Pak. J. Bot., 53(6): 2241-2250, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-6(44)

IDENTIFICATION AND QUANTIFICATION OF BIOCHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF WALNUT POLLENS

VOLKAN OKATAN[1*], İBRAHIM BULDUK[2], BARIŞ KAKI[3], MUHAMMET ALI GÜNDEŞLI[4], SERHAT USANMAZ[5], TURGUT ALAS[5], MURAT HELVACI[5], İBRAHIM KAHRAMANOĞLU[5] AND HANIFEH SEYED HAJIZADEH[6]

[1]*Eskisehir Osmangazi University, Faculty of Agriculture, Department of Horticulture, Eskisehir, Turkey*
[2]*Uşak University, School of Health, Uşak, Turkey*
[3]*Uşak University, Faculty of Economics and Administrative Sciences, Department of Econometrics, Uşak, Turkey*
[4]*Gaziantep University, Nurdagi Vocational School, Nurdagi, Gaziantep, Turkey*
[5]*European University of Lefke, Faculty of Agricultural Sciences and Technologies, Gemikonagi, Northern Cyprus, via Mersin 10 Turkey*
[6]*Department of Horticultural Science, Faculty of Agriculture, University of Maragheh, Maragheh 55136-553, Iran*
**Corresponding author's email: okatan.volkan@gmail.com*

Abstract

The aim of this study was to determine the biochemical composition and antioxidant activity in pollens collected from ten different walnut cultivars. Pollen samples of walnut cultivars ('Aslan', 'Chandler', 'Fernette', 'Fernor', 'Franquet', 'Kaman', 'Oguzlar', 'Pedro', 'Sebin'and'Yalova')were collected during flowering of catkins in Uşak Province of Turkey. Identification and quantification of total phenolic contents, total flavonoids, antioxidant activity, pH, organic acids and phenolic compounds (gallic acid, protocatechuic acid, vanilic acid, caffeic acid and syringic acid) were individually analyzed for each cultivar. The results of observed parameters are significantly varying among the cultivars (p<0.05) and wide range of biochemical compounds were identified from the walnut pollens. Results showed that total phenolic contents varied from 5.050 to 11.030 mg GAE/g and total flavonoids content ranged from 1.530 to 4.123 mgQE/g. Furthermore, the antioxidant capacity of pollen extracts was found to range from 1.510 to 2.003 mg AAE g^{-1}. Among the identified phenolic compounds, gallic acid (10.610-13.410 mg/100g^{-1}) and protocatechuic acid (1.410-3.623 mg/100g^{-1}) were found in highest amounts. Moreover, oxalic acid and citric acid were identified as the dominant organic acids for all cultivars. Results of present study showed that the walnut pollens have high antioxidant capacity which is very beneficial for human health.

Key words: Walnut, Pollen, Antioxidant, Phenolic acids, Organic acids.

Introduction

The walnut species (*Juglans regia* L.) belong to the family of Juglandaceaeare known to be among the oldest domesticated fruit species in the world. Walnut trees have high adaptability to different climatic conditions especially in temperate climates (Bernard *et al*., 2018). Walnut has a wide genetic diversity and a vast differentiation exists in walnut germplasm (Yang, 2005). The vast genetic variation arises mainly from seed based plantations and high heterozygosity (Simsek *et al*., 2010; Bernard *et al*., 2018). Thus, walnut species are highly found in breeding programs and this genetic variability has led to intensive research about walnut germplasm globally (Akça & Şen, 2001; Gunn *et al*., 2010; Poggetti *et al*., 2017). Walnut is a monoecious plant and wind pollinated with male and female reproductive organs on separate flowers on the same tree. Walnut trees produce pistil inflorescences of one to three flowers which are carried laterally on 1 year old branches (Lu *et al*., 2020; Polito *et al*., 2005; Gun *et al*., 2010; Cosmulescu *et al*., 2015a). Total pollen grain production of walnut tree is estimated between 5 and 100 billion in some studies. To have an adequate fruit set, it is utmost necessary to have abundant and healthy flowers at walnut tree. However, walnut trees show high flower and fruit drops which often associated with lack of pollen or pollination (Sutyemez, 2007; Valdebenito *et al*., 2017).

Nowadays, consumer's preferences are changing and they are looking to different characteristics of food rather than size and color. They are gradually more aware of the nutritional composition and health-promoting components of foods. Recent epidemiological reports showed that many phytochemical compounds present in fruits such as nuts are partially responsible for beneficial health effects. Recently, some health-related phytochemical compounds in walnuts have been identified (Fukuda *et al*., 2003; Oliveira *et al*., 2008; Cosmulescu *et al*., 2015a; Kafkas *et al*., 2017). Walnut kernel is a rich source of proteins, fats, vitamins, minerals and polyphenols which makes the fruit indispensable for human nutrition. They are also a good source of flavonoids, sterols, pectic substances, phenolic acids and related polyphenols (Colaric *et al*., 2005; Simsek, 2011; Yerlikaya *et al*., 2012: Polat *et al*., 2015; Beyhan *et al*., 2016). Walnuts are known to have high antioxidant activity (Isanga and Zhang, 2007; Miraliakbari and Shahidi, 2008; Cosmulescu *et al*., 2015b). Antioxidants have high positive impact on the human health by protecting the human body from ROS and other free radicals (Pokorny, 2007; Nunes *et al*., 2012). But the physicochemical characteristics of cultivated and wild plants are significantly vary (Ozkan *et al*., 2019; Gecer *et al*., 2020). In literature, there are various studies related to the phytochemical and antioxidant characteristics potential of walnut kernel (Chevallier *et al*., 2009; Rahimipanah *et al*., 2010; Bujdosó *et al*., 2014). Some study reports on the phenolic profiles of walnut trees describe the total phenolic content in different parts of walnut shoots (Solar *et al*., 2006; Cosmulescu *et al*., 2010a), leaves, and green husk (Stampar *et al*., 2006; Jakopic *et al*., 2009; Cosmulescu *et al*., 2010b; Cosmulescu *et al*., 2015a). But these chemical contents of

walnuts can vary by variety, genotype, ecology, climate, soil conditions and cultural practices (Beyhan *et al*., 2016; Bernard *et al*., 2018). The extraction method also significantly influences the composition (Senica *et al*., 2019). Recently, many researchers turned their attention to bee pollen studies. Bee pollen is the source of many bioactive compounds, which makes the bee pollens a very important food for human nutrition and health. Leja *et al*., (2007) and Moreira *et al.*, (2008) investigated the vast variability of bioactive compounds in pollen species. Many studies have determined the antioxidant and antibacterial activity of monofloral bee pollen samples (Feas *et al*., 2012; Fatrcová-Šramková *et al*., 2013). Nicolson *et al.*, (2018) believe that pollen pollinated by honey bees is very important for human diet because of its high concentration in reducing sugars, amino acids and fatty acids. Bee pollen contains high amounts of proteins, lipids, sugars, fiber, mineral salts, amino acids and vitamins. It also has significant amount of phenolics which show special bioactive properties such as antibacterial, antifungal and antioxidant activities (Bonvehi *et al*., 2001; Margaoan *et al*., 2010; Ulusoy and Kolayli, 2014: Eyduran *et al*., 2015). Bee pollen products are rich in different phenol compounds, antioxidant and biological activities. Therefore, recent studies throughout that the current trend in the world has been focusing on the biochemical compounds and antioxidant characteristics of pollens. Therefore, researchers have mainly focused on the bioactivity of different walnut organs such as kernel and leaves. However, limited information exists in the literature about the biochemical status of the walnut pollens. Considering the large amount of pollen produced by walnutsand lackof studies on pollens, present study conducted to study the total polyphenols, flavonoid contents and antioxidant activity of pollens, collected from ten different walnut cultivars. Results of present study would contribute to human healthand future commercial production.

