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ANTIOXIDATIVE EFFECT OF TURKISH CHESTNUT BEE POLLEN ON DNA OXIDATION SYSTEM AND ITS PHENOLIC COMPOUNDS

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ABSTRACT
Chestnut bee pollen is a high antioxidative natural bee product. In this study the phenolic compounds in chestnut bee pollen were determined by HPLC-DAD system. Ethanol, water and methanol were used for the extraction of antioxidant compounds from chestnut bee pollen. The total phenolic contents and antioxidant capacities of extracts were determined by Folin-Ciocalteu, CHROMAC (Cr(VI) reduction antioxidant capacity) and FRAP (ferric reducing antioxidant power) methods, respectively. When compared the amounts of phenolic compounds and spectroscopic results (total phenolic contents and antioxidant capacities), the most suitable solvent was found ethanol for the extraction of antioxidant compounds from bee pollen. The major phenolic compounds in the chestnut bee pollen were determined as pinocembrin, chrysin, galangin and hyperoside with the contents of 1.246, 0.332, 0.122 and 0.516 mg/g of bee pollen, respectively. And also it was determined that the chestnut bee pollen was inhibited 11% of DNA oxidation in Fenton medium.
Keywords: Chestnut bee pollen, HPLC, CHROMAC, DNA oxidation, phenolic compounds

TÜRK KESTANE ARI POLENİNİN DNA OKSİDASYON SİSTEMİ ÜZERİNDEKİ ANTİOKSİDAN ETKİSİ VE FENOLİK BİLEŞİKLERİ

ÖZ
Kestane arı poleni antioksidan özelliği yüksek doğal bir arı ürünüdür. Bu çalışmada kestane arı poleninde bulunan fenolik bileşikler HPLC-DAD cihazı ile tayin edilmiştir. Kestane poleninden antioksidan bileşiklerin ekstraksiyonu için etanol, su ve metanol çözücüleri kullanılmıştır. Ekstraktların toplam fenolik madde içeriği ve antioksidan kapasiteleri sırasıyla Folin-Ciocalteu, CHROMAC (Cr (VI) indirgen antioksidan kapasite) ve FRAP (demir iyonu indirgeyici antioksidan güç) yöntemleri ile belirlenmiştir. Fenolik madde miktarları ve spektroskopik sonuçlar (toplam fenolik miktarları ve antioksidan kapasiteleri) karşılaştırıldığında arı poleninden antioksidan bileşiklerin ekstraksiyonu için en iyi çözücünün etanol olduğu bulunmuştur. Pinocembrin, chrysin, galangin ve hyperoside sırasıyla 1.246, 0.332, 0.122 and 0.516 mg/g örnek içeriği ile kestane arı poleninde bulunan başlıca fenolik bileşiklerdir. Bununla birlikte Fenton ortamında, kestane poleninin DNA oksidasyonunu %11 oranında önlediği belirlenmiştir.
Anahtar kelimeler: Kestane arı poleni, HPLC, CHROMAC, DNA oksidasyonu, fenolik bileşikler

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INTRODUCTION

Bee pollens are one of the bee products collected by honeybees that have natural nutrient content. Bee pollens are known to contain amino acids, proteins, hormones, enzymes, carbohydrates, minerals, fats, vitamins, phytochemicals and antioxidant substances (Bogdanov, 2004; Eraslan et al., 2010). At the same time bee pollens that have high nutritional and medical value are use in food, cosmetic and medicine industry. Bee pollens are known to be used in the treatment of colds, flu, ulcers, premature aging, anemia, colitis, allergic reactions, enteritis and chronic prostatitis (Kao et al., 2011). Bee pollens are also a rich source of phenolic compounds, which have beneficial antioxidant, anti-carcinogenic, anti-allergic, anti-inflammatory properties (Bravo, 1998; Chang et al., 1977; Leite et al., 2001; Mi et al., 2004; Moroney et al., 1988; Veitch & Grayer, 2011).

