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Phenolic profile and antioxidant activity of bee pollen extracts from different regions of Algeria

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Abstract. Due to its complex biochemical properties, the bee pollen is considered one of the functional foods. Bee pollen collected from pollen grains from different botanical sources offers almost a full diet such as carbohydrates, proteins, amino acids, vitamins, minerals. In this study, methanol extracts of 13 honeybee pollen samples were evaluated for flavonoids, phenolic compounds, and antioxidant capacity. Caffeic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, gallic acid, quercetin, rutin, vanillin, and naringin were identified as main phenolic compounds in pollen extracts by High-Performance Liquid Chromatography (HPLC) analysis. The obtained results are: total phenolic content - 379.8 to 915.6 mg GAE/100 g, total flavonoid content - 207.1 to 550 mg QE/100 g, and antioxidant activity - 808.2 to 3311 mg GAE/100 g in bee pollen extracts.

Keywords: bee pollen; phenols; HPLC; antioxidant properties.

1. Introduction

From the beginning of civilization, bee pollen has been used by man. There are other products, such as royal jelly, propolis, and beeswax, although this is the most popular beehive product. Consumers value these natural products well because of the high number of quality controls they undergo, as well as for their nutritional and medicinal qualities [1].

Pollen is the product of flower pollen agglutination; it is produced by factor honey with nectar and salivary products and stocked at the entrance to the hive [2]. Bee pollen collection is a fairly recent technology, largely based on the simple method of scraping pollen off the bees' legs as they reach the hive. Bee pollen is both nutritious and therapeutic, due to which many of its beneficial properties have been revealed. Modern science has made it possible to classify the useful antimicrobials [3], antifungal [4], antioxidant [5], anti-radiation [6], hepatoprotective [7], chemopreventive [8], anticancer [9] and anti-inflammatory activities [10].

The main primary constituents are carbohydrates and water, a few minor compounds containing organic acids, enzymes, secondary metabolites, minerals, and proteins [11]. The secondary metabolites such as phenols, flavonoids, ascorbic acids, and carotenoids are reported to be responsible for bee pollen's significant health benefits, though present in small quantity [12].

Therefore, secondary metabolite composition is a significant factor in terms of their medicinal benefits, including antioxidant properties. Bee pollen's quality and properties mostly depend on the geographic environment, climatic environment, and floral resource availability [13].

It was proposed that flavonoids and phenolic acids be responsible for biological activities among these compounds. Hence, the phenolic acid content is considered an important index for assessing pollen quality. A large range of analytical approaches, including spectrophotometry, high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC–MS), have been used for the study of phenolic compounds in pollen. Among these methods, HPLC is between the most used techniques in bee product analysis, as it can analyze complex mixtures due to their high selectivity [14].

Thus, the study aimed to assess the phenolic content of thirteen bee pollen samples harvested from different regions of Algeria and their antioxidant activity using a spectrophotometric and high-performance liquid chromatography analysis.

2. Experimental

2.1. Reagents and chemicals

All the solvents, i.e., methanol, acetonitrile, acetic acid, and water, were of HPLC grade and were purchased from Sigma (Sigma–Aldrich, Germany). All the reference compounds were purchased from Alfa Aesar

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(U.S.A.), i.e., gallic acid, chlorogenic acid, vanillic acid, caffeic acid, vanillin, *p*-coumaric acid, rutin, naringin, and quercetin, as well as ammonium molybdate. Sodium phosphate (Na_3PO_4, anhydrous, powder, extra pure), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4) 98 %, aluminum chloride ($AlCl_3$) and sodium carbonate (Na_2CO_3) were purchased from Prolabo (U.S.A.). The Folin-Ciocalteu reagent (FCR) was purchased from Sigma-Aldrich (U.S.A.). All other reagents used were of analytical grade.

2.2. Equipment and chromatographic conditions

An ultrasonic bath model Ultrasons 3000513 (J.P. Selecta, S.A., Barcelona, Spain) was used to extract the active compounds from the samples. Spectrophotometric measurements were performed on a UV-1800 Shimadzu Spectrophotometer (double-beam) equipped with 1 cm quartz cuvettes (Shimadzu Co., Japan).