Materials and Methods

The pollen samples of present study were collected from the "Demirören Village" located in the Uşak city of Turkey. The research district is situated between 38º32' North latitude and 29°20' East longitude at an altitude of 892 meters above sea level. The quantification and identification of phenolic compounds, total phenol (TP), total flavonoid (TF), antioxidant activity capacity (TAC) and pH were carried out in pollen samples which were hand collected from 10 different walnut cultivars (Aslan, Chandler, Fernette, Fernor, Franquet, Kaman, Oguzlar, Pedro, Sebin and Yalova). Sample collection was performed during the flowering of catkins and subjected to natural drying (4 days at room temperature 20-22°C) in Centre Laboratory of Uşak University in 2016.

Ultrasonic assisted extraction: Total phenolic, flavonoid and antioxidant capacity of the pollens were all determined from the extracts prepared with the following method described by Oroian *et al.*, (2020). Ultrasonic assisted extraction was performed in an ultrasonic bath (Bandelin Sonorex with a frequency of 50 kHz). For this purpose, 1 g of the samples was weighed and extracted with 30 ml (70% methanol + 30% de-ionized water) solution for 30 minutes. After extraction, the mixture was filtered through Whatman white band filter paper and the extracts were stored in the refrigerator at +4°C until analysis.

Determination of total phenolic content: Total phenol content (TPC) was determined with the Folin-Ciocalteu method developed by Kähkönen *et al.*, (1999). For this purpose, 0.2 mL of sample and 0.5 mL of Folin-Ciocalteu reagent (diluted 10 times with water) were added to the test tubes. The solution was then kept in the dark for 5 minutes and 1 mL of sodium carbonate (7.5% w / v) was then added. The resulting solution was kept in the dark for 1 hour. The absorbance of the solution was then read at 765 nm by UV-VIS spectrophotometer (SHIMADZU UV-1800). The phenolic content was then calculated as gallic acid equivalents. GAE g^{-1} of dry sample by using the standard curve of gallic acid (150, 300, 450, 600, 750 ppm, $Y = 0.0001x + 0.0026$, $R^2 = 0.9998$). All measurements were performed in triplicate and the results were expressed as mg gallic acid g^{-1} dry sample (DW).

Determination of total flavonoids content: Total flavonoid content was determined with a modified aluminum chloride colorimetric method described by Chang *et al.*, (2002). Briefly, 50 µL of extract was taken into the test tube. It was mixed with 950 µL of methanol, 4 mL of purified water and 300 µL of 5% $NaNO_2$ solution. After incubation, 300 µLof 10% $AlCl_3$ solution was added and the mixture was allowed to stand for 6 minutes. Next, 2 mL of 1 M NaOH solution was added and the final volume of the mixture was filled up to 10 mL with distilled water. The mixture was allowed to stand for 15 minutes and the absorbance value at 510 nm was measured. The total flavonoid content was then calculated by using the quercetin calibration curve and the results were expressed as mg quercetin equivalent per day weight (mg QE g^{-1}).

Determination of antioxidant activity: The scavenging activities of methanolic extracts of walnut pollens against DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals were measured according to the method described by Thaipong *et al*., (2006) and Cosmulescu *et al.*, (2015a) with some modifications. Briefly, 50 µL of sample extracts were mixed with 3 mL of methanolic solution containing DPPH radicals (40 mg L^{-1}). The mixture was kept in dark for 60 minutes, and the absorbance was measured at 515 nm. The absorbance results were transformed using the calibration curve of the ascorbic acid standard and expressed as the equivalent of ascorbic acid. Percentage inhibition of radical DPPH was calculated based on control reading by the following equation.

$$\text{DPPH-Percentage \% of reduction power} = (A_0 - A_1)/A_0 \times 100$$

In the above formula, A_0 represents the absorbance of control (standard solution) and the A_1 is the absorbance of sample. All assays were conducted in triplicate.

Identification of phenolic compounds: Analytical quantification of phenolic substances was performed by

HPLC Agilent 1260 series. The HPLC system equipped with C18 (4.6 mm × 150 mm, 5 µm) column, with a flow rate of 0.5 mL min^{-1}, injection volume of 10 µL and a run time of 40 min. Mobile phase A was water containing 0.02% trifluoroacetic acid (TFA), and mobile phase B was methanol containing 0.02% trifluoroacetic acid (TFA). The gradient conditions were as follows: 0-5 min, 25% B; 5-10 min, 25-30% B; 10-16 min, 30-45% B; 16-18 min, 45% B; 18-25 min, 45-80% B; 25-30 min, 80% B; 30-40 min, 80-25% B. The temperature of the column kept at 25°C. The detection wavelengths of DAD were set at four selected positions: 254, 275, 305 and 320 nm.

Determination of organic acids and ph: Oxalic acid, ascorbic acid and acetic acid contents of walnut pollens were determined according to the method described by Fadavi *et al.*, (2005). The organic acid compositions of the samples were determined by Agilent 1260 model HPLC from the fruit juices. The juice samples were firstly passed through white band filter paper and then 25 micron syringe filter. For this purpose, ACE 5 C18 column (5 µm, 250 mm x 4.6 mm) and UV Detector were used. In the analysis performed in isocratic flow, 2% KH_2PO_4 solution with orthophosphoric acid and pH adjusted to 2.3 was used as mobile phase. Organic acids were determined at a wavelength of 214 nm in the analysis performed at a flow rate of 0.9 mL min^{-1} and injection volume of 10 µl at 30°C. Analysis run time is 20 min. The amounts of organic acid components in the samples were calculated according to standard organic acid analysis results.The pH of the samples was determined by Mettler Toledo brand digital pH meter at 20°C.

Statistical analysis

The data obtained from the studies was summarized as means ± standard deviation. Parametric tests showed that the data is non-parametric. Thus, Kruskal Wallis test was used for the comparison of the different cultivars (p<0.05, 0.01 and 0.001). In case of the determination of a significant difference, Bonferroni correction was used for pairwise comparisons. Furthermore, Pearson correlation was performed to determine the correlations among the parameters observed. Statistical analysis was performed with SPSS 24.00 and R 3.6.1 software programs.

Results and Discussion

Total phenolic, total flavonoid content, antioxidant activity and pH: The total phenolic content, total flavonoid content, antioxidant activity and pH of the pollens of ten tested walnut cultivars are given in Table 1. The results indicated significant differences forall bioactive compounds and pH among the 10 walnut cultivars (p<0.05). Total phenolic contents of walnut pollens ranged from 5.050 mg GAE g^{-1} DW in Pedro to 11.030 mg GAE g^{-1} FW in Yalova. Yalova cultivar found to have the highest total phenolic contents. On the other hand, 'Pedro' and Fernette cultivars were found to have the lowest total phenol contents. Numerous studies have been carried out on the total phenolic contents not on the pollens but on the fruits of different walnut cultivars. In such studies, Kornsteiner *et al.*, (2006) reported that the total phenolic contents ranged from 1.020 to 2.052 mg 100 g^{-1}in different walnut cultivars. In another study, Cosmulescu *et al.*, (2015a) found out that the total phenol content of walnut pollens varied from 1.080 to 1.764 mg GAE 100 g^{-1}. Results of present study are found to be higher than the reports of Kornsteiner *et al*., (2006), but lower than the results of Cosmulescu *et al*., (2015a). Other previous studies (Anderson *et al*., 2010; Fatrcová-Šramková *et al*., 2013, Beyhan *et al*., 2016) carried with the walnut fruits reported a range of 1.071-2.370 mg GAE 100 g^{-1}, 3.193-13.837 mg GAE 100 g^{-1} and 1.107- 1.876 mg GAE 100 g^{-1}, respectively. Abe *et al*., (2010) reported total phenols of nuts such as walnut 50-2.499 mg GAE 100 g^{-1} in descending order walnuts (2.499) > pecans (703) > peanuts (597) > pistachios (576) > cashew nuts (381) > almonds (114)> hazelnuts (111) > Brazil nuts (106) mg GAE 100 g^{-1}. The total phenolic contents of pollens determined in current study are found to be similar, but some cultivars higher than those reports (Carpes *et al*., 2013; Ulusoy and Kolayli, 2014; Kafkas *et al*., 2017).

