Hrana u zdravlju i bolesti, znanstveno-stručni časopis za nutricionizam i dijetetiku (2018) 7 (1) 1-5
Food in Health and Disease, scientific-professional journal of nutrition and dietetics (2018) 7 (1) 1-5

BOTANICAL ORIGIN AND ANTIOXIDANT CAPACITY OF BEE POLLEN FROM EASTERN CROATIA

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original scientific paper

Summary

Bee products are considered to be a good resource of bioactive substances such as flavonoids, phenolic acids or terpenoids. Bee pollen is collected and transported by the bees as granules or pollen-loads and reserved as nutrient resource for honeycomb. Because of its nutritional value and healthful properties, bee pollen is valuable product that can increase the beekeepers' income. In this work botanical origin and antioxidant capacity of bee pollen collected in eastern Croatia in April and May 2018 were examined. Botanical origin determined by palynological analysis showed that eight out of twelve analysed samples had > 45% of the pollen grains coming from one family while in one sample *Amorpha fruticosa* pollen grains dominated by 99%. Total phenolic content, total flavonoids and antioxidant capacity determined by the ferric reducing antioxidant power (FRAP) were determined spectrophotometrically. Total phenolic content varied between 7.08 – 15.27 mg GAE/g, total flavonoids were from 1.34 to 4.25 mg QE/g while FRAP value ranged from 51.97 to 83.56 μmol Fe^{2+}/g. The highest antioxidant capacity was determined in *Amorpha fructicosa* and *Salix* spp. unifloral bee pollen samples.

Keywords: botanical origin, bee pollen, antioxidant capacity, total flavonoids, total phenolic content

Introduction

The most famous products produced by the honey bees are honey and propolis. Honey bees produce also, not less valuable products, like pollen, beebread, royal jelly, known for their positive influence on human health (Kaškoninene et al., 2015). According to Campos et al. (2010) bee pollen is defined as a food, but due to small quantities that are generally consumed, it should be classified as functional food or a food supplement.

Honey bees collect pollen grains from the anthers of the flowers and on their way to comb the grains are sticked to the thorax hairs. With addition of the bee salvia, pollen is formed in the balls which are carried to the hive. Harvesting is done by using the pollen trap and the grains must be dried before human consumption (Coe, 2007).

With certain concentration of phytochemicals such as phytosterols and phenolic compounds, pollen could be considered as beneficial for human health. It has significant antioxidant activity that mostly depends on phenolic compounds but researches showed that there are large deviations due to different botanical and geographical origin of pollen grains (Aličić et al., 2014). Beside antioxidant activity recent studies showed that bee pollen has antibacterial properties against *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* (Fatrcová-Šramková et al., 2013), proteasome activation properties (Graikou et al., 2011) and ant-inflammatory activity (Campos et al., 2010). Studies showed that some types of honey have antifungal activity like heather honey (*Erica* spp.), so it is to be expected that *Erica* spp. pollen also could possess this activity (Feás and Estevinho, 2011). Palynological origin of the bee pollen is factor that affects its chemical composition and therapeutic effect. Bee pollen sample is, in most cases, composed of different botanical origin pollen species. This diversity could be seen in research of Nogueira et al. (2012) where pollen species of *Boraginaceae*, *Cytisus* spp., *Castanea* spp., *Cistus* spp. and *Trifolium* spp. were the most frequent but none of the botanical families are represented in all the samples studied. Some authors detected predominant pollen type's characteristic for some country or region like in Lithuanian pollen (Čeksteryte et al., 2013). In Croatia, studies carried out on bee pollen are scarce. Taking into account that botanical origin and composition are connected with geographical region, this research investigated bee pollen from eastern Croatia from the aspect of botanical origin and antioxidant capacity.

Materials and methods

Twelve samples of bee pollen from eastern Croatia were collected in the beekeeping season 2018 in the period of April and May. After collection, the samples were frozen at -18 °C to preserve biological and chemical properties.

