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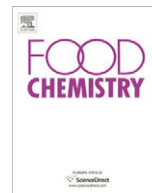

In vitro antioxidant capacity of honeybee-collected pollen of selected floral origin harvested from Romania

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ABSTRACT

Total phenolic phytochemical concentration was measured in 12 honeybee-collected pollens of selected floral species as well as their antioxidant capacity. The content of total polyphenols was measured spectrophotometrically using the Folin–Ciocalteu reagent with gallic acid as standard. The antioxidant properties were evaluated by 2,2-diphenyl-picrylhydrazyl radical scavenging capacity (DPPH) assay, Trolox equivalent antioxidant Capacity procedure and Ferric ion reducing antioxidant power assay. A great variability regarding the correspondence between the antioxidant activity and the content of total polyphenols of honeybee-collected pollens with different botanical origin was found. Antioxidant activities were different for each floral species and were not clearly associated to their total phenolic content.

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1. Introduction

In recent years there has been a remarkable increment in scientific research dealing with natural antioxidants and their potential health benefits. Oxidative stress, the consequence of an imbalance between ROS (reactive oxygen species) generation and antioxidants in the organism, initiates a series of harmful biochemical events which are associated with diverse pathological processes which can lead to various cellular damages and diseases (Sastre, Pallardo, & Vina, 2003). Antioxidants are considered as possible protection agents reducing oxidative damage to important biomolecules, including lipoprotein and DNA (deoxyribonucleic acid) from ROS (Gulcin, Buyukokuroglu, Oktay, & Kufrevioglu, 2003).

The growing interest in the physiological benefits of natural antioxidants has been matched by acceleration in the development of analytical and biological methodologies for measurement of both the levels and antioxidant potential of these compounds.

There is increasing evidence from epidemiological, *in vivo*, *in vitro*, and clinical trials clearly suggesting that the polyphenolic compounds present in natural foods may reduce risk of chronic disease such cancer, anti-inflammatory, cardiovascular and neuro-degenerative diseases (Luthria, 2006).

Phenolic compounds, such as flavonoids, phenolic acid and tannins, are considered to be a major contributor to the antioxidant potential of foods. In this respect, antioxidant capacity of phenolic compounds extracted from various foodstuffs, egg, Vegetables (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002), honey, propolis and royal jelly (Buratti, Benedetti, & Cosio, 2007) and wine (Lee, Kim, Joo, & Lee, 2003), have been intensively studied using *in vitro* methods.

The antioxidant activity of polyphenols is mainly due to their redox properties, which can play an important role in neutralising free radicals, quenching oxygen, or decomposing peroxides (Nijveldt et al., 2001). In addition to their individual effects, antioxidants interact in synergistic ways and have sparing effect in which one may protect another against oxidative destruction (Damintoti, Mamoudou, Simpore, & Traore, 2005). The best-described property of almost every group of flavonoids, which are the predominant phenolic class present in honeybee-collected pollen, is their capacity to act as antioxidants (Kroyer & Hegedus, 2001). One way is the direct scavenging of free radicals. Flavonoids are oxidised by radicals, resulting in a more stable, less-reactive radical. In other words, flavonoids stabilize the reactive oxygen species by reacting with the reactive compound of the radical (Nijveldt et al., 2001).

There are various methods available in the assessment of the antioxidant capacity of samples, they provide useful data, and however, they are not sufficient to estimate a general antioxidant

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ability of the sample (Filipiak, 2001). These methods differ in terms of their assay principles and experimental conditions. Consequently, in different methods, particular antioxidants have varying contributions to total antioxidant potential (Cao & Prior, 1998).

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems (Gulcin, Elias, Gepdiremen, Boyer, & Koksal, 2007). The DPPH method (Brand-Williams, Cuvelier, & Berset, 1995) consist in the reaction of DPPH (2,2-diphenyl-1-picrylhydrazyl) a stable free radical, which accepts an electron or hydrogen radical to become a stable molecule, and, accordingly, is reduced in presence of an antioxidant. DPPH radical are widely used for the preliminary screening of compounds capable to scavenging activated oxygen species since they are much more stable and easier to handle than oxygen free radical (Tominaga et al., 2005). The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate). Due to its operational simplicity, the TEAC assay has been used in many research laboratories for studying antioxidant capacity, and TEAC values of many compounds and food samples are reported. The mechanisms of both methods are similar, in that the absorption spectra of the stable, free radical changes when the molecule is reduced by an antioxidant or a free radical species. The FRAP assay measures the ferric-to-ferrous iron reduction in the presence of antioxidants and is very simple and convenient in terms of its operation (Cao & Prior, 1998).