Table 1. Comparison of ten cultivars in terms of biochemical properties.

Cultivars	Chemical Characteristics (Mean ± standard deviation)			
	Total phenol (GAE g^{-1})	pH	DPPH (mg AAE g^{-1} DW)	Total flavonoid (mg QE g^{-1})
Aslan	6.977 ± 0.006^{B}	6.243 ± 0.012^{AB}	1.810 ± 0.010^{AB}	3.740 ± 0.026^{A}
Chandler	7.250 ± 0.010^{B}	6.147 ± 0.006^{AB}	1.810 ± 0.011^{AB}	3.447 ± 0.006^{AB}
Fernette	6.587 ± 0.006^{B}	6.120 ± 0.010^{B}	**2.003 ± 0.015^{A}**	2.777 ± 0.006^{C}
Fernor	7.380 ± 0.010^{B}	6.483 ± 0.006^{AB}	1.517 ± 0.029^{C}	3.297 ± 0.006^{B}
Franquet	6.603 ± 0.015^{BC}	7.030 ± 0.010^{A}	1.710 ± 0.010^{B}	2.790 ± 0.010^{C}
Kaman	7.633 ± 0.015^{B}	6.183 ± 0.006^{AB}	1.907 ± 0.012^{A}	3.613 ± 0.015^{AB}
Oguzlar	6.867 ± 0.035^{B}	6.250 ± 0.010^{AB}	1.703 ± 0.006^{B}	3.227 ± 0.006^{B}
Pedro	***5.050 ± 0.010^{C}***	***6.117 ± 0.006^{B}***	***1.510 ± 0.010^{C}***	***1.530 ± 0.010^{D}***
Sebin	9.420 ± 0.010^{AB}	**7.047 ± 0.042^{A}**	1.617 ± 0.015^{BC}	3.423 ± 0.015^{AB}
Yalova	**11.030 ± 0.010^{A}**	6.127 ± 0.006^{B}	1.707 ± 0.012^{B}	**4.123 ± 0.006^{A}**
p value	*0.001*	*0.001*	*0.001*	*0.001*
Average	7.480 ± 1.594	6.375 ± 0.354	1.729 ± 0.154	3.197 ± 0.688

Different capital letters used to indicate significant difference among cultivars at p<0.05 (Kruskal Wallis test). The bold values represent the highest and bold italic values are the lowest

Similar with total phenolic, the pH was also found to significantly vary among the cultivars. The lowest pH content was determined in Pedro pollens as 6.117, where the highest pH was recorded in Sebin as 7.047 (Table 1). Similarly, Pedro cultivar was found to have the lowest antioxidant activity (1.510 mg AAE g^{-1} DW) and the highest value was obtained from the Fernette cultivar (2.003 mg AAE g^{-1} DW). The antioxidant capacity of different tissues and/or organs of walnut trees were previously studied in numerous studies. The results of current study are found to be higher than the results of Kornsteiner *et al*., (2006) who reported 1.025 mg GAE 100 g^{-1} antioxidant activity. In some of examples, Cosmulescu and Trandafir (2012) reported a range from 16.40 to 50.72 mg AAE g^{-1}, Rupasinghe *et al*., (2006) noted a variation from 214 to 468 GAE 100 g^{-1} and Rop *et al*., (2009) suggested a range from 86 to 413 GAE 100 g^{-1} in their studies. It is a well-known phenomenon that the chemical content and antioxidant capacity of plants are significantly affected by several factors. Biotic and abiotic stress conditions, cultural practices, plant organs and cultivars have great influence on the contents and composition of the biochemical compounds (Gundogdu *et al*., 2017; Okatan, 2020). The findings of this study are mostly in agreement with those of other researchers. The minor differences are possibly attributed to environmental conditions and geneticfactors of the studied cultivars and organs.

Total flavonoid contents of walnut pollens were observed between 1.530 (Pedro) and 4.123 (Yalova) mg QE $g^{-1}$and the concentration was found significantly vary among the cultivars (Table 1). Yalova cultivar had the highest average in terms of total flavonoids, while Pedro showed the lowest. In a previous study, Cosmulescu *et al*., (2015a) reported that the total flavonoid contents in walnut pollen ranged from 7.32 to 7.95 mg QE g^{-1}. In another study, Ghasemi *et al*., (2011) also reported total phenolic values in green husks of walnuts (1.515–10.811 mg GAE 100 g^{-1}). Similar variation was previously recorded by Stanciu *et al*., (2011) for flavonol and flavones content of bee pollens to be ranged from 0.224 (Rosa canina) to1.008 mg QE g^{-1} (mixture of bee pollen from *Brassica napus* and *Taraxacum officinale*). Depending on the species and bee bread, Kao *et al*., (2011) reported a flavonol content of tea (*Camellia sinensis*) to be ranged from 1.94 to 8.12 mg QE g^{-1}. In terms of the content of flavonoids, the results obtained by other researchers differ depending on the study area, pollen composition and the material analyzed.

Results showed that the highest correlation coefficients were obtained between antioxidant activity and total phenolic content. Yalova cultivar had the highest average in terms of total phenol and flavonoids (p<0.05). Sebin, Aslan and Chandle varieties are high in terms of phenol and flavonoids. Pedro and Fernette varieties were found to have the lowest average in terms of these two characteristics (p<0.05). Sebin and Fernor were the highest average in terms of pH and low value in terms of the antioxidant activity (p<0.05). To observe the correlation and relationship among the ten cultivars, a PCA analysis was performed and the results are given in (Fig. 1). PCA analysis showed that chemical properties explain the total variation with two main components at a high rate of 80.3%. In terms of chemical properties, especially Yalova variety had the highest values while Pedro had the lowest values. The positive correlation between phenol and flavonoids and the negative correlation between pH and DPPH are evident in the PCA graph.

Identification and characterization of phenolic compounds: Chromatographic separations were resulted with the successful identification and quantification of five free phenolic acids: gallic acids, pyrocathecin, vanilic acid, caffeic acid andsyringic acid. Significant differences were obtained for the phenolic compounds among the different pollens of walnut cultivars (Table 2). Among the studied phenolic compounds, gallic acid was the highest and predominant in the pollens of all walnut cultivars. Results of present study are in conjunction with the reports of Vu *et al*., (2018). The highest concentration of gallic acid was recorded inFernette cultivar with 13.410 µg g^{-1} and was followed by the Franquet cultivar with 12.510 µg g^{-1}. The second predominant phenolic compound was pyrocathecin, the highest value was measured in Fernor cultivar (3.510 µg g^{-1}). The other important phenolic acid is the vanilic acid which was found in the highest content in the Fernor cultivar with a value of 3.623 µg g^{-1} whereas the lowest content was observed from Oguzlar cultivar (1.403 µg g^{-1}). The other phenolic acid was caffeic acid which was recorded highest in the pollens of Pedro cultivar and lowest in the Aslan cultivar with the values of 1.607 µg g^{-1}, 1.393 µg g^{-1}, respectively. The measured amount of syringic acid was the highest in the Fernette cultivar (1.830 µg g^{-1}) and the lowest in the Pedro cultivar (1.010 µg g^{-1}).