The chromatographic system for analysis of phenolic acids and flavonoids was carried out with Shimadzu model prominence liquid chromatography, thermostatic column compartment, online degasser and a UV detector model SPD-20A (operating at 268 nm). An analytical column used was a Shim-pack VP-ODS C18 (4.6 mm×250 mm, 5 µm) (Shimadzu Co., Japan).

2.3. Plant material

Thirteen dehydrated bee pollen samples were collected by beekeepers from thirteen locations in North and South Algeria (Table 1 and Figure 1), during the period from 2016 to 2018. After collection, each sample was separately crushed in a commercial blender and homogenized.

Figure 1. Geographical locations from which bee pollen samples were collected

Table 1. The date and place of harvest of bee pollen.

Sample code	Date of harvest	Place of production
P01	2017	Bouira
P02	2017	Mitidja
P03	2017	Skikda
P04	2017	Constantine
P05	2016	Tipaza
P06	2016	El Bayadh
P07	2016	Tipaza
P08	2016	Bouira
P09	2016	Laghouat
P10	2016	Tizi Ouzou
P11	2016	Boumerdès
P12	2016	Tizi Ouzou
P13	2018	El-Oued

2.4. Preparation of crude extract

According to Khosravi et al. [15], with slight modification, 2 mL of methanol were added to 200 mg of bee pollen samples, then the mixture was placed in ultrasonic bath under the conditions: at room temperature for 30 min, to obtain the extract. Each of the extracts was transferred to the centrifuge at 3000 rpm/min. The supernatant was then separated from the residue by filtration, using Whatman No. 1 filter paper, and then evaporated with a rotary evaporator at 45 °C. The extracts thus obtained were weighed up and stored at 4 °C in a brown bottle before further use.

2.5. Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu reagents according to Beretta's method [16, 17]. In brief, 0.25 mL of Folin-Ciocalteu phenol reagent was mixed with 500 µL (1 $mg \cdot mL^{-1}$) of bee pollen extract. After 3 min, 1 mL of 7.5 % aqueous sodium carbonate solution was added to this mixture. The reaction was held for 30 min in the dark, after which the absorbance was read at λ = 760 nm.

Gallic acid was used as the standard to produce the calibration curve (0.01- 0.0375 $g \cdot L^{-1}$). The mean of three readings was used, and the total phenolic content was expressed in mg of gallic acid equivalents (GAEs) ($mg \cdot kg^{-1}$).

2.6. Determination of total flavonoids content

A colorimetric assay estimated the total flavonoid content in bee pollen based on the procedure of LIANDA Regina [18]. Specific flavonoids in bee pollen were determined as follows: 1 mL (1 $mg \cdot mL^{-1}$) of bee pollen extract was combined with 1 mL of 2% $AlCl_3$ solution. Then the mixture was incubated for 30 min at room temperature. The absorbance was estimated at 430 nm. A calibration curve of quercetin was prepared, and flavonoid contents were determined from the calibration curve's linear regression equation. The findings were expressed as mg of the corresponding quercetin (Qu Es) per g of extract. All the samples and the standards were analyzed in triplicate.

2.7. Standard preparation for HPLC analysis

Stock solutions of phenolic acids: gallic acid, chlorogenic acid, vanillic acid, caffeic acid, vanillin, *p*-coumaric acid, rutin, naringenin, and quercetin were prepared in methanol, at a concentration of 1 mg/mL and then further diluted with methanol (HPLC grade) to get standard calibration curve. All supplementary solutions were filtered with 0.45 µm Millipore nylon filter disk.

2.8. Sample preparation for HPLC analysis

The bee pollen extracts (10 mg) were dissolved through sonication in HPLC grade methanol (10 mL), then filtered with 0.45 µm Millipore nylon filter disk. Then 20 μL of the sample was analyzed in the HPLC system.

2.9. Mobile phase preparation

The mobile phase was consisting of 0.2% acetic acid in water (A) and acetonitrile (B). The mobile phase contents were filtered before use through a 0.45 µm membrane filter, sonicated and pumped from the solvent reservoir to the column at a flow rate of 1 mL/min. A

binary gradient linear system was used for elution, as described below (Table 2).

Table 2. Gradient program for elution of phenolic compounds.

