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COMPARISON OF PHENOLIC ACID AND FLAVONOID CONTENTS OF SUNFLOWER (*HELIANTHUS ANNUUS* L.) BEE POLLEN AND BEE BREAD TAKEN FROM THE SAME HIVES

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ABSTRACT. In this study, individual phenolic compounds in sunflower bee pollen and bee bread taken from the same hives were compared. Quantitatively individual phenolic compound content analyses were performed with high pressure liquid chromatography coupled with diode array detector (HPLC-DAD). Eight different phenolic compounds analyzed in bee pollen and bee bread samples, namely caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid, rutin, naringenin, quercetin, and kaempferol. The amounts of phenolic compounds detected in the samples in bee pollen and bee bread are as follows, respectively: caffeic acid: 4.10 ± 0.63 mg/kg, 2.07 ± 0.38 mg/kg; *p*-coumaric acid: 9.52 ± 1.21 mg/kg, 4.26 ± 0.46 mg/kg; ferulic acid: 0.24 ± 0.03 mg/kg, 5.48 ± 0.14 mg/kg; cinnamic acid: 0.00 mg/kg, 6.19 ± 0.92 mg/kg; rutin: 132.09 ± 1.13 mg/kg, 63.47 ± 3.11; naringenin: 82.91 ± 3.59 mg/kg, 329.11 ± 2.95 mg/kg; quercetin: 460.81 ± 14.23 mg/kg, 936.14 ± 12.21 mg/kg; kaempferol: 2.90 ± 0.31 mg/kg, 183.80 ± 4.24 mg/kg. Differences in the amounts of individual phenolic compounds in bee pollen and bee bread are thought to be due to the conversion of phenolic compounds to aglycone forms by enzymes of bacteria and/or secretions of bees.

KEY WORDS: Phenolic compounds, Bee pollen, Bee bread, Sunflower, Türkiye

INTRODUCTION

Pollen grains, produced by the anthers of flowering plants, possess a hard, waxy, and polymeric outer wall. Although its main function in the ecosystem is to act as a vessel for the transport of genetic information during plant reproduction, it has also been widely consumed by humans as medicine due to its rich nutritional and bio-active metabolite content [1]. The use of pollen as an astringent and a sedative was first recorded by a physician named Maimonides, who lived in the years 1135-1204. During the 1200's, Ibn al-Baytar noted the beneficial effects of pollen for the stomach and for swellings caused by allergic reactions to certain foods [2]. Aside from humans, various organisms such as bees, bugs, and birds feed on pollen due to its high nutritional value. With the increased use of pollen traps, plant pollen collected by forager bees and brought to the hive became more widely available [3], which is known as 'bee pollen' (BP).

Honey bees have a symbiotic relationship with plants as they transport pollen aiding in plant reproduction, in return feeding on some of this nutritious product. They visit plants intensively, especially in spring, to collect pollen grains that stick to their bodies. Some of the waxy grains that stick to the hairy legs of honey bees are enriched with saliva and stored as pellets in hind leg pockets called "corbiculae" [4]. After these pellets are transferred to empty or semi-filled cells in the hive, young worker bees inside the hive add hypopharyngeal glandular secretion, nectar, honey, microorganisms, and various enzymes and top it with a layer of honey. The cells are then capped with wax, initiating the process that will form a more nutritionally valuable product known as 'bee bread' (BB). The pH of BB, which is a modified form of BP, is approximately 4, so it can

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be preserved as food for a long time without spoiling [5-7]. BB is a product of fermentation by microorganisms of the BP and bee secretions [8, 9]. *Lactobacillus* and *Bifidobacterium* species, usually present in bee secretions and nectar [10-12] carry out lactic acid fermentation on the stored pollen [13, 1].

Pollen may have a variable metabolic profile depending on the plant species [14], although it typically has large amounts of proteins, essential amino acids, nucleic acids, carbohydrates, and lipids [15-19]. Apart from these core metabolites, pollen is also abundant in secondary metabolites, which are produced as part of the plant's defense mechanisms, in reaction to environmental stress, or during reproductive processes [20]. Since secondary metabolites can also be used as biochemical markers to identify the botanical origin of bee products, research interest in the secondary metabolite profiles of BP and BB has grown as the therapeutic potential of secondary metabolites has come to light [5].