The results of current work are in agreement with some other previous studies, which used three different extraction methods and identified gallic acid, syringic acid, sinapic acid, chlorogenic acid, pyrocathecin acid, vanillicacid and caffeic acids from the different organs walnut cultivars (Amaral *et al*., 2004; Jakopic *et al*., 2009; Bujdosó *et al*., 2014; Vu *et al*., 2018). In a very similar study for present research, Chrzanowski *et al*., (2011) studied the phenolic acids of male walnut flowers and reported three acids which arevanillic acid (359.5 µg·g^{-1}), syringicacid (427.0 µg g^{-1}) and caffeic acid (456.9 µg g^{-1}). Similar to the results of current work, Bujdosó *et al*., (2014) reported the amounts of gallic acid, pyrocathecinacid, syringic acid and vanillic acid as 601, 3.646, 489 and 6.000 mg g^{-1}, respectively. Moreover, Vu *et al*., (2018) reported similar findings for the different organs walnut trees where they noted three phenolics as: gallic acid 1.01-4.29 µg g^{-1}, vanillic acid 7.32-9.92 µg g^{-1} and syringic acid 6.43-14.26 µg g^{-1}. The contents of the phenolic acids in current work were found to be higher than the previous studies carried with the different organs and/or tissues of the walnut trees. Principal component analysis (PCA) studies suggested that the Fernette, Fernor and Franquet are the richest cultivars in terms of the phenolic acids content (Fig. 2). On the other hand, among these ten cultivars, Pedro was found to have the lowest phenolic acid concentrations.

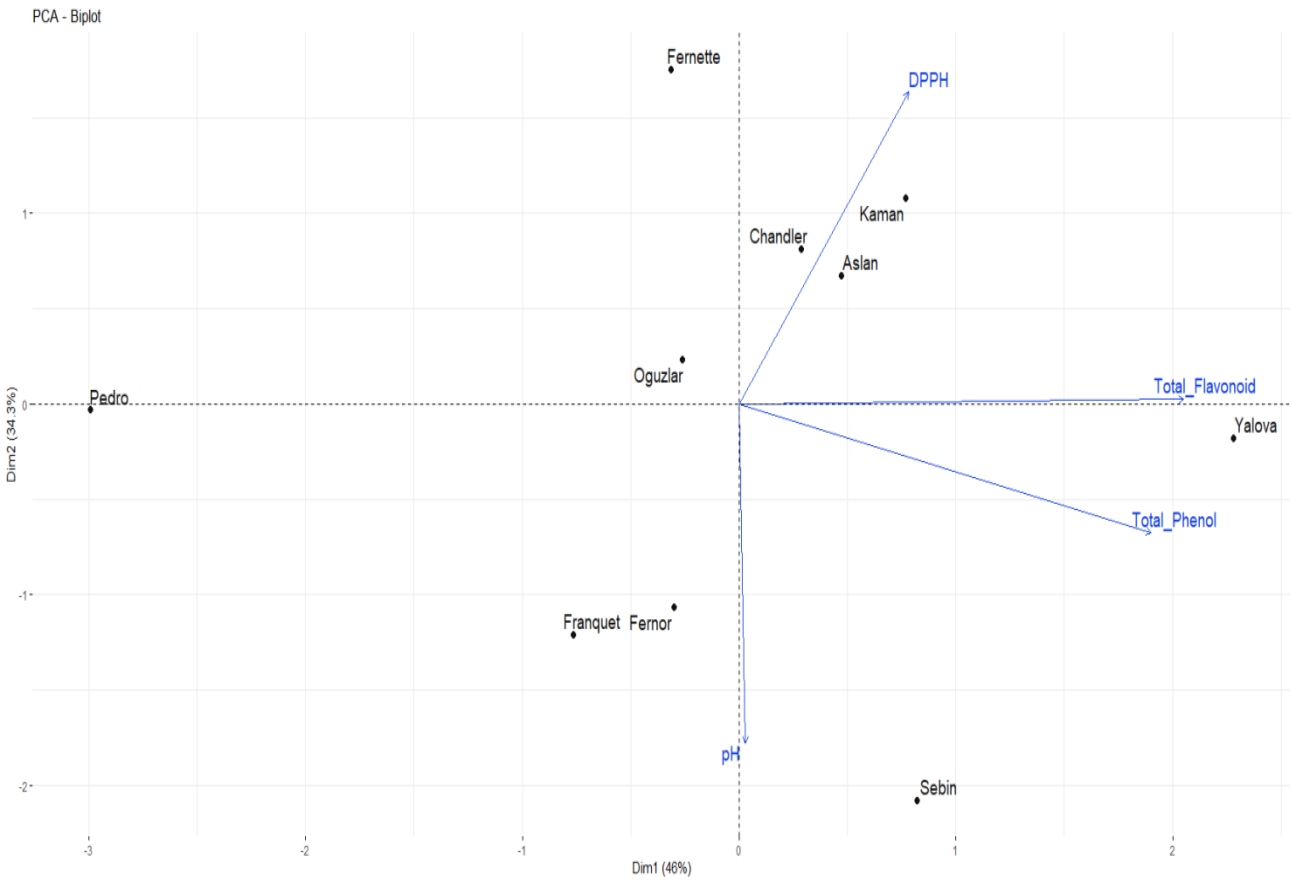

Fig. 1. PCA analysis for chemical properties.

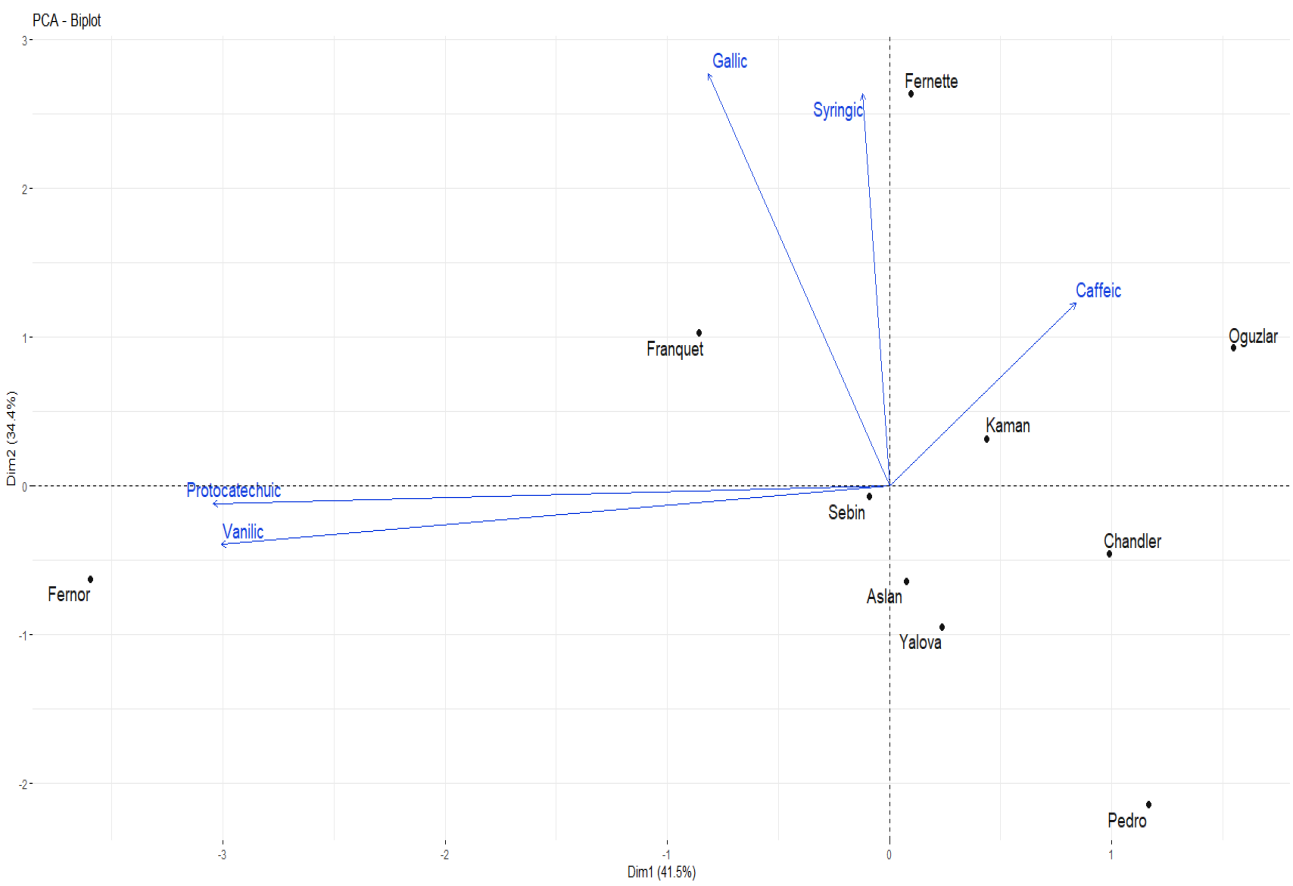

Fig. 2. PCA analysis for phenolic acids.