Fruits and vegetables that naturally contain phenolic compounds can be consumed as a functional food. These compounds can be transferred from plants to the bee pollens. Phenolic compounds, such as phenolic acids and flavonoids, are considered to be useful for human health because they have the ability to deactivate free radicals and decrease the risk of degenerative diseases by reducing oxidative stress (Rzepecka-Stojko et al., 2014; Silva et al., 2004). There are various methods available for the assessment of the antioxidant capacity that provides useful data.

DNA is an important biomolecule for the cellular functionality of human and all organisms. It is known that many diseases such as neurodegenerative, cardiovascular, cancer diseases and ageing problems are related to the oxidation of DNA (Dawbaa et al., 2017). So the detection of DNA damage has got great importance. DNA damage can be caused by exogenous and endogenic factor such as UV and ionizing radiations, chemicals and reactive oxygen species (Chen et al., 2012).

The aim of this study was to analyze phenolic compounds quantitatively in chestnut bee pollen from Turkey by HPLC-DAD system and to investigate antioxidative effect of chestnut bee pollen on DNA oxidation in Fenton medium.

MATERIAL and METHOD

Chemicals and reagents

Deoxyribonucleic acid (DNA) from calf thymus, activated, lyophilized powder, type XC was purchased from Sigma (Germany). Ethanol and methanol in HPLC grade, formic acid, phosphoric acid, sodium dihydrogen phosphate and hydrochloric acid were purchased from Merck (Darmstadt, Germany). Iron(II) sulfate-7-hydrate extra pure, potassium dichromate, 1,5-diphenylcarbazide, Folin-Ciocalteau reagent, potassium sodium tartrate, sodium hydroxide, copper(II) sulfate pentahydrate, sodium carbonate and gallic acid, syringic acid, kaempferol, pinocembrin, chrysin and galangin in HPLC grade were supported from Sigma-Aldrich (St. Louis, MO., USA). Hydrogen peroxide trace Select Ultra ≥30% was purchased from Fluka (Buchs, Switzerland). Trolox was purchased from Acros organics (New Jersey, USA). Water was purified with Purelab Option-Q from Elga Laboratory (UK).

Chestnut pollen

Chestnut (*Castanea sativa*) bee pollen sample was collected from beekeepers in Cumalıkızık, Bursa (Turkey) in 2016. The sample was stored at -24°C for analysis. 10 g of bee pollen was used for melissopalynological analysis. Preparations were made according to the Louveaux *et al.* (1978) method and stained with glycerine gelatine colored by means of basic fuchsine and slides analyzed by light microscopy. The number of pollen grains was expressed as grains per square centimeter of microscope cover glass (22 × 22 mm).

Extraction of chestnut bee pollen

3 g of chestnut bee pollen was extracted for 30 min at 65°C with 30 mL of ethanol by ultrasonic assisted extraction. pH was adjusted to 2 with 10 M HCl. The ethanol extract (E) was filtered through filter paper. C18 column was used for solid phase extraction. For conditioning of SPE column, 10 mL of distilled water and 5 mL of 10 M HCl were passed from column. 5 mL of

ethanol extract (E) was passed from column and the antioxidant compounds were holded on the column. These components were eluted from the column with 3 mL of methanol and this sample is called as EM. Sample E was passed through SPE column and the same procedure explained above was applied except the final elution solvent was ethanol and this sample is called as EE. 3 g of chestnut bee pollen was extracted along 30 min at 65°C with 30 ml of distilled water by ultrasonic assisted extraction. pH was adjusted to 2 with 10 M HCl. The water extract was filtered through filter paper and this sample is called as W. As in the case of sample EE the same procedure was applied for water extract and the elution solvent was ethanol and this sample is called as WE.