Time (min)	A	B
0	90	10
6	86	14
16	83	17
23	81	19
28	77	23
35	77	23
38	60	40
50	90	10

The effluent was detected at 268 nm. The sample was injected at room temperature, and the injecting volume was 20 µL. Until evaluate injection, the column was balanced with the mobile phase for 15-20 min.

The detection limit (LOD) values and the quantification limit (LOQ) are given in Table 3.

Table 3. Retention times (Rt), detection, and quantification limits for phenolic compounds.

Compound	Rt (min)	LOQ (µg/mL)	LOD (µg/mL)	Range (µg/mL)
Gallic acid	5.29	0.36939	0.12189	2.5-300
Chlorogenic acid	13.392	0.41308	0.13631	1.25–200
Vanillic acid	15.531	0.09577	0.03192	1.25–250
Caffeic acid	16.277	0.44863	0.14804	0.5-320
Vanillin	21.46	0.11350	0.37833	4.75–350
p-Coumaric acid	23.817	0.09385	0.03097	4.5-200
Rutin	28.37	0.39912	0.13304	2.5–50
Naringin	34.788	0.52711	0.17570	4.25-80
Quercetin	45.047	0.13475	0.04491	2.5–200

2.10. Total antioxidant capacity

Phosphomolybdenum method was tool for assessing total antioxidant ability [19]. 0.1 mL (1 mg·mL^{-1}) of bee pollen extract was mixed with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Mixtures were cooled to room temperature after an incubation of 90 min at 95 °C, and the absorbance of the mixture was measured at 695 nm. In the case of blank, 0.1 mL of methanol, the sample was used instead. Experiments were approved out in triplicate. The total antioxidant capacity can be calculated as gallic acid equivalent (GAE) by the following equation [19]:

$$\mathrm{TAC} = \frac{C \times V}{m}$$

Where: **TAC** is the total antioxidant capacity in mg GAE/100 g of the extracts, ***C*** is the concentration of gallic acid established from the calibration curve in mg/mL, ***V*** is the volume of the extract solution in mL, and ***m*** is the weight of the extract in g.

Gallic acid is used as a standard compound. The total antioxidant capacity was expressed as *m* GAE /100 g using the standard curve equation: y = 5.642x + 0.128, where y is the absorbance at 695 nm, and x is total antioxidant capacity of the extract.

3. Results and discussion

Determination of total phenolic and flavonoid contents of different extracts of bee pollen was done by using Folin–Ciocalteu colorimetric and $AlCl_3$ methods, separately.

Folin–Ciocalteu colorimetric approach has been used to estimate total polyphenol contents. Phenolics, including phenolic acids and flavonoids, form a complex of blue color with a phosphomolybdic – phosphotungstic acid reagent (Folin–Ciocalteu reagent) at a maximum absorbance of 765 nm. The total phenolic content of each bee pollen extract was reported as mg gallic acid equivalent per g dried extract. Bee pollen includes many phenolic compounds, the nature and the quantity of which change widely according to the botanic origin [20, 21]. Total phenolic compounds ranged from 379.8 to 915.6 mg / 100 g for our samples. The largest amount was in the sample P8, followed by each of the samples P10 and P12, with a ratio of 898.6 and 861.3 mg / 100 g, while the rest of the samples had a ratio of phenols in it medium-ranged between 501.9 - 661.2 mg / 100 g, and was the lowest value in the sample P02, as shown in Figure 2.

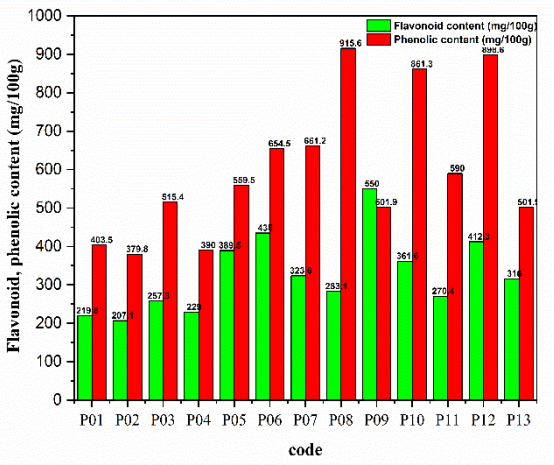

Figure 2. The variation of total flavonoid and phenolic content for different type bee pollen samples.