Table 2. Comparison of cultivars in terms of phenolic acids.

Cultivars	Gallic (µg g^{-1} DW)	Pyrocathecin (µg g^{-1} DW)	Vanilic (µg g^{-1} DW)	Caffeic (µg g^{-1} DW)	Syringic (µg g^{-1} DW)
Aslan	11.813 ± 0.012^{AB}	1.917 ± 0.015^{B}	2.103 ± 0.006^{A}	***1.393 ± 0.012^{B}***	1.403 ± 0.015^{B}
Chandler	11.010 ± 0.010^{AB}	1.710 ± 0.017^{BC}	1.910 ± 0.017^{AB}	1.573 ± 0.046^{A}	1.517 ± 0.015^{B}
Fernette	**13.410 ± 0.011^{A}**	2.010 ± 0.011^{AB}	2.080 ± 0.026^{A}	1.703 ± 0.006^{A}	**1.830 ± 0.044^{A}**
Fernor	12.113 ± 0.012^{A}	**3.623 ± 0.025^{A}**	**3.510 ± 0.036^{A}**	1.420 ± 0.021^{AB}	1.417 ± 0.015^{B}
Franquet	12.510 ± 0.010^{A}	2.410 ± 0.012^{A}	2.410 ± 0.010^{A}	1.510 ± 0.012^{A}	1.717 ± 0.015^{A}
Kaman	11.810 ± 0.013^{AB}	1.907 ± 0.012^{B}	1.810 ± 0.010^{AB}	1.410 ± 0.010^{AB}	1.707 ± 0.012^{A}
Oguzlar	12.413 ± 0.015^{A}	***1.410 ± 0.010^{C}***	***1.403 ± 0.006^{B}***	1.517 ± 0.029^{A}	1.623 ± 0.021^{A}
Pedro	***10.610 ± 0.010^{B}***	1.710 ± 0.011^{BC}	1.927 ± 0.031^{AB}	**1.607 ± 0.012^{A}**	***1.010 ± 0.010^{C}***
Sebin	11.297 ± 0.006^{AB}	2.310 ± 0.016^{A}	2.213 ± 0.015^{A}	1.597 ± 0.015^{A}	1.580 ± 0.020^{B}
Yalova	10.910 ± 0.017^{B}	1.907 ± 0.012^{B}	2.017 ± 0.015^{A}	1.313 ± 0.012^{B}	1.613 ± 0.012^{AB}
p value	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*
Average	11.790 ± 0.830	2.091 ± 0.589	2.138 ± 0.531	1.504 ± 0.116	1.542 ± 0.222

Differentcapital letters used to indicate significant difference among cultivars at $p<0.05$ (Kruskal Wallis test). The bold values represent the highest and bold italic values are the lowest

Table 3. Comparison of cultivars in terms of organic acids.

Cultivars	Oxalic	Ascorbic	Acetic
Aslan	5.743 ± 0.006^{BC}	1.350 ± 0.010^{B}	**4.173 ± 0.012^{A}**
Chandler	5.413 ± 0.015^{BC}	1.627 ± 0.006^{A}	3.880 ± 0.010^{AB}
Fernette	***4.453 ± 0.012^{C}***	1.677 ± 0.006^{A}	***3.163 ± 0.015^{C}***
Fernor	6.350 ± 0.017^{A}	**2.463 ± 1.712^{A}**	4.017 ± 0.006^{A}
Franquet	5.307 ± 0.006^{BC}	1.543 ± 0.006^{AB}	3.347 ± 0.023^{BC}
Kaman	5.230 ± 0.020^{BC}	1.453 ± 0.006^{B}	3.433 ± 0.012^{BC}
Oguzlar	6.163 ± 0.029^{AB}	1.237 ± 0.006^{BC}	4.010 ± 0.010^{A}
Pedro	6.137 ± 0.006^{AB}	***1.270 ± 0.035^{C}***	3.710 ± 0.010^{B}
Sebin	6.207 ± 0.012^{A}	1.720 ± 0.010^{A}	3.757 ± 0.012^{B}
Yalova	**7.227 ± 0.006^{A}**	1.513 ± 0.006^{AB}	3.323 ± 0.006^{BC}
p value	*0.001*	*0.002*	*0.001*
Averages	5.823 ± 0.739	1.585 ± 0.562	3.681 ± 0.334

Different capital letters used to indicate significant difference among cultivars (Kruskal Wallis test). The bold values represent the highest and bold italic values are the lowest

Organic acids: The organic acids in pollens of ten tested walnut cultivars are given in Table 3. Statistically significant differences were observed for the organic acid contents among the walnut cultivars. The highest organic acid was found to be oxalic acid, which was followed by the acetic acid and ascorbic acid. The ranges of these three acids were found to be 4.453-7.227, 1.270-2.463 and 3.163-4.017 respectively (Table 3). The highest content of oxalic acid was measured in Yalova cultivar and was followed by Fernor cultivar. This Fernor cultivar was also found to have the highest ascorbic acid content. The organic acid content of the fruits play a decisive role in the fruit flavor by affecting the sugar/acid balance of the fruits. In addition organic acids in fruits often occur in a free form or are combined as esters or glycosides (Cemeroğlu, 2007; Gundogdu *et al*., 2017). Results of present study are consistent with those of detected by different Researchers (Ozgen *et al*. 2009; Gundogdu *et al*., 2011; Rao *et al*., 2016).

The comparison of the ten cultivars in terms of the organic acid contents is shown in (Fig. 3). Results showed that the Fernette cultivar had the lowest oxalic acid and acetic acid concentrations. However, the ascorbic acid content of the Fernette cultivar was found to be significantly similar with the Fernor cultivar, which has the highest content. Moreover, Yalova cultivar was found to have the highest content of oxalic acid, while the ascorbic acid and acetic acid values were in medium level as compared with other cultivars. PCA analysis resulted with a better comparison of the ten cultivars and suggested that the Fernette, Kaman and Franquet cultivars have the lowest organic acids concentration while the Fernor cultivar was found to have the highest results for organic acids.

The correlation of the biochemical compounds tested from the pollens of different walnut cultivars is given in (Fig. 4). Results suggested that there is a low negative correlation between pH and DPPH. Total phenol was then found to have moderate positive correlation with oxalic acid contents where it had high positive correlation with total flavonoids. On the other hand, DPPH was negatively correlated with oxalic acid content and positively correlated with syringic acid. However, both these two correlations were moderate. The correlation between the oxalic acid and gallic acid was found to be moderately negative while the correlation between the ascorbic acid and protocatechuic acid was moderately positive. Similarly, the correlation between gallic acid and syringic acid was moderately positive. According to the results obtained, the highest correlation was observed between the protocatechuic acid and vanilic acid.