Antioxidant capacity analysis

The antioxidant capacities of bee pollen extracts were determined with chromium reducing antioxidant capacity (CHROMAC) (Nasır et al., 2017; Şahin and Demir, 2013) and ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1996) methods. In CHROMAC method, the extracts of chestnut bee pollen (100 μL), 400 μL of distilled water and 3.5 mL of phosphate buffer (pH 2.8) were taken to the test tube and the test tube was mixed. 0.5 mL of 1,5-diphenylcarbazide ($3.4x10^{-4}$ mol/L) was reacted with the sample, after incubation for 1 min with 0.5 mL of $K_2Cr_2O_7$ (50 mg/L). The absorbance of the sample was analyzed at 540 nm, after waiting in the dark for 50 min. The antioxidant capacity was calculated as milligrams of trolox equivalent (TE) per g of chestnut bee pollen by using a standard calibration graph of trolox.

The FRAP method relies on the reduction of the Fe^{3+}-TPTZ (ferrictripyridyltriazine) complex to the ferrous form at low pH. Briefly, the extract of chestnut bee pollen (250 μL) and 2.75 mL of the TPTZ were mixed and incubated at 37°C for 30 min. 500 μL of the sample was mixed with 500 μL of distilled water and was stored in the dark for 8 min. The blue color samples were analyzed using a Varian Cary 50 UV-Vis spectrophotometer at 593 nm. The results were calculated as millimolar of trolox equivalent (TE) per g of chestnut bee pollen by using a standard calibration graph of trolox.

Total phenolic content analysis

The total phenolic content of bee pollen extract was evaluated according to Folin-Ciocalteu (FC) method. The FC method relies on the transfer of electrons from phenolic compounds and other reducing compounds to molybdenum. The FC method was performed according to the literature (Aybastıer et al., 2013). Briefly, the extract of chestnut bee pollen (100 μL), 1.9 mL of distilled water and 2.5 mL of Lowry C solution were taken to the test tube. The mixture was stirred before Folin reagent (0.25 mL) was added and the samples were stored in the dark for 30 min. The samples were analyzed using a UV-Vis spectrophotometer (Varian Cary-50) at 750 nm. The concentration of total phenolic content was calculated as milligrams of gallic acid equivalent (GAE) per g of chestnut bee pollen by using a standard graph of gallic acid.

HPLC analysis

The chromatographic analysis of the chestnut bee pollen was performed on the HPLC system (Agilent 1200 Series) equipped with a diode array detector, vacuum degasser, binary pump and autosampler. XBridge C18 (4.6 × 250 mm, 3.5 μm) column was used, injection volume was 20 μL, flow rate was 0.5 mL/min. HPLC separation of phenolics was performed with a gradient elution using two mobile phase system (phase A: 1% formic acid; phase B: 100% methanol). The elution programs were: 0-10 min 5% B, 10-15 min 15% B, 15-20 min 30% B, 20-30 min 40% B, 30-50 min 45% B, 50-52 min 60% B, 52-60 min 80% B, 60-70 min 80% B, 70 min 5% B (Güneş et al., 2016).

Quantitative analysis of the phenolic compounds in extracts of chestnut bee pollen were determined by HPLC-DAD. Gallic acid, syringic acid, hyperoside, kaempferol, isorhamnetin, pinocembrin, chrysin and galangin were determined in the chestnut bee pollen. The calibration curves were prepared to determine the amounts of these compounds found in the chestnut bee pollen. Chromatograms of chestnut bee pollen extracts were interpreted in comparison with each phenolic standards. The calibration data of phenolic compounds that can

be assigned in chestnut bee pollen were given in Table 1.

Effect of antioxidants on the DNA oxidation

Antioxidant ability of chestnut bee pollen was examined to investigate the prevention of oxidation of calf thymus DNA in Fenton reaction medium. 3 mL of ethanol extract of chestnut bee pollen was evaporated to dryness under nitrogen atmosphere and then the residue was dissolved 3 mL of water. DNA of calf thymus was purchased and 5 mg of DNA was resolved in 10 mL of ultra-distilled water. Four different samples were prepared to study the antioxidant effects on DNA oxidation; (1) 20 μg/mL DNA solution, (2) Fenton (150 μM Fe^{+2} and 300 μM H_2O_2) solution, (3) Fenton and DNA (20 μg/mL) solutions and (4) Fenton, DNA (20 μg/mL) solutions and aqueous extract of chestnut bee pollen (1000 μL). Sample solutions were scanned every 10 min along a time range of 1 hour at a range of 200-400 nm.