Honeybee-collected pollen is an apicultural product which is composed of nutritionally valuable substances and contains considerable amounts of polyphenolic compounds, mainly flavonoids, which may act as potent antioxidants (Kroyer & Hegedus, 2001). The antioxidant activity of honeybee-collected pollen has been recognised as a free radical scavenger and as a lipid peroxidation inhibitor as previously Almaraz-Abarca et al. (2004) reported.

In the current study, antioxidant potential of bee pollen of selected floral origin was evaluated, in comparison with the level of total phenolic and flavonoid content.

2. Materials and methods

2.1. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman carboxylic acid), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulphate ($K_2S_2O_8$), sodium carbonate, Folin–Ciocalteu's phenol reagent and gallic acid were purchased from Sigma–Aldrich (St. Louis, MO). Iron(III) chloride 6-hydrate ($FeCl_3 \cdot 3H_2O$), iron(II) sulfate 7-hydrate ($FeSO_4 \cdot 7H_2O$) and acetic acid (CH_3COOH) were obtained from BDH (Poole, UK). 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) was purchased from Fluka Chemie AG (Bushs, Switzerland). Hydrochloric acid (HCl) and methanol were obtained from Merck (Darmstadt, Germany). All chemicals used in the experiments were of analytical grade.

2.2. Equipments

Spectrophotometric measurements were performed by Synergy™ HT Multi-Detection Microplate Reader with 96-well plates (BioTek Instruments, Inc., P) and spectrophotometer (double-beem) 1700 Schimadzu. In order to perform the analysis, the following equipments were also used: Microscope Nikon Eclipse 50i 40×, rotary evaporator Buchi R-215, ultrasonic bath Bandelin.

2.3. Botanical origin identification of the pollen pellets

Samples of honeybee-collected pollen were purchased from local beekeepers from Transylvania area of Romania in 2007. The floral origin of honeybee-collected pollen pellets was identified by colour and light microscope examination by palynological analysis according to the acetolysis method (Erdtman, 1969). The microscope examination was performed under normal lighting at 400× magnification. Pollen types were identified by comparison with pollen reference slides made by the authors of the present work, and then compared with available pollen atlases (Erdtman, 1969; Ricciardelli & d'Albore, 1998; Sawyer, 1981). The pollen reference slides were prepared from anthers of flowers and the plant taxon was identified upon the botanic atlas (Popovici, Moruzi, & Toma, 1973).

2.4. Preparation of extracts

Each samples of bee pollen (2 g) were individually extracted three times with 15 ml of methanol solvent at the room temperature for 1 h. After sonication (15 min), maceration and filtration, the filtrate was evaporated to dryness under vacuum. The resulting dried extracts were dissolved in methanol and stored until analysis (4 °C).

2.5. Determination of total phenolic content

The content of total polyphenols was estimated according to the Folin–Ciocalteu method proposed by Singleton, Orthofer, and Lamuela-Raventos (1999) using gallic acid as reference standard (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005). The method was adapted to the 96 well microplate reader. Briefly, the 125 µL Folin–Ciocalteu reagent (0.2 N) was added to 25 µL of bee pollen extracts and mixed for 5 min. After the addition of 100 µL sodium carbonate (Na_2CO_3) solution (75 g/L) the extracts was incubated for 2 h. The absorbance at 760 nm was then measured against a methanol blank. A standard curve of gallic acid was created using an adequately range of gallic acid solutions from 0.01 to 0.25 mg/mL. The results were expressed as Gallic Acid Equivalent (mg GAE g^{-1} dry matter sample).