Phenolic chemicals, especially flavonoids and phenolic acids, are of special interest among these secondary metabolites. These compounds are defined by the existence of a minimum of one benzene ring and one or more hydroxyl groups [21]. Their biological activity is mostly ascribed to the hydroxyl and/or methoxy functional groups [22, 23], with multiple studies focusing their antioxidant, antibacterial, and anticancer characteristics [3, 24, 25].

Comparative information on both products obtained from the same region is insufficient, even though a small number of research have looked at the phenolic content of BP and BB derived from sunflowers [26-28].

In order to compare and quantitatively assess the distinct phenolic component profiles of sunflower (*Helianthus annuus*) BP and BB derived from the same honeybee colonies, the study focused on flavonoids and phenolic acids.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile high pressure liquid chromatography (HPLC grade, Product No. 34851), formic acid (HPLC grade, Product No. 5.43804), methanol (HPLC grade, Product No. 34885), dichloromethane (Suitable for HPLC, Product No. 34856), caffeic acid (HPLC grade, Product No. C0625), *p*-coumaric acid (HPLC grade, Product No. C9008), rutin (HPLC grade, Product No. R5143), cinnamic acid (HPLC grade, Product No. 140-10-3), quercetin (HPLC grade, Product No. Q4951), naringenin (HPLC grade, Product No. PHL89738), kaempferol (HPLC grade, Product No. 60010), and ferulic acid (HPLC grade, Product No. 46278) were purchased from Sigma-Aldrich Company (Sternheim, Germany).

Instruments and equipment

Rotary evaporator: Buchi (Switzerland), magnetic stirrer: Isolab (Germany), orbital shaker: Onilab (USA), ultrasonic sonicator: Isolab (Germany), HPLC system: Shimadzu Nexera-i LC-2040C 3D Plus HPLC with PDA detector (Shimadzu, Kyoto, Japan), HPLC column: Phenylhexyl 4.6 × 150 mm, 3 µm (UP), C18 column (GL Sciences Inter Sustain, Japan), refrigerator: Bosch (Türkiye).

Sample collection

Bee pollen and bee bread samples were provided by a local beekeeper from an apiary placed near a sunflower field in Tokat, Türkiye (latitude: 40.3139°N, longitude: 36.5544°E), which were then stored in a refrigerator at +4 °C before analyses.

Sample preparation

Prior to extraction, fresh samples were ground using liquid nitrogen in a porcelain mortar. 10 mL of methanol/dichloromethane mixture (3:1) was added to 1 g of the ground samples and incubated at orbital shaker at room temperature for 2 h. Finally, the samples were centrifuged at 3000 g and the supernatant was used for quantitative analysis of individual phenolic compounds.

Preparation of stock solutions of samples

The extracts were dissolved in the mobile phase mixture at the original gradient ratio (solvent A: solvent B = 9:1, v/v) to create stock solutions with a concentration of 1 mg/mL for quantitative measurement by HPLC. Peak regions from the HPLC chromatograms were compared to those of the respective phenolic standards in order to perform quantification. The results were computed and represented as mg/kg of sample and displayed in tables and graphs.

High pressure liquid chromatography method

The quantitative analysis was carried out using an HPLC machine consisting of a Shimadzu LC 20AT pump, SPD-M20A DAD detector, and a CTO-2-AC column oven. Separation was carried out on a Dionex, Acclaim Polar Advantage 120 Å (150×4.60 mm, 3 µm) C16 reverse phase column. Table 1 shows the pump program used with a mobile phase consisting of solvent A (2.5% formic acid/deionized water) and solvent B (acetonitrile). The flow rate and temperature during the analysis were set at 1 mL/minute and 40 °C, respectively. The extract was filtered through a 0.22 µm nylon using a syringe and 20 µL was injected into the high pressure liquid chromatography coupled with diode array detector (HPLC-DAD) device. A wavelength between 190 and 800 nm was scanned for detection, resulting in a reading at 280 nm. The individual phenolic compounds were identified based on their retention times and a comparison to the each phenolics standard spectrum.