Table 1. Calibrations of phenolic compounds contained in chestnut bee pollen

Phenolic compounds	Concentration range (mg/L)	Calibration equation	Regression coefficient (R^2)
Gallic acid	0.5 – 8.0	y=52.5085x-30.5770	0.9996
Syringic acid	0.1 – 10.0	y=74.3861x-0.1617	0.9990
Hyperoside	0.1 – 10.0	y=96.5243x-28.6102	0.9987
Kaempherol	0.1 – 2.0	y=82.9653x-6.6938	0.9993
Isorhamnetin	0.1 – 10.0	y=205.4008x-54.4131	0.9990
Pinocembrin	0.1 – 10.0	y=64.3752x+4.8661	0.9996
Chrysin	0.1 – 10.0	y=89.4119x-7.1368	0.9998
Galangin	0.1 – 4.0	y=85.7689x-6.3087	0.9997

RESULTS AND DISCUSSION

Antioxidant properties of chestnut bee pollen

The antioxidant capacities and total phenolic contents of chestnut bee pollen are reported in Table 2. The total phenolic contents of chestnut bee pollen determined by the FC method are between 43.48±0.77 and 4.12±0.09 mg of GAE per g of chestnut bee pollen. Antioxidant capacity values are between 20.24±1.15 and 3.15±0.04 mM of TE per g of chestnut bee pollen for the FRAP method and 3.70±0.05 and 34.18±0.02 mg of TE per g of chestnut bee pollen for the CHROMAC method. According the literatures, the antioxidant capacities of chestnut bee pollen determined by the FRAP method were found 82.31±2.41 mM of TE per g of dried chestnut bee pollen weight (DW) (Yildiz et al., 2013a) and 124.62±4.88 mM of TE per g of chestnut bee pollen (Yildiz et al., 2013b). And also the total phenolic contents of chestnut bee pollen determined by the FC method were 28.87±2.48 mg of GAE per g DW (Yildiz et al., 2013a) and 52.12±2.14 mg of GAE per g of sample (Yildiz et al., 2013b). When the antioxidant capacity and total phenolic content values were compared to these literatures, these antioxidant capacity values are higher than our results but the total phenolic content values are found to be similar due to different sample preparation and extraction conditions. Phenolic compounds such as flavonoid and phenolic acid, possess a unique profile for each plant. For this reason, the concentration and species difference of phenolic compound in pollen are due to the change of plant origin. Furthermore, environmental factors are an important influence on these differences in phenolic compounds.

There is no study about antioxidant capacity of chestnut bee pollen determined by CHROMAC

method without chestnut honey in our knowledge. Güneş et al.(2016) have used the CHROMAC method to determine the antioxidant capacities of chestnut honeys. The antioxidant capacity results were found between 10.80±0.01 and 22.00±1.00 mg of TE kg of chestnut honey. In the direction of this data the antioxidant capacity of chestnut bee pollen was observed to be higher than the antioxidant capacity of chestnut honey.

Table 2. Total phenolic content (mg GAE/g chestnut bee pollen) determined by Folin method and antioxidant capacities determined by CHROMAC (mg TE/g chestnut bee pollen) and FRAP (mM TE/g chestnut bee pollen) methods of chestnut bee pollen

Sample	Total phenolic content	CHROMAC	FRAP
E	43.48±0.77	34.33±0.02	20.24±1.15
EM	13.91±0.54	4.69±0.06	5.58±0.11
EE	14.77±0.61	5.22±0.07	5.82±0.10
W	28.45±0.76	19.34±0.04	14.36±0.79
WE	4.12±0.09	3.70±0.05	3.15±0.04

Mean of two determinations±SD; E: ethanol extract, EM: methanol extract and EE: ethanol extract after SPE of ethanol extract, W: water extract, WE: ethanol extract of after SPE of water extract.