2.6. Determination of total flavonoid content

Total flavonoids were measured by the aluminium chloride colorimetric assay developed by Zhishen, Wengcheng, and Jianming (1999) using quercetin as reference standard, as described by (Kim, Jeong, & Lee, 2003). An aliquot (1 ml) of appropriately diluted sample or standard solutions of quercetin (0.001–0.25 mg/mL) was added to a 10 ml volumetric flask containing 4 ml distilled water. At zero time, 0.3 ml 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 ml 10% $AlCl_3$ was added. At 6 min, 2 ml 1 M NaOH was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 ml of distilled water and thoroughly mixed. Absorbance of the mixture, pink in colour, was determined at 510 nm versus prepared reagent blank. Total flavonoid content was expressed as mg Quercetin Equivalent (mg QE g^{-1} dry matter sample).

2.7. Determination of antioxidant capacity

2.7.1. Determination of DPPH scavenging activity

The scavenging activity (H/e-transferring ability) of bee pollen extracts against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was evaluated spectrophotometrically by a slightly modified method of Brand-Williams et al. (1995) as described by Velazquez, Tournier, Mordujovich de Buschiazzo, Saavedra, and Schinella (2003), with adaptation on the micro-plate reader. Briefly, an aliquot (40 µL) of appropriately diluted extracts of bee pollen was mixed with 200 µL DPPH solution (0.02 mg/mL). Samples were kept for 15 min at room temperature and then the absorbance was measured at 517 nm. Absorbance of blank sample containing the same

amount of solvent and DPPH solution was prepared and measured daily. The percentage of absorbance inhibition at 517 nm was calculated using the equation below:

$$\text{Inhibition }(\%) = \left(1 - \frac{\text{Asample}}{\text{Ablank}}\right) \times 100 \quad (1)$$

The extent of decolourisation is calculated as percentage reduction of absorbance, and this is determined as a function of concentration and calculated relatively to the equivalent trolox concentration (0.1–0.01 mM). The radical scavenging activity is expressed in millimol of equivalent Trolox per gram of sample (mmol Trolox g^{-1} dry matter sample).

2.7.2. Determination of Trolox equivalent antioxidant capacity (TEAC)

For Trolox equivalent antioxidant Capacity assay, the procedure followed the method of Re et al. (1999) with some modifications. The TEAC assay is based on the scavenging of the 2,2'-azinobis-(3-ethylbenzothiazoline- 6-sulphonic acid) (ABTS) radical ($ABTS^{\cdot+}$) converting it into a colourless product. The degree of decolourisation induced by a compound is related to that induced by trolox, giving the "TEAC value". The $ABTS^{\cdot+}$ cation radical was produced by the reaction between 7 mM ABTS solution and 2.45 mM potassium persulphate solution, stored in the dark at room temperature for 16 h. The solution is stable for 3 days. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.700 ± 0.025 at 734 nm with ethanol. For the assay the resulting solution was mixed with 17 μL of sample. The absorbance was read at 30 °C after exactly 6 min. The extent of inhibition of the sample, calculated using the formula mentioned in the DPPH method, was then compared with a standard curve made from the corresponding readings of Trolox (0.4–0.04 mM). Results were expressed in millimol of equivalent Trolox per gram of sample (mmol Trolox g^{-1} dry matter sample).

2.7.3. Determination of ferric reducing/antioxidant power

Total antioxidant potential of the sample was evaluated using the ferric reducing ability (FRAP) assay as a measure of "antioxidant power". The ferric reducing/antioxidant power FRAP is a simple, direct test of antioxidant capacity. This method was initially developed to assay plasma antioxidant capacity by Benzie and Strain (1996) and adapted to a manual assay by Varga, Matkovics, Sasvári, and Salgó (1998) (Szollosi & Varga, 2002). FRAP assay measures the change in absorbance at 593 nm owing to the formation of the blue coloured Fe^{II}-tripycridyltriazine compound from the colourless oxidised Fe^{III} form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 mL of 300 mmol/L acetate buffer pH 3.6 (3.1 g sodium acetate $CH_3COONa \cdot 3H_2O$ and 16 mL acetic acid glacial CH_3COOH per litre), with 1 mL of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L hydrochloride acid and 1 mL of 20 mmol/L ferric chloride solution in distilled water. All solutions were used on the day of preparation. The sample consisted in 300 μL FRAP reagent, 10 μL of bee pollen extract and 30 μL deionized water, in order to obtain a final dilution of the sample in the reaction mixture of 1:34. The sample was incubated at 37 °C throughout the monitoring period (4 min). The antioxidant capacity of the samples under study was calculated with reference to the reaction signal given by aqueous solutions of Fe^{II} solution of known concentration (0.1–1 mmol/L of $FeSO_4 \cdot 7H_2O$). The results were corrected for dilution and calculated using a standard calibration curve (r^2 = 0.9965) and expressed as FRAP value (mmol Fe^{II} g^{-1} dry matter sample).