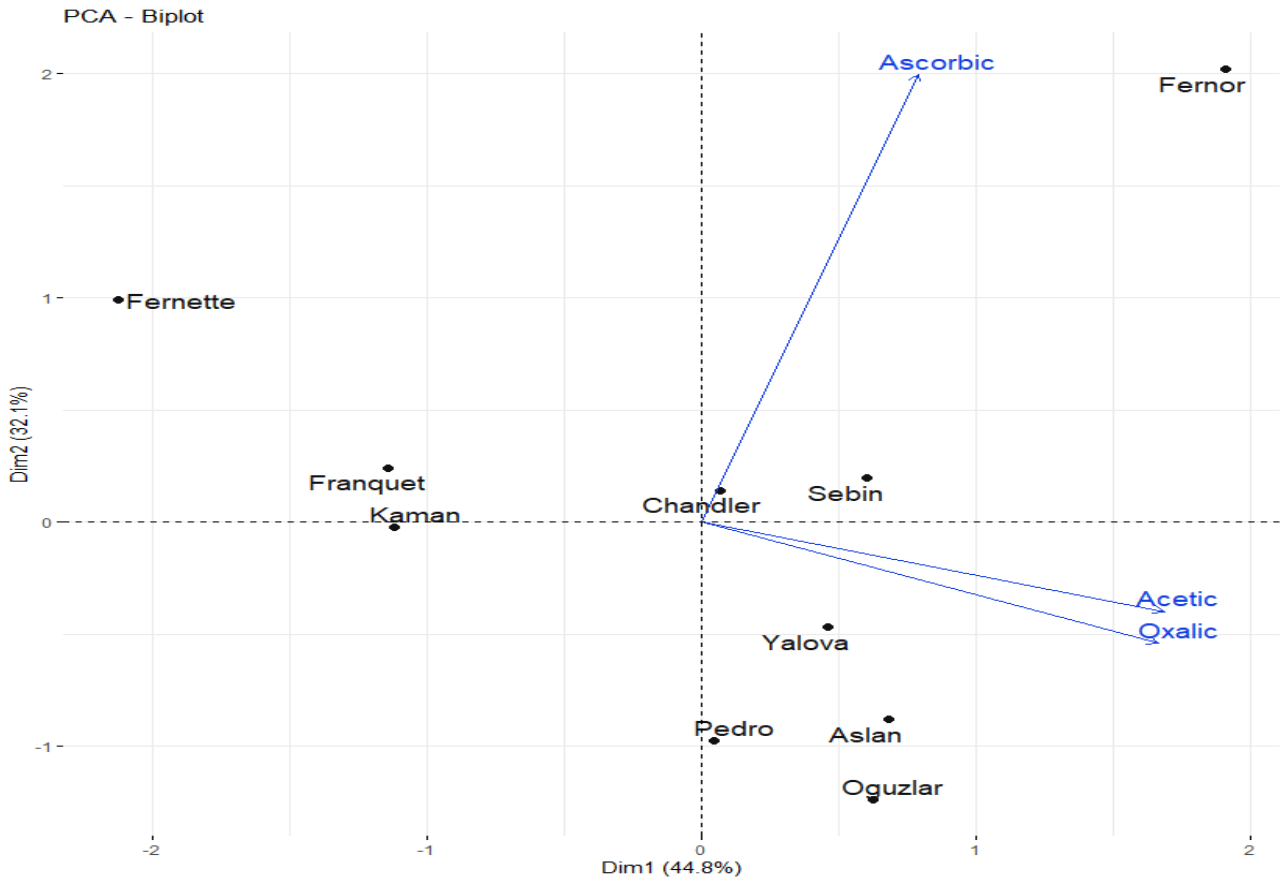

Fig. 3. PCA analysis for organic acids.

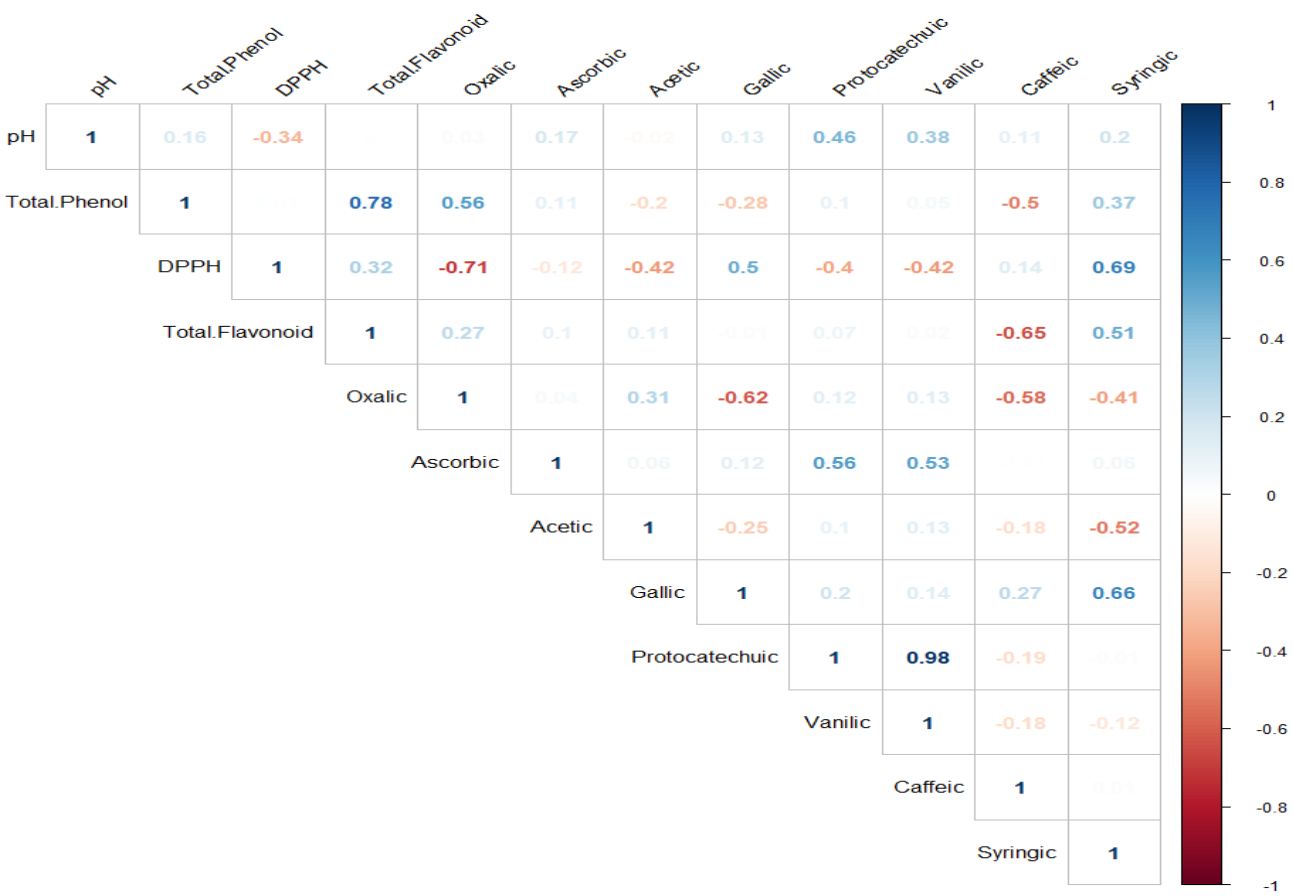

Fig. 4. Pearson's correlations among variables.

Conclusions

Biochemical compounds including total phenols, total flavonoid, antioxidant activity, phenolic and organic acid composition found in the pollens of the walnut cultivars were found to have a large variation. Results of present study not only showed the variation among the walnut cultivars, but also represent the first evidence that pollens of walnut trees have high concentration of the biochemical compounds in pollens (especially the high antioxidant activity) as compared with the other organs and/or tissues of walnut trees. So it is concluded from present study that the walnut pollensare a valuable phytochemical source with significant nutritional and health benefits.

Acknowledgements

We carried out the bioactive analysis in the Scientific Analysis Technological Application and Research Center of Uşak University. Thus we are very grateful to the Scientific Analysis Technological Application and Research Center of Uşak University for bioactive analysis for this manuscript.

References

Abe, L.T., F.M. Lajolo and M.I. Genovese. 2010. Comparison of phenol content and antioxidant capacity of nuts. *Ciência e Tecnologia de Alimentos Campinas*, 30: 254-259.

Akça, Y. and S.M. Sen. 2001. Study on the selection of superior walnut trees in Hizan (Bitlis) populations. *Acta Hort.*, 544: 115-118.