When spectroscopic methods of data were compared, high correlations were observed between methods. The correlation coefficient (R^2) between total phenolic content and CHROMAC methods was found to be 0.9706, between total phenolic content and FRAP methods was found to be 0.9888 and between CHROMAC and FRAP methods was found to be 0.9876. The high correlation between the methods proves that the phenolic content analyzes are compatible with each other.

Determination of phenolic compounds in chestnut bee pollen

Phenolic compounds in chestnut bee pollen were determined by HPLC-DAD system. The chromatogram of ethanol extract was shown in Figure 1. In the chromatogram of ethanol extract the first peak appearing was syringic acid at 26.8 min, it was followed by hyperoside at 33.9 min, kaempferol at 55.0 min, isorhamnetin at 56.6 min, pinocembrin at 60.3 min, chrysin at 61.3 min and galangin at 62.2 min. The peaks observed outside these peaks indicate the presence of other phenolic compounds but these phenolic compounds could not be assigned. The amounts of phenolic compounds in chestnut bee pollen were shown in Table 3. The results showed that the most abundant amount was that of pinocembrin found in the ethanol extract of chestnut bee pollen with 1.246±0.125 mg/g of sample.

There was no big difference between the amounts of phenolic compounds in ethanol and methanol extracts obtained after SPE method. The detected compounds in the ethanolic extract that pinocembrin, chrysin and galangin were also found in the methanolic extract with the similar amounts. Hyperoside is the only compound found in methanolic but not in ethanolic extract. The amounts of phenolic compounds in the extracts of SPE were found to be lower than those of ethanolic extract. Some compounds were not found in methanolic and ethanolic extracts of SPE. This could be due to the low recovery and the insufficient sorption power of the column.

Hyperoside and pinocembrin were also found in the water extract but with much lower amount in

comparison to the ethanolic extract. Gallic acid was the only one compound found in the water extract but it could not be found in the ethanolic extract. The amount of gallic acid (1.340±0.134 mg/g chestnut bee pollen) was observed to be the highest among other contents. Upon the application of SPE to the obtained water extract of chestnut bee pollen, gallic acid was only found in the eluate. The low recovery and low sorption properties of the column are thought to be the cause of such low content.