2.8. Statistical analysis

All determinations were performed in triplicate and results are expressed as mean ± standard deviation calculated using spreadsheet software Microsoft Excel. The data were analysed by an analysis of variance ($p \leqslant 0.05$) and means separated by Duncan's multiple range test. The relationship between antioxidant content and the antioxidant capacity of different monofloral bee pollen samples, as well as between different antioxidant capacity assays, was analysed by Pearson correlation coefficients. The results were processed by STATPlus2008 software.

3. Results and discussion

3.1. Botanical identification

The pollen loads studied here were first separated by colour from the complex mixtures of pollen pellets from different species of plants, resulting monochromatic pollen loads with uniform colour. The microscopic examination was the principal tool for the selection of the honeybee-collected pollen pellets coming from one species. In each microscopic preparation, pollen was determined, when possible, into genus, species or family. The selected pollen load samples studied here presented the following colours: grey yellow (*Capsella bursa pastoris* L.), red orange (*Helianthus annuus* L., *Matricaria chamomilla* L., *Taraxacum officinale* Web.), light green (*Crataegus monogyna* J.), light yellow (*Pinus* sp., *Carex* sp.), violet (*Carduus* sp.), brown (*Onobrychis viciifolia* Scop.), grey (*Centaurea cyanus* L.), pink (*Knautia arvensis* (L.) Coulter)), maroon (*Salix* sp.).

3.2. Total phenol content of honeybee-collected pollen of selected floral origin

There was a wide range of phenolic concentration in the honeybee-collected pollen analysed, as shown in Table 1. The highest polyphenol concentration was determined in the methanol extracts of bee pollen from *Salix* sp. (16.4 mg GAE g^{-1}) followed by *T. officinale Web.* bee pollen (16.2 mg GAE g^{-1}), *C. cyanus* L. bee pollen (16.0 mg GAE g^{-1}), *C. monogyna* J. bee pollen and (7.7 mg GAE g^{-1}) *C. bursa pastoris* L. bee pollen (15.2 mg GAE g^{-1}). The lowest level of total polyphenol content was determined in bee pollen from *K. arvensis* (L.) Coulter bee pollen with value of 4.4 mg GAE g^{-1}. Similar values were obtained for *Pinus* sp. and *Carex* sp. bee pollen with value of 6.4 mg GAE g^{-1}. The total phenolic content was significantly ($p \leqslant 0.05$) higher (3.7 times) in *Salix sp.* than in *K. arvensis* (L.) Coulter bee pollen.

3.3. Total flavonoid content of honeybee-collected pollen of selected floral origin

Total flavonoid content showed discrepancies in the examined honeybee-collected pollen of selected floral origin. The highest levels were quantified in *Salix* sp. bee pollen (13.6 mg QE g^{-1}) followed by *M. chamomilla* L. (12.7 mg QE g^{-1}) and *C. cyanus* L. bee pollen (11.8 mg QE g^{-1}). The bee pollen from *Pinus* sp. contained the lowest total flavonoid content (0.6 mg QE g^{-1}). Similar lower values were determined in *K. arvensis* (L.) Coulter bee pollen (2.8 mg QE g^{-1}) and *T. officinale Web.* bee pollen (3.8 mg QE g^{-1}). The flavonoid content differs significantly between samples ($p \leqslant 0.05$), with exception of *Carduus* sp., *O. viciifolia* Scop. and *C. bursa pastoris* L. bee pollens.