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Botanical origin of bee pollen

The modified method of Barth et al. (2010) was used for microscopic slide preparation. Two grams of pollen loads mixture were weighted into a 12 mL centrifuge tube, mixed with 70% ethanol just to complete 10 mL. The mixture was vortexed and placed for five minutes in ultrasonic bath for 25 minutes. After 3 minutes centrifugation at 1500 rpm, sediment was resuspended with ethanol and the procedure was repeated. Solution of water and glycerol (1:1) was added to sediment in amount of 7 mL and left for 30 minutes. Solution was mixed and dissolved two times with same mixture of water and glycerol. Sediment was stirred with Pasteur pipette and spread on microscopic slide. Microscopic slide was covered with 22×22 mm cover slide and sealed. At least 500 pollen grains were counted in each microscopic slide and identified using a 400 × magnification. CMS Celle's Melissopalynological Collection (von der Ohe, K, 2003) and Ponet Pollen databank was used for botanical origin identification.

Bee pollen extracts

Ten grams of mixed bee pollen loads were weighted in 100 mL volumetric flask, filled with methanol to the label and ultra-sonicated for 60 minutes. Suspension was filtered and 100 mL volumetric flask was filled with methanol. Each sample was made in duplicate and stored at -18 °C before analyses.

Total phenolic content

The phenolic content was evaluated by the Folin-Ciocalteu method (FC) described by Singleton et al. (1999). Water in the amount of 6 mL and 0.5 mL FC reagent were added to 100 μL of the prepared sample solution. In the period of 1-8 minutes 1.5 mL of 20% Na_2CO_3 was added and 10 mL volumetric flask was filled with water and left to incubate for two hours in dark. The absorbance was measured at 760 nm and calibration curve was constructed by testing solutions of gallic acid in concentrations from 0.02 to 0.5 mg/mL (98%).

Flavonoid content

The content of total flavonoids was determined in accordance with the method described by Pascoal et al. (2014) with quercetin as a reference standard (Kim et al., 2003). An aliquot of pollen extract was mixed with 5% $NaNO_3$, 10% $AlCl_3$ and 1 M NaOH . The absorbance of the prepared solution was measured at 510 nm. The results were calculated from the equation of the calibration line made with different concentrations of quercetin solution (0.001 – 0.5 mg/mL).

Antioxidant activity by FRAP method

The antioxidant activity of the compounds present in the prepared pollen load solution was determined according to the method described by Benzie and Strain (1999.). FRAP reagent was made by mixing acetate buffer (pH=3.6, 300 mmol/L), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) reagent (98%) and $FeCl_3 \times 6\ H_2O$. The mixture containing 3 mL FRAP reagent and 0.1 mL of pollen extract was incubated for 5 minutes at 37 °C and afterwards the absorbance was measured at 593 nm. Calibration curve was made with solutions of $FeSO_4 \times 7\ H_2O$ (0.1 – 0.8 mmol/L).

General

For bee pollen botanical origin analysis an upright research microscope (B-800 Series; Optika Microscopes, Ponteranica, Italy) and a Sigma 2-16 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) were used. Methanol (Merck KGaA, Darmstadt, Germany) was used for preparation of bee pollen extracts.
Analyses of total phenolic content were conducted using the FC reagent (Reagecon, Shannon, Ireland), Na_2CO_3 (Panreac, Barcelona, Spain) and gallic acid (98%) (Sigma-Aldrich, Switzerland). For determination of flavonoid content $NaNO_3$ (Gram-mol, Zagreb, Croatia), $AlCl_3$ (Kemika, Zagreb, Croatia), NaOH (Gram-mol, Zagreb, Croatia) and quercetin (Sigma-Aldrich, Switzerland) were used. FRAP reagent for antioxidant activity was made of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) reagent (98%) (Sigma-Aldrich, Switzerland) and $FeCl_3$ x 6 H_2O (Riedel-de Haën, Germany). For calibration curve $FeSO_4$ x 7 H_2O (Kemika, Zagreb, Croatia) was used. UV-Vis spectrophotometer UV-1800 (Shimadzu Corp., Kyoto, Japan) was used in all analyses.

Results and discussion

The botanical origin shows the presence of different plant sources attended by the bees to produce the bee pollen. This description permitted to classify them as unifloral/ bifloral/ mixed multifloral bee pollen as in the Table 1. Eight out of twelve pollen samples correspond to unifloral samples according to predominant botanical species percentage (> 45%). Samples P4 and P7 had highest percentage of predominant pollen, especially P4 sample which was almost fully unifloral *Amorpha fructicosa* (Fig. 1) sample. *Brassica* spp., which is characteristic

botanical species for area of investigation in this study, was present in just three samples. The results indicate that *Asteraceae* and *Apiaceae* pollen type, which were present in majority of samples, together with *Amorpha fructicosa* and *Salix* spp. are important source of pollen collection in this area in the collection period.