Table 1. Gradient elution program for HPLC analysis.

Time (min)	% solvent A	% solvent B
0.00-3.00	99	1
3.01-20.00	90	10
20.01-65.00	70	30
66.00-80.00	0	100

Statistical analysis

StatPlus software (version 5.8.4) was used for statistical analysis. To provide a summary of the mean, standard deviation, and range of the variables under study, descriptive statistics were computed. Additionally, the mean amounts of phenolic components in bee pollen (BP) and bee bread (BB) were compared using a paired two-sample t-test. The difference was statistically significant at $p < 0.01$.

RESULTS AND DISCUSSION

Totally, eight phenolic compounds (caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid, rutin, naringenin, quercetin, and kaempferol) were identified in sunflower BP and BB as reported in Table 2. The two products showed great differences in terms of phenolic substances they contained, even though their botanical origins were the same and they were taken from the same hives, indicating that the content of BP was changed significantly during its fermentation to BB.

The phenolic content in milligrams per kilogram of BP and BB was calculated from peak areas and displayed in Table 1 as the average of 3 readings with the respective standard deviation.

Table 2. The mean quantities of phenolic acids and flavonoids in bee pollen (BP) and bee bread (BB) (mg/kg pollen).

Compound	Sample		P value
	BP ± SD	BB ± SD	
Caffeic acid	4.10 ± 0.63	2.07 ± 0.38	0.01
p-Coumaric acid	9.52 ± 1.21	4.26 ± 0.46	0.01
Ferulic acid	0.24 ± 0.03	5.48 ± 0.14	0.001
Cinnamic acid	0.00 ± 0.00	6.19 ± 0.92	0.001
Rutin	132.09 ± 1.13	63.47 ± 3.11	0.01
Naringenin	82.91 ± 3.59	329.11 ± 2.95	0.001
Quercetin	460.81 ± 14.23	936.14 ± 12.21	0.01
Kaempferol	2.90 ± 0.31	183.80 ± 4.24	0.001

Phenolic compounds detected in BP and BB samples are as shown in Table 2. As mentioned above, these compounds differ individually between the two products. For the flavonoids, the quercetin values show a drastic increase to 936.14 ± 12.21 mg/kg in BB when compared to 460.81 ± 14.23 mg/kg in BP. Naringenin and kaempferol also showed a similar trend and increased in BB to 329.11 ± 2.95 mg/kg and 183.80 ± 4.24 mg/kg from 82.91 ± 3.59 mg/kg and 2.90 ± 0.31 mg/kg in BP respectively (Figure 2). The only decreased flavonoid during the transition from BP to BB is rutin, which is 132.09 ± 1.13 mg/kg in BP and 63.47 ± 3.11 mg/kg in BB samples. As for phenolic acids, cinnamic acid is absent in BP while it measures about 6.19 ± 0.92 mg/kg in BB samples. Similarly, the ferulic acid content in BB is quite high compared to BP. The other two acids, namely caffeic acid and *p*-cumaric acid, were found to be higher in BP than in BB samples (Table 2, Figure 2). Cinnamic acid, which is not found in BP but is prominently present in BB, may be one of the intermediate products of ferulic and *p*-coumaric acids.

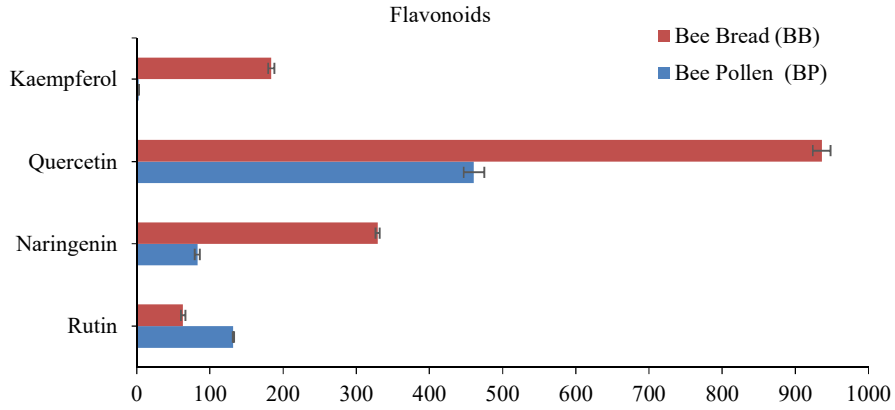


Figure 1. Change of flavonoid levels in bee pollen and bee bread.