Depending on the pollen species, the participations of flavonoids in total phenols significantly differ ($p \leqslant 0.05$), the highest participation was determinate in the case of *O. viciifolia* Scop. bee pollen (91.2%), followed by *M. chamomilla* L. (90.7%), *H. annuus* L. (89.5%) and *Salix* sp. bee pollens (82.9%). In comparison, the flavonoid fraction represents only 9.4% of total phenols in *Pinus* sp. bee pollen and 23.5% in *T. officinale* Web. bee pollen.

Table 1
The phenolic content and antioxidant capacity of honeybee-collected pollen of selected floral origin*.

Botanical name of floral species	Total phenolics** (mg GAE g^{-1})	Total flavonoids** (mg Qe g^{-1})	Antioxidant capacity**		
			DPPH value (mmol Trolox g^{-1})	TEAC value (mmol Trolox g^{-1})	FRAP value (mmol Fe^{II} g^{-1})
Capsella bursa pastoris L.	15.2 ± 0.3 g	9.4 ± 0.2 ef	1.342 ± 0.02 g	2.365 ± 0.02 f	2.412 ± 0.02 h
Helianthus annuus L.	11.4 ± 0.2 d	10.2 ± 0.3 g	0.454 ± 0.01 d	1.860 ± 0.02 d	1.491 ± 0.02 e
Crataegus monogyna J.	15.3 ± 0.3 g	10.8 ± 0.3 h	1.313 ± 0.01 f	2.785 ± 0.03 h	2.014 ± 0.03 g
Pinus sp.	6.4 ± 0.1 b	0.6 ± 0.03 a	0.135 ± 0.01 a	0.546 ± 0.01 a	0.697 ± 0.02 c
Matricaria chamomilla L.	14.0 ± 0.3 f	12.7 ± 0.1 j	1.348 ± 0.02 g	4.466 ± 0.04 k	5.355 ± 0.04 l
Carduus sp.	12.9 ± 0.2 e	9.6 ± 0.1 f	1.432 ± 0.02 h	2.747 ± 0.03 g	3.382 ± 0.04 j
Taraxacum officinale Web.	16.2 ± 0.2 h	3.8 ± 0.1 c	0.348 ± 0.02 c	1.499 ± 0.02 c	0.327 ± 0.01 b
Onobrychis viciifolia Scop.	10.2 ± 0.2 c	9.3 ± 0.1 e	0.684 ± 0.01 e	1.883 ± 0.02 e	1.040 ± 0.03 d
Centaurea cyanus L.	16.0 ± 0.3 h	11.8 ± 0.2 i	2.615 ± 0.03 j	3.638 ± 0.04 i	1.980 ± 0.03 f
Knautia arvensis (L.) Coulter	4.4 ± 0.1 a	2.8 ± 0.02 b	0.274 ± 0.01 b	0.938 ± 0.02 b	0.255 ± 0.02 a
Salix sp.	16.4 ± 0.3 i	13.6 ± 0.2 k	2.814 ± 0.03 k	6.838 ± 0.04 l	3.760 ± 0.05 k
Carex sp.	13.9 ± 0.1 f	8.8 ± 0.1 d	1.533 ± 0.02 i	3.770 ± 0.03 j	3.055 ± 0.05 i

* Samples analysed in triplicate.
** Means followed by the same letters are not significantly different ($p \leqslant 0.05$).

3.4. Antioxidant capacity of honeybee-collected pollen of selected floral origin

The antioxidant capacity determination results of an extract depend greatly on the methodology used, that is the oxidant and the oxidisable substrate used in the measurement. Therefore, it is important to compare different analytical methods varying in their oxidation initiators and targets in order to understand the biological activity of an antioxidant and to obtain accurate data for a better comparison with other literature (Cao & Prior, 1998; Stratil, Klejdus, & Kuban, 2006 cited by Santas, Carbo, Gordon, & Almajano, 2008).

Recent investigations show differences between the test systems in determining antioxidant capacity. Use of at least two methods is recommended to assess and compare the antioxidant capacity of a sample (Sakanaka & Ishihara, 2008).

The present study present different *in vitro* tests based either on the capacity to scavenge free radicals (DPPH, TEAC) or on the ability of reducing oxidants (ferric ions) the ferric-reducing ability (FRAP) (Table 1).