Table 1. Botanical origin of collected bee pollen samples

Sample	Predominant pollen (>45%)		Secondary pollen (16-45%)	Important minor pollen (3-15%)	Minor pollen (<3%)	Classification
	Specie	%	Specie	Specie	Specie	
P1	*Apiaceae*	61.3	*Prunus* spp.	/	*Rubus* spp., *Papaver* spp., *Taraxacum* spp., *Castanea sativa* Mill., *Poaceae*, *Fraxinus* spp., *Juglans regia*, *Jasione montana*	Unifloral
P2	*Apiaceae*	59.5	*Prunus* spp.	*Humulus* spp.,	*Poaceae*, *Lotus* spp., *Fraxinus* spp., *Juglans regia*, *Brassica* spp.	Unifloral
P3	*Genista* spp.	51.4	/	*Rhamnaceae*, *Cornus sanguinea*, *Rubus* spp., *Prunus* spp., *Loranthus europaeus*, *Malus* spp., *Potentila* spp.	*Fraxinus* spp., *Bellis perenis*, *Campanulaceae*, *Violaceae*	Unifloral
P4	*Amorpha fructicosa*	99.0	/	/	*Cornus sanguinea*, *Juglans regia*	Unifloral
P5	*Salix* spp.	47.7	*Cerastium* spp.	*Prunus* spp., *Taraxacum* spp., *Rubus* spp., *Brassicaceae*, *Malus* spp.	*Poaceae*, *Castanea sativa* Mill., *Humulus* spp., *Pinus* spp., *Tilia* spp., *Bellis perenis*, *Trifolium* spp., *Taxus* spp., *Juglans regia*	Unifloral
P6	/	/	*Trifolium* spp., *Brassica* spp., *Apiaceae*	*Robinia pseudoacacia* L., *Prunus* spp., *Malus* spp., *Cornus sanguinea*, *Castanea sativa* Mill.	*Pinus* spp.	Multifloral
P7	*Amorpha fructicosa*	83.4	/	*Malus* spp., *Brassica* spp., *Prunus* spp.	*Trifolium* spp.	Unifloral
P8	/	/	*Asteraceae* type, *Helianthus annuus*, *Taraxacum* spp.	*Chicorium* spp.	*Zea mays*, *Carex* spp., *Iris pseudocorus*	Multifloral
P9	*Asteraceae* type	58.2	*Helianthus annuus*	*Taraxacum* spp., *Zeam mays*	*Apiaceae*, *Artemisia* spp.	Unifloral
P10	/	/	*Asteraceae t*ype, *Helianthus annuus*	*Taraxacum* spp., *Zeam mays*, *Trifolium* spp., *Cornus sanguinea*, *Artemisia* spp.	*Apiaceae*	Multifloral
P11	*Asteraceae* type	52.9		*Asteraceae* other, *Taraxacum* spp., *Bellis* spp., *Helianthus annuus*, *Trifolium* spp., *Euphorbia* spp.	*Poaceae*, *Apiaceae*, *Acacia* spp., *Cirsium* spp.	Unifloral
P12	/	/	*Tilia* spp.	*Bellis* spp., *Myrtaceae*, *Artemisia* spp., *Apiaceae*, *Amorpha fructicosa*, *Asteraceae* other, *Trifolium* spp., *Helianthus annuus*, *Prunus* spp., *Cichorium* spp.	*Cornus sanguinea*	Multifloral