Amaral, J.S., R.M. Seabra, P.B. Andrade, P.Valentão, J.A. Pereiraand and F. Ferreres. 2004. Phenolic profile in the qualitycontrol of walnut (*Juglansregia* L.) leaves. *Food Chem*., 88(3): 373-379.

Bernard, A., F. Lheureuxand and E. Dirlewanger. 2018. Walnut: past and future of genetic improvement. *Tree Gen. & Genom.,* 14: 1.

Beyhan, O., S. Gozlekci, M. Gundogdu and S. Ercişli. 2016. Physico-chemical and antioxidant characteristics in fruits of walnut (*Juglansregia* L.) genotypes from inner Anatolia. *Not. Bot. Hort. Agrobot.*, 44(2): 586-592.

Bonvehi, S.J., S.M. Torrento and C.E. Lorente. 2001. Evaluation of polyphenolic and flavonoid compounds in honeybee-collected pollen produced in Spain. *J. Agric. Food Chem.*, 49(4): 1848-1853.

Bujdosó, G., G. Végvári, V. Hajnal, G. Ficzek and M. Tóth. 2014. Phenolic profile of the kernel of selected persian walnut (*Juglans regia* L.) cultivars. *Not. Bot. Hort. Agrobot.*, 42(1): 24-29.

Carpes, S.T., S.M. De Alencar, I.S. Cabral, T.L.C. Oldoni, G.B. Mourao, C.W.I. Haminiuk and M.L. Masson. 2013. Polyphenols and palynological origin of bee pollen of *Apismellifera* L. from Brazil., characterization of polyphenols of bee pollen. *CyTA. J. Food*, 11: 150-161.

Cemeroglu, B. 2007. Gıda Analizleri [Food Analysis]. Food Technology Society Publication, Ankara, Turkey, 168-171 (in Turkish).

Chang, C., M. Yang, H. Wen and J. Chern. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.*, 10: 178-182.

Chevallier, A., O. Bray, J.P. Prunet and M. Giraud. 2009. Factors influencing walnut blight symptoms emergence and development. *Acta Hort*., 861: 473-478.

Chrzanowski, G., B. Leszczynski, P. Czerniewicz, H. Sytykiewicz, H. Matok and R. Krzyzanowski. 2011. Phenolic acids of walnut (*Juglans regia* L.). *Herb. Polon.*, 57(2):

Colaric, M., R. Veberic, A. Solar, M.Hudina and F. Stampar. 2005. Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of *Juglans regia* L. *J. Agric. Food Chem.,* 53: 6390-6396.

Cosmulescu, S. and I. Trandafir. 2012. Anti-oxidant activities and total phenolics contents of leaf extracts from 14 cultivars of walnut (*Juglans regia* L.). *J. Hort. Sci. & Biotechnol.*, 87(5): 504-508.

Cosmulescu, S., A. Baciu, M. Botu, G. Achim, I. Trandafir and M. Gruia. 2010b. Polyphenols-potential markers for determining genetic diversity in walnut (*Juglans regia* L.). *Lucrari Stiintifice Universitatea Agrara de Stat din Moldova, Facultatea de Horticultura*, 24(1): 19-25.

Cosmulescu, S., I. Trandafir and V. Nour. 2015b. Mineral composition of fruit in black and red currant. *South West. J. Hort., Biol. & Environ.*, 6: 43-51.

Cosmulescu, S., I. Trandafir, Gh. Achim, M. Botu, A. Baciu and M. Gruia. 2010a. Phenolics of green husk in mature walnut fruits. *Not. Bot. Hort. Agrobot.,* 38(1): 53-56.

Cosmulescu, S., I. Trandafir, V. Nour and M. Botu 2015a. Total phenolic, flavonoid distribution and antioxidant capacity in skin, pulp and fruit extracts of plum cultivars. *J. Food Biochem.*, 39: 64-69.

Eyduran, S.P., S. Ercisli, M. Akin, O. Beyhan and M.K. Gecer. 2015. Organic acids, sugars, vitamin c, antioxidant capacity, and phenolic compounds in fruits of white (*Morus alba* L.) and black (*Morus nigra* L.) mulberry genotypes. *J. Appl. Bot. Food Qual.*, 88: 134-138.

Fadavi A, M. Barzegar, M.H. Azizi and M. Bayat. 2005. Note. Physicochemical composition of ten pomegranate cultivars (*Punica granatum* L.) grown in Iran. *Int. J. Food Sci. Tech.*, 11(2): 113-119.

Fatrcová-Šramková, K., J. Nôžková, M. Kacániová, M. Máriássyová, K. Rovná and M. Stricík. 2013. Antioxidant and antimicrobial properties ofmonofloral bee pollen. *J. Environ. Sci. & Heal., Part B* 48:133-138.

Feas, X., M.P. Vazquez-Tato, L. Estevinho, J.A. Seijas and A. Iglesias. 2012. Organicbee pollen: botanical origin, nutritional value, bioactive compounds, antioxidant activity and microbiological quality. *Molecules,* 17: 8359-8377.

Fukuda, T., H. Ito and T. Yoshida. 2003. Antioxidant polyphenols from walnuts (*Juglans regia* L.). *Phytochem.*, 63: 795-801.

Gecer, M.K., T. Kan, M. Gundogdu, S. Ercisli, G. Ilhan and H.I. Sagbas. 2020. Physicochemical characteristics of wild and cultivated apricots (*Prunus armeniaca* L.) from Aras valley in Turkey. *Genet. Resour. Crop Evol.,* 67: 935-945.

Ghasemi, K., Y. Ghasemi, A. Ehteshamnia, S.M. Nabavi, S.F. Nabavi, M.A. Ebrahimzadeh and F. Pourmorad. 2011. Influence of environmental factors on antioxidant activity, phenol and flavonoids contents of walnut (*Juglans regia* L.) green husks. *J. Med. Plants Res.,* 5(7): 1128-1133.

Gun, A., V. Erdoğan, M.E. Akçay, A. Fidanci and I. Tosun.2010. Pistillate flower abscission in turkish walnut cultivars and its reduction by AVG conference: 2nd International Symposium on Sustainable Development (ISSD'10), At: Sarajevo. Volume: *Proce., Sci. & Technol.*, 3: 29-34.

Gundogdu, M., F. Muradoglu, R.I.G. Sensoyand and H. Yilmaz. 2011. Determination of fruit chemical properties of *Morusnigra* L., *Morus alba* L. and *Morus rubra* L. by HPLC. *Sci. Hort.*, 132: 37-41.

Gundogdu, M., I. Canan, M.K. Gecer, T. Kan and S. Ercisli. 2017. Phenolic compounds, bioactive content and antioxidant capacity of the fruits of mulberry (*Morus* spp.) germplasm in Turkey. *Folia Hort.*, 29(2): 251-262.

Gunn, B.F., M. Aradhya, J.M. Salick, A.J. Miller, Y. Yongping, L. Lin and H. Xian. 2010. Genetic variation in walnuts (*Juglans regia* and *J. sigillata*; Juglandaceae): species distinctions, human impacts, and the conservation of agrobiodiversity in Yunnan, China. *Amer. J. Bot.,* 97(4): 660-671.

Isanga, J. and G.N. Zhang. 2007. Biological active components and nutraceuticals in peanuts and related products: Review. *Food Rev. Int.,* 23: 123-140.

Jakopic, J., R. Veberic and F. Stampar. 2009. Extraction of phenolic compounds from green walnut fruits in different solvents. *Acta Agri. Slovenica*, 93(1): 11-15.

Kafkas, E., A. Burgut, H. Ozcan, A. Ozcan, M. Sutyemez, S. Kafkas and N. Türemis. 2017. Fatty acid, total phenol and tocopherol profiles of some walnut cultivars: a comparative study. *Food & Nutr. Sci.*, 8(12): 1074.