In the DPPH assay, antioxidants will react with a nitrogen-centered radical (2,2-diphenyl-1-picrylhydrazyl) which is with a characteristic absorption at 517 nm and converted into 1,1,-diphenyl-2-picryl hydrazine, at a very rapid rate. On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid (Jayaprakasha & Patil, 2007). Honeybee-collected pollen from different floral sources differed significantly ($p \leqslant 0.05$) in their DPPH values. The DPPH values of methanol extracts of the 12 monofloral bee pollens ranged from 0.135 (bee pollen from *Pinus* sp.) and 2.814 mmol Trolox g^{-1} dry matter sample (bee pollen from *Salix* sp.).

TEAC method can measure the antioxidant capacity determined by the decolourization of the $ABTS^{\cdot+}$ through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. In $ABTS^{\cdot+}$ decolourisation method, *Salix* sp. bee pollen extract possessed the highest TEAC value of 6.838 mmol Trolox g^{-1}, followed by *M. chamomilla* L., *Carex* sp. and *C. cyanus* L. bee pollens with 4.466 mmol Trolox g^{-1}, 3.770 and 3.638 mmol Trolox g^{-1}, respectively. In terms of ABTS decolourisation action, amongst the monofloral bee pollen extracts, the lowest antioxidant potential was performed by *Pinus* sp. bee pollen (0.546 mmol Trolox g^{-1}) followed by *K. arvensis* (L.) Coulter bee pollen witch registered TEAC values of 0.938 mmol Trolox g^{-1}. For the TEAC assay, the difference of antioxidant capacities was very significant ($p \leqslant 0.05$).

The ferric reducing/antioxidant power (FRAP) assay, in contrast to other tests of total antioxidant power, is a simple, speedy and robust assay (Prior, Wu, & Schaich, 2005). At low pH (optimum pH 3.6) Fe^{III}–TPTZ complex is reduced by antioxidants to its intense blue coloured form Fe^{II}–TPTZ which has maximum absorbance at 593 nm. Methanol extracts of bee pollen from different floral species differed significantly ($p \leqslant 0.05$) in their FRAP values. The FRAP values of honeybee-collected pollens ranged from 5.355 to 0.255 mmol Fe^{II} g^{-1}. In the present study, the highest antioxidant potentials amongst the methanol extracts of the honeybee-collected pollen was registered for the *M. chamomilla* L. bee pollen (5.355 mmol Fe^{II} g^{-1}), followed by the *Salix* sp. bee pollen (3.760 mmol Fe^{II} g^{-1}) and *Carex* sp. bee pollen (3.055 mmol Fe^{II} g^{-1}). The bee pollen extracts from *K. arvensis* (L.) Coulter, *T. officinale* W. and *Pinus* sp. species exhibits the lower antioxidant potentials (0.255, 0.327 and 0.697 mmol Fe^{II} g^{-1}, respectively).

3.5. Relationship between antioxidant capacity and phenolic content

Free radical scavenging of phenolic compounds is an important property underlying their various biological and pharmacological activities (Damintoti et al., 2005). Total phenol content and total antioxidant capacity differs significantly amongst 12 selected monofloral bee pollens analysed. In the present investigation, was found a great variability regarding the correspondence between the DPPH scavenging activity, Trolox equivalent antioxidant capacity respectively the reducing/antioxidant power and the content of total phenolics and flavonoids.

Bee pollen of *Salix alba* L. contains the highest quantities of polyphenols (16.4 mg GAE g^{-1}), respectively flavonoids (13.6 mg QE g^{-1}) and demonstrated high antioxidant capacity in all antioxidant systems evaluated (DPPH: 2.814 mmol Trolox g^{-1}, TEAC: 6.838 mmol Trolox g^{-1} and FRAP: 3.670 mmol Fe^{II} g^{-1}).

It can be observed that high antioxidant content (total phenolics 16.4 and 16.0 mg GAE g^{-1}; total flavonoids 13.6 and 11.8 mg QE g^{-1}) are accompanied by high DPPH scavenging activity (2.814, respectively 2.615 mmol Trolox g^{-1}) and TEAC values (6.838, respectively 3.638 mmol Trolox g^{-1}) only in the case of bee pollen from *Salix* sp. and *C. cyanus* L. (Table 1).