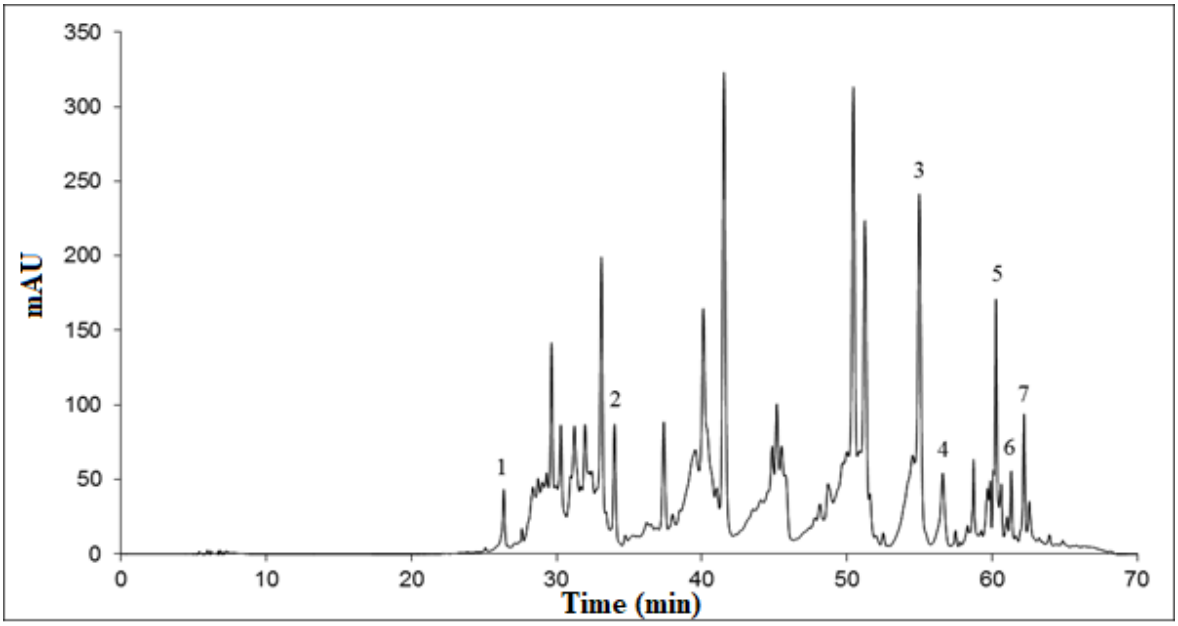

Figure 1. The chromatogram of chestnut bee pollen; 1, syringic acid; 2, hyperoside; 3, kaempherol; 4, isorhamnetin; 5, pinocembrin; 6, chrysin; 7, galangin.

Table 3. The amounts of phenolic compounds in chestnut bee pollen (mg per g chestnut bee pollen)

Phenolic compounds	E	EE	EM	W	WE
Gallic acid	nd	nd	nd	1.340±0.134	0.034±0.003
Syringic acid	0.011±0.001	nd	nd	nd	nd
Hyperoside	0.121±0.012	nd	0.072±0.007	0.120±0.012	nd
Kaempherol	0.516±0.052	nd	nd	nd	nd
Isorhamnetin	0.062±0.006	nd	nd	nd	nd
Pinocembrin	1.246±0.125	0.489±0.049	0.119±0.012	0.198±0.020	nd
Chrysin	0.332±0.033	0.134±0.013	0.153±0.015	nd	nd
Galangin	0.122±0.012	0.111±0.011	0.055±0.006	nd	nd

Mean of two determinations±SD; nd: not detected, E: ethanol extract, EM: methanol extract and EE: ethanol extract after SPE of ethanol extract, W: water extract, WE: ethanol extract of after SPE of water extract.

Güneş et al. (2016) have used the same method to determine the phenolic content of chestnut honeys. Gallic acid content was found to be considerably low in chestnut honeys as well as in chestnut bee pollen. Flavonoids such as chrysin and pinocembrin were generally found in high amounts in chestnut honeys. Kaempferol was found higher amount except pinocembrin and chrysin in chestnut bee pollen. Syringic acid and galangin were observed lower amount in chestnut bee pollen as in the chestnut honeys. Hyperoside and isorhamnetin were found in this study on chestnut bee pollen while not observed in chestnut honeys. When the amounts of phenolic compounds in general are compared the phenolic compounds in the chestnut bee pollen appeared to be higher than in the chestnut honeys. According to the results, chestnut bee pollen is believed to be a valuable natural source due to its phenolic compound content.

Determination of the effect of antioxidants on DNA oxidation

DNA damage occurs oxidatively in living cells by various mechanisms (Dizdaroglu, 2012). DNA damage leads to various mutations and diseases such as cancer unless repaired. It is known that natural vegetables and fruits protect DNA damage by the antioxidants, if they contain. The effects of bee pollen which is a natural product with high antioxidant properties, on DNA damage were investigated in this study. The results were shown in Figure 2. DNA concentrations were calculated by absorbance at 260 nm using molar extinction coefficient of DNA, after oxidation with and without bee pollen solutions. The amount of 20 μg/mL of pure DNA solution was decreased to 10.2 μg/mL in Fenton environment due to oxidation. When the bee pollen extract was added in the same conditions, the amount of DNA was determined to be 12.4 μg/mL. The percentage of damage was determined initially by the amount of DNA in the medium. While the oxidative damage of DNA by Fenton environment was found to be 49%, this percentage was decreased to 38% by the effect of chestnut bee pollen. According to the results obtained, chestnut bee pollen was found to prevent DNA oxidation damage by 11% under oxidative environment.

