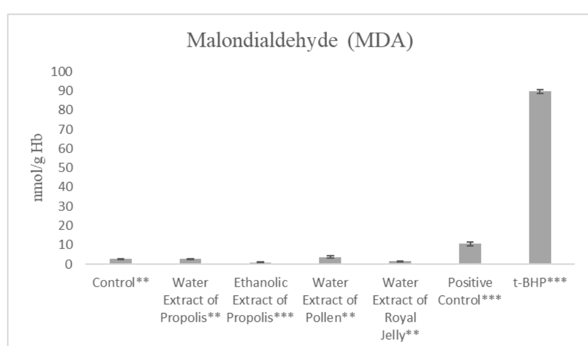
*One-Way ANOVA p=0,0001 ***There is a significant difference compared to both control and t-BHP (p<0,05).*

Figure 1. Effect of Bee Products on TOS in t-BHP-Induced Erythrocyte Damage



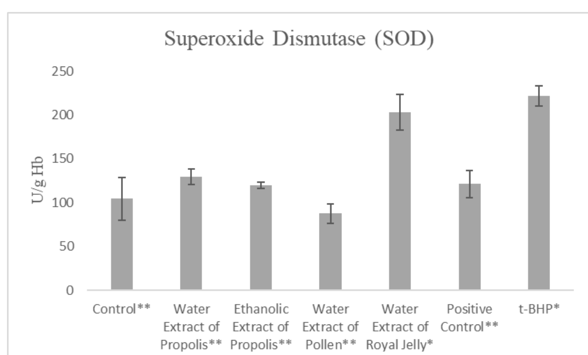
*One-Way ANOVA p=0,0001 *There is a significant difference compared to control (p<0,05), **There is a significant difference compared to t-BHP (p<0,05), ***There is a significant difference compared to both control and t-BHP (p<0,05).*

Figure 2. Effect of Bee Products on TAS in t-BHP-Induced Erythrocyte Damage



*One-Way ANOVA p=0,0001 **There is a significant difference compared to t-BHP (p<0,05), ***There is a significant difference compared to both control and t-BHP (p<0,05).*

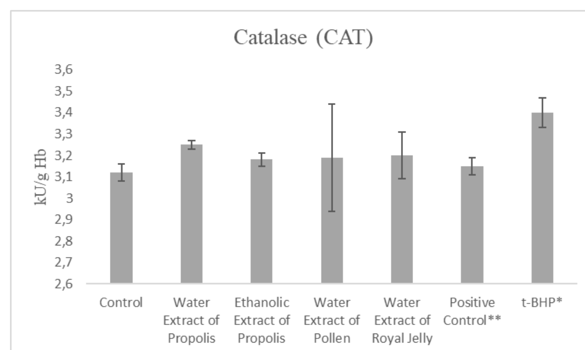
Figure 3. Effect of Bee Products on MDA in t-BHP-Induced Erythrocyte Damage



*One-Way ANOVA p=0,0001 *There is a significant difference compared to control (p<0,05), **There is a significant difference compared to t-BHP (p<0,05).*

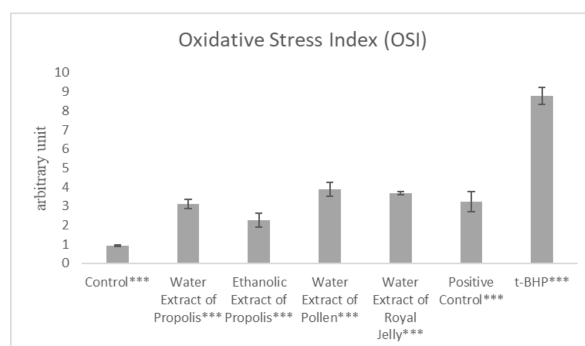
Figure 4. Effect of Bee Products on SOD in t-BHP-Induced Erythrocyte Damage

MDA levels of groups treated with bee products approached to control levels. All bee products decreased TOS levels and increased TAS levels according to those of t-BHP.