In the case of the *K. arvensis* L. and *Pinus* sp. bee pollens, the lower antioxidant capacity (DPPH: 0.274 mmol Trolox g^{-1} and 0.135 mmol Trolox g^{-1}, respectively; TEAC: 0.938 mmol Trolox g^{-1} and 0.546 mmol Trolox g^{-1}, respectively; FRAP: 0.255 mmol

Fe^{II} g^{-1} and 0.697 mmol Fe^{II} g^{-1}, respectively) is reflected by the lower antioxidant content: polyphenols (4.4 and 6.4 mg GAE g^{-1}) and flavonoids (2.8 and 0.6 mg QE g^{-1}).

Low DPPH, TEAC and FRAP values obtained in the case of *H. annuus* L. bee pollen (0,454 mmol Trolox g^{-1}, 1.860 mmol Trolox g^{-1} and 1.491 mmol Fe^{II} g^{-1}) is not reflected by their polyphenol content (11.4 mg GAE g^{-1}), respectively flavonoid content (10.2 mg QE g^{-1}). Similarly, high flavonoid content of bee pollen of *O. viciifolia* Scop. (9.3 mg QE g^{-1}) is not reflected in their low antioxidant capacities (DPPH: 0.684 mmol Trolox g^{-1}, TEAC: 1.883 mmol Trolox g^{-1} and FRAP: 1.040 mmol Fe^{II} g^{-1}).

Bee pollen of *T. officinale* Web. despite the high polyphenol content (16.2 mg GAE g^{-1}) demonstrate low antioxidant capacity (DPPH: 0.348 mmol Trolox g^{-1} and FRAP: 0.327 mmol Fe^{II} g^{-1}) according to their flavonoid content (3.8 mg QE g^{-1}).

The total polyphenol content of honeybee-collected pollen ("bee pollen") and their extracts was previously determinate by Kroyer and Hegedus (2001). In the bee pollen extracts (ethanol, methanol-water 1:1 and water) the amount of total polyphenols ranged between 21.4–24.6 mg g^{-1}, with the highest content in the ethanol extract. As a result, the best antiradical activity against the DPPH was pursued by the ethanol extract (53 %).

In a previous study, Almaraz-Abarca et al. (2004) determined the total flavonol content in a mixture of bee pollen and their constituent pollens, and the values ranged between 3.5–0.1 mg/g dry matter of pollen. In addition, using a modified Campos method (1997), the antiradical activity was expressed as the amount of antioxidant needed to decrease by 50% the initial DPPH concentration (EC_{50}). Comparing the antiradical activity and the flavonol content of the samples, no correlation seems to exist between the total extract of the mixture of bee pollen and those of its constituent pollens, in terms of their flavonol content.

Leja, Mareczek, Wyzgolik, Klepacz-Baniak, and Czekonska (2007) determined the phenolic constituents (total phenols, phenylpropanoids, flavonols and anthocyanins) and antioxidant ability in bee pollen of 12 plant species. Great variability of phenolic content was observed in the investigated species of pollen, levels of total phenols ranged between 82.4 and 12.9 mg g^{-1}. Flavonol content showed discrepancies in the examined pollen samples and ranged between 1.7 and 13.4 mg g^{-1}. Great differences in the radical-scavenging activity (8.6–91.5% of DPPH neutralisation) and in the hydroxyl radical-scavenging activity (10.5–98% inhibition of deoxyribose degradation) were observed and were not direct correlated with the content of phenolic compounds in all examined samples.

In the present study, antioxidant capacities were different for each floral species and were not clearly associated to their total phenolic content in the case of all bee pollen samples (Table 2). The lower correlation coefficients between total phenol content and the antioxidant capacities of the monofloral bee pollens analysed arises from the anomalous behaviour of *H. annuus* L., *T. officinale* W. and *O. viciifolia* Scop. bee pollens, as mentioned above. The above results are in agreement with Leja et al. (2007), the direct correlation was questionable in some species, as mentioned above.