Many studies have found that dark-colored bee pollen has a higher total phenolic compound content [22]. Geldof and Meda have shown a link between antioxidant activity and phenolic content overall [22, 23]. Katarzyna Komosinska-Vassev and others suggest that the determination of bee pollen's total phenolic content is a good parameter for assessing its consistency and potential for therapy [24].

Aluminum chloride forms acid-stable complex with the keto and/or hydroxyl groups in the A or C ring of flavonoids in the $AlCl_3$ colorimetric process; besides it, forms labile acid groups with ortho-dihydroxyl groups of flavonoid A or B rings. The $AlCl_3$ complexes of flavonoid compounds show high absorbance at 420 nm, and flavonoids absorb better at 420 nm with more functional groups [25]. The total flavonoid contents of different bee pollen samples were reported as mg quercetin equivalent per g dried extract. As presented in Table 1, the results show that all of the Algeria bee pollen samples had a higher content of flavonoids, where the number of flavonoids ranged between 550.0 -

207.1 mg / 100 g. The most considerable amount was in the sample P09, followed by both the samples P12 and P06. The percentage of flavonoids was average in the samples P10, P07, P05, and P13 ranging between 389.5-316 mg / 100 g, as represented the lowest value in the sample P02. Based on these data, it can be concluded that the P12 extract possesses a high content of phenolics and flavonoids.

Phosphomolybdenum assay has been used to estimate the total antioxidant potential of the different specimens. The method of phosphomolybdenum was used based on the theory of reducing Mo (VI) to Mo (V) by the various antioxidants present in the extracts and, eventually, the formation of green phosphate / Mo (V) complexes [26].

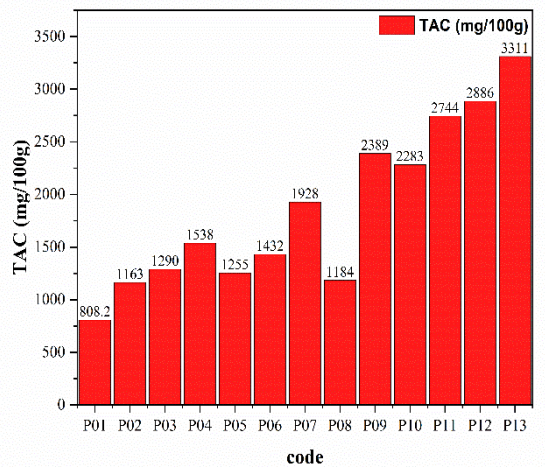

Figure 3. The variation of total antioxidant capacity for different types of bee pollen samples.

Table 4. Phenolic compounds content identified by HPLC in methanolic pollen extract.