Kähkönen, M.P., A.I. Hopia, J.V. Heikki, J.P. Rauha, K. Pihlaja, T.S. Kujala and M. Heinonen. 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.,* 47: 3954-3962.

Kornsteiner, M., K.H. Wagner and I. Elmadfa. 2006. Tocopherols and total phenolics in 10 different nut types. *Food Chem*., 98: 381-387.

Leja, M., A. Mareczek, G. Wyzgolik, J. Klepacz-Baniak and K. Czekonska. 2007. Antioxidative properties of bee pollen in selected plant species. *Food Chem*., 100: 237-240.

Lu, B., L.N. Chen, J.B. Hao, Y. Zhang and J.C. Huang. 2020. Comparative transcription profiles reveal that carbohydrates and hormone signalling pathways mediate flower induction in Juglans sigillata after girdling. *Ind. Crops Prod.*, 153: 112556.

Margaoan, R., L. Marghitas, D. Dezmirean, C. Mihai and O. Bobis. 2010. Bee collected pollen. General aspects and chemical composition. Bulletin of University of Agricultural Sciences and Veterinary Medicine ClujNapoca. *J. Anim. Sci. Biotechnol.,* 67: 254-259.

Miraliakbari, H. and F. Shahidi. 2008. Oxidative stability of tree nut oils. *J. Agric. Food Chem.,* 56: 4751-4759.

Moreira, L., L.G. Dias, J.A. Pereira and L. Estevinho. 2008. Antioxidant properties, total phenols and pollen analysis of propolis samples from Portugal. *Food Chem. Toxicol.,* 46: 3482-3485.

Nicolson, S.W., S.D.S.D. Neves, H. Human and C.W. Pirk. 2018. Digestibility and nutritional value of fresh and stored pollen for honey bees (*Apis mellifera* scutellata). *J. Ins. Physiol.*, 107: 302-308.

Nunes, P.X., S.F. Silva, R.J. Guedes and S. Almeida. 2012. Biological oxidations and antioxidant activity of natural products. In: (Ed.): Venketeshwer, R. Phytochemicals as Nutraceuticals- Global Approaches to their Role in Nutrition and Health. Rijeka, Croatia: *InTech*, 1-20.

Okatan, V. 2020. Antioxidant properties and phenolic profile of the most widely appreciated cultivated berry species: A comparative study. *Folia Hort.*, 32(1): 79-85.

Oliveira, I., A. Sousa, J.S. Morais, I.C. Ferreira, A. Bento, L. Estevinho and J.A. Pereira. 2008. Chemical composition, and antioxidant and antimicrobial activities of three hazelnut (*Corylus avellana* L.) cultivars. *Food & Chem. Toxic.*, 46(5): 1801-1807.

Oroian, M., F. Ursachi and F. Dranca. 2020. Ultrasound-assisted extraction of polyphenols from crude pollen. *Antioxidants*, 9(4): 322.

Ozgen, M., S. Serçe and C. Kaya. 2009. Phytochemical and antioxidant properties of anthocyanin-rich *Morus nigra* and *Morus rubra* fruits. *Sci. Hort.*, 119(3): 275-279.

Ozkan, G., S. Ercisli, A. Zeb, G. Agar, H.I. Sagbas, G. Ilhan and M. Gundogdu. 2019. Some morphological and biochemical characteristics of wild grown Caucasian Whortleberry (*Vaccinium arctostaphylos* L.) genotypes from Northeastern Turkey. *Not. Bot. Hort. Agrobot*., 47(2): 378-383.

Poggetti, L., P. Ermacora, G. Cipriani, F. Pavan and R. Testolin. 2017. Morphological and carpological variability of walnut germplasm (*Juglans regia* L.) collected in North-Eastern Italy and selection of superior genotypes. *Sci. Hort.,* 225: 615-619.

Pokorny, J. 2007. Are natural antioxidants better–and safer–than synthetic antioxidants?. *Eur. J. Lipid Sci. & Technol.*, 109(6): 629-642.

Polat, M., V. Okatan and S.F. Güçlü. 2015. Determination of some physical and chemical properties of walnut (*Juglans regia* L.) genotypes grown in the central district of Bitlis/Turkey.-Scientific Papers. *Series B. Hort.,* 59: 81-86.

Polito, V., J. Grant and H. Johnson. 2005. Walnut pollination and pistillate flower abortion. *University of California Fruit and Nut Research Info Center,* p. 133.

Rahimipanah, M., M. Hamedi and M. Mirzapour. 2010. Antioxidant activity and phenolic contents of Persian walnut (*Juglans regia* L.) green husk extract. *Afri. J. Food Sci. & Techn.,* 1: 105-111.

Rop, O., T. Jurikova, J. Mlcek, D. Kramarova and Z. Sengee. 2009. Antioxidant activity and selected nutritional values of plums (*Prunus domestica* L.) typical of the White Carpathian Mountains. *Sci. Hort.,* 122(4): 545-549.

Rupasinghe, H.P.V., S. Jayasankar and W. Lay. 2006. Variation in total phenolics and antioxidant capacity among European plum genotypes. *Sci. Hortic.* 108(3): 243-246.

Senica, M., F. Stampar and M. Mikulic-Petkovsek. 2019. Different extraction processes affect the metabolites in blue honeysuckle (*Lonicera caerulea* L. subsp. edulis) food products. *Turk. J. Agric. For.,* 43: 576-585.

Simsek, M. 2011. A study on selection and ıdentification of table fig types in East Edge of Firat River. *Asian J. Ani. & Vet. Adv*., 6(3): 265-273.

Simsek, M., A. Osmanoglu and H. Yildirim. 2010. Evaluation of selected almond types in Kocakoy and Hani Counties. *Afr. J. Agri. Res.*, 5(17): 2370-2378.

Solar, A., M. Colarič, M. Hudina and F. Stampar. 2006. Phenolic content of walnut fruit as affected by cultivar and developmental stage. *Acta Hort.,* 705: 231-240.

Stampar, F., A. Solar, M. Hudina, R. Veberic and M. Colaric. 2006. Traditional walnut liqueurcocktail of phenolics. *Food Chem.*, 95: 627-631.

Stanciu, O.G., L.A. Marghitas, D. Dezmirean and M.G. Campos. 2011. A comparison between the mineral content of flower and honeybee collected pollen of selected plant origin (*Helianthus annuus* L. and *Salix* sp.). *Rom. Biotechnol. Lett*., 16: 6291-6296.

Sutyemez, M. 2007. Determination of pollen production and quality of some local and foreign walnut genotypes in Turkey. *Turk. J. Agric. For.,* 31: 109-114.

Thaipong, K.U. Boonprakob, K. Crosby, L. Cisneros-Zevallos and D.H. Byrne. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J. Food Comp. Anal.,* 19: 667-669.

Ulusoy, E. and S. Kolayli. 2014. Phenolic composition and antioxidant properties of Anzer bee pollen. *J. Food Biochem.*, 38: 73-82.

Valdebenito, D., D. Farías, E. Oyanedel, M. Castro, B. Lampinen, A. Tixier and S. Saa. 2017. The morphology of a walnut (*Juglans regia* L.) shoot is affected by its position in the canopy and correlated to the number and size of its fruits. *Sci. Hort.*, 220: 303-309.

Vu, D.C., P.H. Vo, M.V. Coggeshall and C.H. Lin. 2018. Identification and characterization of phenolic compounds in black walnut kernels. *J. Agri. & Food Chem*., 66(17): 4503-4511.

Yang, L.X. 2005. Effect of water extracts of larch on growth of Manchurian walnut seedings. *J. For. Res.,* 16(4): 285-288.

Yerlikaya, C., S. Yucel, Ü. Erturk and M. Korukluoğlu. 2012. Proximate composition, minerals and fatty acid composition of *Juglans regia* L. genotypes and cultivars grown in Turkey. *Braz. Arch. Biol. Technol.,* 55(5): 677-683.

(Received for publication 27 June 2020)