*One-Way ANOVA p=0,001 *There is a significant difference compared to control (p<0,05), **There is a significant difference compared to t-BHP (p<0,05).*

Figure 5. Effect of Bee Products on CAT in t-BHP-Induced Erythrocyte Damage



*One-Way ANOVA p=0,0001 ***There is a significant difference compared to both control and t-BHP (p<0,05).*

Figure 6. OSI Levels of Bee Products in Erythrocyte

Statistacally significant effects of bee products on levels of SOD and CAT were not observed. All bee products prevented the increase of OSI value in erythrocytes and showed protective activity (p<0,05). Free radicals are atom or molecules that have one or more unpaired electrons. Because of this structure, they are highly unstable and therefore very reactive and attack other biomolecules in order to become stable. As a result, it is accepted that the damage of functional biomolecules contributes to the development of chronic and degenerative diseases such as cancer, autoimmune disorders, aging, cataracts, rheumatoid arthritis, cardiovascular and neurodegenerative diseases.[29] According to the radical theory in human physiology, it is claimed that free radicals are involved in almost all cellular degradation processes and lead to cell death. Antioxidants are molecules that can slow down or prevent the oxidation

of other molecules and thus prevent such changes.[30] Many scientific studies have noted that bee products have a wide variety of beneficial health effects, including antioxidant, antibacterial, anti-inflammatory, antitumor, antiviral properties, and others.[31, 32] One of the most important properties of bee products is their antioxidant capacity, where they contribute to the prevention of some diseases by protecting cells against oxidative damage caused by various free radicals. In our study, we first treated the erythrocyte cells with bee products and then treated them with t-BHP and carried out MDA, TAS, TOS, SOD and CAT measurements. As a result of our study, protective effects of bee products against the formation of MDA, which is the final product of lipid peroxidation, have been revealed. In addition, TOS values were positively correlated with MDA, while TAS values were negatively correlated (data not shown). Although there was a significant difference in the determination of SOD and CAT enzyme activities, no statistically significant result was found. There was only a significant difference between control and t-BHP. The extraction of bioactive compounds depends on the type and quantity of solvent, on temperature and time, and on the process used to interact with raw propolis, pollen and royal jelly. A recent study revealed that propolis prevents erythrocyte membrane fragility in individuals with hereditary spherocytosis (HS) disease.[33] In a study by Mujica et al. reported that the daily consumption propolis solution [(3% propolis dissolved in propylene glycol) (twice daily, 15 drops each time, 90 days)] are effectiveness on humans, they found that serum MDA levels decreased, GSH and High-density lipoprotein (HDL) levels increased.[34] On the other hand, in a study conducted by Jasprica et al. on the consumption of propolis (48.75 mg once a day, 30 days) of people, decreased MDA and increased SOD activities were found in men, while no significant differences in women.[35] A recent study investigating the effectiveness of water extract of propolis against cerebral ischemia-induced oxidative damage in mice revealed that SOD and MDA levels decreased and GPx levels were increased.[36] Ishikawa et al, reported that MDA, which is an indicator of lipid peroxidation, reduces plasma concentration in oral administration of pollen to mice [37]. Slamenova et al, showed that t-BHP-induced SOD enzyme activity significantly increased in human HepG2 cells compared to normal cell lines [38]. Bonamigo et al, reported that the protective efficacy of ethanolic extracts of Brazilian propolis against lipid peroxidation induced by 2,2'-azobis (2-aminopropane) hydrochloride (AAPH) in human erythrocytes has been demonstrated [39].

CONCLUSION AND RECOMMENDATIONS

The following conclusions have been reached with this study: i) Bee products protect erythrocytes from oxidative damage without affecting the activities of antioxidant enzymes, ii) Propolis (with ethanol and water) is effective at the level of 100-200 µg/ml, iii) 5 mg/ml level for pollen and royal jelly is effective. It was concluded that bee products protect RBCs from oxidative damage. This study can be evaluated with the same method in different cell suspensions or cell culture with different oxidant molecules and contribute to the literature. Instead of the protective effect of bee products, the curative effect can be evaluated by modifying the method. As a result of this study, it is predicted that the production and marketing strategies of bee products will develop.

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