The phenolic composition of BP and BB may differ in many aspects such as product type or geographical origin [29]. Our results differed significantly from the phenolics of sunflower pollen from Serbia [27], indicating regional differences. Only two, caffeic acid and kaempferol, were

similar in both studies. Some phenolics such as protocatechuic acid, 5-O-caffeoylquinic acid and isorhamnetin were detected in Serbia but not in our study; *p*-coumaric acid and ferulic acid were detected in our study but not in Serbia [27]. Cinnamic acid could not be detected in sunflower BP in either study, which is detected only in BB in our study.

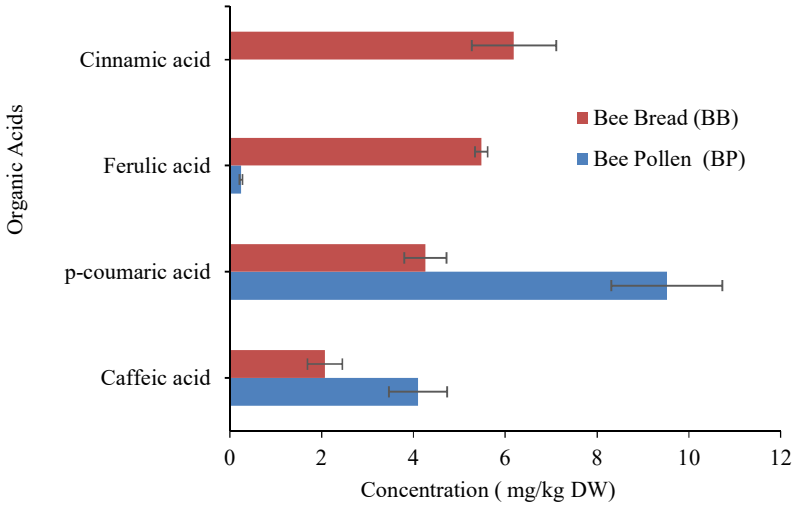


Figure 2. Change of organic acid levels in bee pollen and bee bread.

According to one research [29], the antioxidant activities of flavonoids decrease in the following order: kaempferol > quercetin > luteolin > apigenin. They attributed the lower antioxidant activity of sunflower pollen compared to other monofloral pollens to the lack of kaempferol in sunflower pollen. In our study, the amounts of kaempferol and quercetin were found low in BP but high in BB, indicating that the antioxidant activity is potentially higher in BB than BP.

The high abundance of quercetin, naringenin and kaempferol in BB is probably due to the enzymatic conversion of flavonoid glycosides into aglycones during the conversion of BP to BB. Previous studies have shown that some phenolics are converted to others by the enzymes of microorganisms such as *Lactobacillus* bacteria [9, 11, 15]. The decrease in caffeic acid and *p*-coumaric acid in BB is also most likely due to their degradation by the enzymes of such bacteria.

CONCLUSION

It is known that microorganisms cause changes in BP content through fermentation during the conversion process of BP to BB [1, 13]. These transformations most likely occur through enzymes produced by these bacteria and/or secretions of bees. Since many of these enzymes have glycosidase activity, they can increase the formation of aglycone forms of phenolic compounds. In the present study some of the phenolics decreased while others increased significantly. Particularly, the increase in biologically active flavonoids such as quercetin, kaempferol, naringenin is remarkable. This increase in antioxidant compounds makes BB a more functional food with various biological activities, which needs to be supported by more detailed studies. Due to its high phenolic content, BP has strong biological activities such as antimicrobial, anti-inflammatory and antioxidant effects and it provides various benefits to human health. Of course,

BB surpasses BP in terms of nutritional value and functional potential. Therefore, BP and BB deserve to be among the most popular natural products of recent years.

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