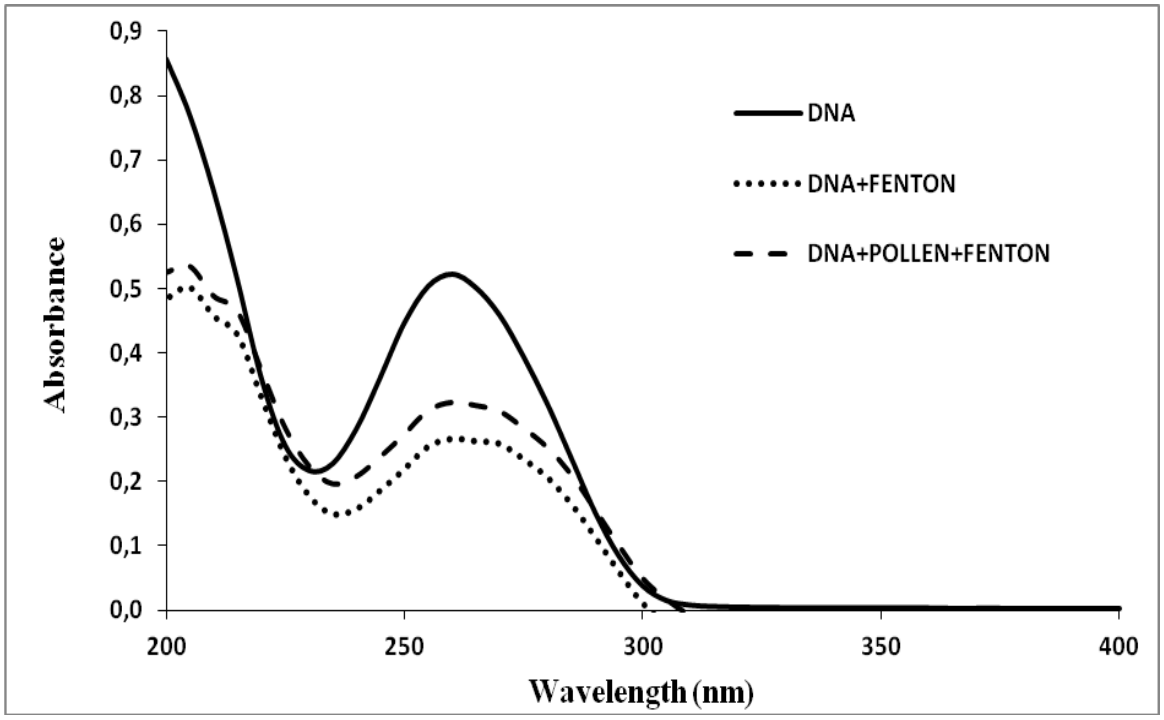

Figure 2. The spectrum of 20 μg ml^{-1} of DNA, DNA and DNA with chestnut bee pollen extract after Fenton oxidation at 200-800 nm.

The ability of phenolic compounds to inhibit DNA damage was observed. The chestnut bee pollens contain natural phenolic compounds that are important due to their antioxidant properties. Therefore, they are recommended to be consumed as part of the normal daily nutritional elements.

CONCLUSIONS

This is the first study about determination of phenolic compounds quantitatively in chestnut bee pollen from Turkey in our knowledge. Solvent and SPE extraction methods were used for the extraction of phenolic compounds from the chestnut bee pollen. When compared these methods with the amount of phenolic compounds and antioxidant capacity values, the best results were found in the ethanolic extract. There are a number of methods in the literature because of the many different structures and properties that make up the antioxidant capacity. Unfortunately, there is no single antioxidant capacity determination method that will detect all of these compounds. For this reason, two methods (CHROMAC and FRAP) were used to determine antioxidant capacity in chestnut bee pollen. High correlation between them was showed the accuracy of the methods. Since CHROMAC method is a newly developed method, it is important to apply to different matrices. Because of the rich content of bee pollen such as phenolic compounds, carotenoids and flavonoids and bee pollen can inhibit DNA oxidation. The ability of phenolic compounds in chestnut bee pollen to inhibit DNA damage was observed as 11% under oxidative environment. Finally it is suggested that the consumption of chestnut bee pollen can be recommended as a dietary supplement for humans.

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