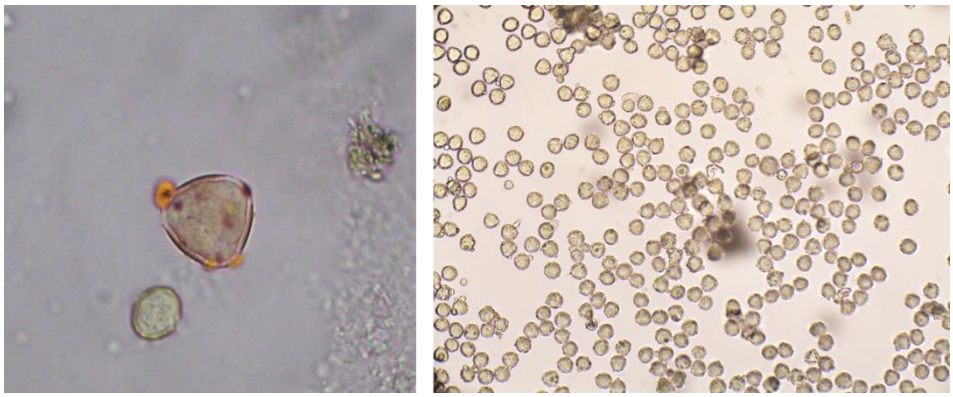

Fig. 1. Pollen grain of *Amorpha fructicosa* (800× magnification) and microscopic view of P4 sample (400× magnification)

Total phenolic content of analysed samples ranged between 7.08 – 15.27 mg GAE/g of pollen. Results were similar to those obtained by Kroyer and Hegedus (2001) and Mărghitaş et al. (2009) where values were between 7.4 – 9.7 GAE/g and 4.4 – 16.4 GAE/g were reported, respectively. In comparison to researches of Carpes et al. (2009), Araujo et al. (2017) and Velásquez et al. (2017) where results ranged between 19.28 – 48.90 GAE/g, 33.73 – 75.60 GAE/g and 6.86 – 52.99 GAE/kg, respectively, the results obtained in this study were lower. Velásquez et al. (2017) reported the highest values of total phenolic content in samples with predominant *Prunus* spp. pollen. Samples P1 and P2 with *Prunus* spp. as secondary pollen had also higher results for total phenolic content, but still significantlylower than those obtained in the above mentioned research. The highest total phenolic content had unifloral *Amorpha fructicosa* sample P7 (Table 2) while the lowest result was observed for P10 sample which was multifloral one.

Table 2. Average values and standard deviation of total phenolic content, total flavonoids and antioxidant capacity by FRAP determined in bee pollen samples

Sample	Total phenolic content [mg GAE/g]	Total flavonoids [mg QE/g]	FRAP [µmol Fe^{2+}/g]
P1	13.47	2.49	74.43
P2	12.45	2.06	60.42
P3	8.20	2.28	66.07
P4	12.62	2.30	60.18
P5	11.63	3.01	83.56
P6	12.84	1.77	67.86
P7	15.27	2.13	81.49
P8	12.35	3.58	54.67
P9	12.04	3.48	53.27
P10	7.08	3.39	51.97
P11	9.47	4.25	75.64
P12	8.79	1.34	57.57
Minimum	7.08	1.34	51.97
Maximum	15.27	4.25	83.56
Average	11.35	2.67	65.59
Standard deviation	2.43	0.86	11.03

Total flavonoid content ranged between 1.34 – 4.25 mg QE/g (Table 2). Mărghitaş et al. (2009), Araujo et al. (2017) and Carpes et al. (2009) reported higherresults than those obtained in this research (2.8 – 13.6 mg QE/g, 1.42 – 9.05 mg QE/g and 2.10 – 28.33 mg QE/g, respectively). The bee pollen of unifloral sample P11 (*Asteraceae* type) had the highest flavonoid content while the lowest value was again in mixed multifloral sample. Total flavonoid content of bee pollen was analyzed also by Feás et al. (2012) and Pascoal et al. (2014) but results were exspresed in mg catechin equivalent (CAE)/g of pollen, what enables the comparison of the results.

FRAP values ranged between 51.97 – 83.56 μmol Fe^{2+}/g (Table 2). The highest value was observed in unifloral *Salix* spp. sample (P5). Unifloral samples P7, P11 and P1 had also high FRAP values, while multifloral sample P10 had the lowest FRAP value as well as lowest total phenolic content. Other studies also showed correlation between total phenolic content and FRAP (Ulusoy and Kolayli, 2004; Borycka et al., 2016) thus the antioxidant power showed by bee pollen samples can be attributed to their phenolic content.

Conclusions

The obtained results indicate that bee pollen samples seem to be as different as honeys of different botanical origin. Due to the diversity and complexity of the bee pollen, future study should take into account more bee pollen samples for better understanding correlation between botanical origin and antioxidant properties of bee pollen. It will be interesting to broad the research with more parameters and samples from other regions of Croatia as well as from different collection periods.

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