Code	GA (µ/g)	CLA (µ/g)	V A (µ/g)	CA (µ/g)	VAN (µ/g)	p-C A (µ/g)	RUT (µ/g)	NAR (µ/g)	QR (µ/g)	Total (mg/g)
P01	7.274 ±0.145	27.251 ±0.545	41.384 ±0.827	247.857 ±4.957	109.862 ±3.295	1205.766 ±30.144	2120.501 ±53.0125	ND	1033.663 ±25.841	4.793 ±0.119
P02	23.701 ±0.474	47.366 ±0.947	8.073 ±0.161	2.757 ±0.055	1596.775 ±47.903	267.59 ±6.689	356.736 ±8.918	164.538 ±4.113	1695.654 ±42.391	4.163 ±0.104
P03	13.002 ±0.260	76.187 ±1.523	12.044 ±0.240	ND	6249.18 ±187.475	12.599 ±0.314	46.013 ±1.150	198.802 ±4.970	266.922 ±6.673	6.874 ±0.171
P04	83.846 ±1.676	39.639 ±0.792	ND	10.215 ±0.204	33.938 ±1.0181	1007.887 ±25.197	ND	92.471 ±2.311	1166.543 ±29.163	2.434 ±0.060
P05	116.548 ±2.330	31.35 ±0.627	ND	ND	498.163 ±14.944	331.314 ±8.282	160.133 ±4.03	1343.299 ±33.582	186.31 ±4.657	2.667 ±0.066
P06	61.882 ±1.237	13.773 ±0.275	ND	ND	16.466 ±0.493	866.62 ±21.665	ND	58.558 ±1.463	289.942 ±7.248	1.307 ±0.032
P07	85.32 ±1.706	ND	ND	ND	ND	210.138 ±5.253	ND	939.656 ±23.491	534.395 ±13.359	1.769 ±0.044
P08	74.899 ±1.497	50.182 ±1.003	9.093 ±0.181	5.864 ±0.117	68.345 ±2.05	45.677 ±1.141	ND	69.198 ±1.729	4049.905 ±101.247	4.373 ±0.109
P09	50.847 ±1.016	30.048 ±0.622	3.783 ±0.075	3.977 ±0.079	339.232 ±10.176	8145.242 ±203.631	12.109 ±0.302	ND	1925.814 ±48.145	10.511 ±0.262
¨P10	648.558 ±12.971	334.834 ±6.698	145.882 ±2.917	ND	10488.72 ±314.66	9312.071 ±232.801	6751.996 ±168.799	3087.187 ±77.179	20749.05 ±518.726	51.518 ±1.287
P11	67.072 ±1.341	53.81 ±1.077	ND	ND	30.246 ±0.90738	52.211 ±1.305	ND	4485.019 ±112.1255	625.289 ±15.632	5.313 ±0.132
P12	87.8 ±1.756	41.643 ±0.832	33.09 ±0.662	ND	102.389 ±3.0716	235.842 ±5.896	ND	1564.084 ±39.102	613.187 ±15.329	2.678 ±0.066
P13	130.418 ±2.608	28.977 ±0.579	4.778 ±0.095	ND	2035.299 ±61.058	28.33 ±0.708	41.614 ±1.04	37.638 ±0.94	845.876 ±21.146	3.152 ±0.078

Identified compounds are: GA — gallic acid; CLA — Chlorogenic acid; VA — Vanillic acid; CA — Caffeic acid; V — Vanillin; *p*-CA — *p*-coumaric acid; RUT — rutin; NAR — naringin; QR—quercetin. ND: not determined.

Figure 3 shows the amount of antioxidant activity ranged between 808.2 - 3311 mg / 100 g. The most considerable amount was in the sample P13, followed by each of the samples P09, P10, P11, and P12 with average values ranging between 2283 - 2886 mg/100 g, while the amount of antioxidant activity the rest of the other samples ranged between 1163 - 1928 mg/100 g. The lowest value was in the sample P01.

Many reports [27-29] confirmed that high antioxidant capacity of bee pollen methanolic extracts correlated with the high amount of polyphenols, mainly flavonoids and phenolic acids. It cannot be excluded that non-phenolic compounds contribute to the entire antioxidant potential of these extracts.

Through the obtained results shown in Fig. 1, it was found that there are important quantities of phenolic compounds and flavonoids in the pollen samples. However, the presence of these compounds differed between the studied samples, where the results in Table 4 showed that all samples contain gallic acid (GA), *p*-coumaric acid (*p*-CA), and quercetin (QR). The sample P07 does not contain vanillin (VAN) and chlorogenic acid (CLA). As for the naringin (NAR), its absence was observed in samples P01 and P09.

We also note that the samples P04, P05, P06, P07, P11, and the samples P04, P06, P08, P07, P12, P11 do not contain vanillic acid (VA) and rutin (RUT) respectively, while caffeic acid (CA) was observed in most samples except (P08, P04, P02, P01, P09). From the comparison of these results, it was found that the sample (P02) only contained the nine studied phenolic compounds.

4. Conclusions

In this study, the phenolics profile and Algerian bee pollen's antioxidant properties were investigated in detail. This study showed that Algerian bee pollen samples have high antioxidant potential, as indicated by their high flavonoid and phenolic contents "bioactive agents" especially quercetin, and the most common compounds in the products was gallic acid, *p*-coumaric acid, and quercetin. It can be considered that bee pollen is a strong source of economic nutrition that can be used as supplements for human food.

Conflict of interest

The authors declare no conflict of interest.

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