A better interdependence was obtained between the flavonoid fraction of the bee pollen analysed and their antioxidant capacities in all *in vitro* tests, resulting significant positive correlation coefficients (Table 2). As the previous results indicate, the flavonoid components play a significant role in the free radical scavenging capacity of bee pollen (Almaraz-Abarca et al., 2004). The relationship is not clearly related to the high total flavonoid content in the case of all bee pollen species. The results of the present study supported previous conclusions, that the free radical scavenging effectiveness is determined by its particularly phenolic or non-phenolic constituents with their variable structure and actions (Leja et al. 2007).

Table 2
Pearson correlation matrix*.

	TFC	TF	DSA	ASA	FRAP
TFC	1				
TF	0.685	1			
DSA	0.680	0.776	1		
ASA	0.650	0.806	0.880	1	
FRAP	0.502**	0.748	0.635	0.794	1

* TFC: Total phenolic content; TF: Total flavonoid content; DSA: DPPH scavenging activity; ASA: ABTS radical cation scavenging activity, FRAP: Ferric reducing/antioxidant power.
** Significant at $p < 0.05$.

Good positive correlation was found between DPPH and ABTS scavenging activity ($r^2 = 0.880$) and between the ABTS scavenging activity and the Ferric reducing/antioxidant power ($r^2 = 0.635$) of the methanol extracts of monofloral bee pollens studied (Table 2). This suggested that the compounds which could scavenge DPPH radical in the bee pollen extracts were also able to scavenge ABTS radical cation. The Ferric reducing/antioxidant power and the ABTS scavenging activity were good correlated ($r^2 = 0.794$). Because the redox potential of Fe^{III}-TPTZ (0.7 V) is comparable with that of $ABTS^{\cdot+}$ (0.68 V), similar compounds react in both the TEAC and FRAP assays (Prior et al., 2005).

4. Conclusions

In the present investigations, great variability regarding content of total phenols, total flavonoids and antioxidant capacity in examined bee pollen samples was found. Each pollen type has its own specificity, mainly linked to the floral species or cultivars (Zauralow, 1983 cited by Nagai, Inoue, Suzuki, Myoda, & Nagashima, 2005).

It is known that only flavonoids of a certain structure and particularly hydroxyl position in the molecule, determine antioxidant properties. In general, these properties depend on the ability to donate hydrogen or electron to a free radical. Detailed examination of phenolic composition in bee pollen extracts is required for the comprehensive assessment of individual compounds exhibiting antioxidant activity.

In addition, the redox properties of polyphenol compounds, especially flavonoids, play an important role in absorbing and neutralising free radicals, quenching oxygen and decomposing peroxides (Damintoti et al., 2005). This various mechanisms of antioxidant activity permit a wide range of free radicals scavenging and lipo-peroxidation assays in order to evaluate the complete antioxidant potential (Sancez-Moreno, 2002). On the other hand, different antioxidants respond differently in various measurement methods which involve specific reaction conditions and mechanisms of action. This may explain the various results for DPPH, FRAP and TEAC assay, in regard with the antioxidant content of bee pollen samples analysed. A specific polyphenolic compounds, or an association of them, may have different actions as antioxidant against various free radicals.

The results of this study confirm that antioxidant activities from different assay methods strongly depend on the oxidation conditions used in the particular oxidation test. Antioxidant activity is not necessarily correlated with high amounts of phenolic compounds. Total phenolic content, measured by the Folin–Ciocalteu procedure, does not give a full idea of the nature of the phenolic constituents in the extracts (Martha-Estrella, Niokhor, & Stevanovic, 2008). In addition, it may be that antioxidant activity of specific monofloral bee pollen extracts is not limited to phenolics.

As a previous study indicate (Almaraz-Abarca et al., 2004), the results of this preliminary study, conducted on bee pollen from different plant sources from Romania, demonstrated that the polyphenolic composition, rather that the concentration, could be the determinant factor.

Since the previous studies on bee pollen antioxidant capacities not included the *in vitro* TEAC and FRAP tests, the simultaneous comparison between different pollen species made in the present study may contribute to a better characterisation of their particular antioxidant properties. Our results suggested that the antioxidant capacity of samples might be associated with their specific compounds.

In conclusion, future analysis is required, not only in testing other different systems of evaluating the antioxidant activity, but also in separation and identification the specific bioactive compounds in bee pollens with different botanical origin, in order to elucidate the differences between various samples.

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