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ANTIOXIDANT, ANTIMICROBIAL ACTIVITY AND HEAVY METALS CONTENT IN POLLEN OF *Papaver somniferum* L.

ZAWARTOŚĆ METALI CIĘŻKICH ORAZ AKTYWNOŚĆ ANTYOKSYDACYJNA I ANTYBAKTERYJNA PYŁKU *Papaver somniferum* L.

Abstract: An aim of the study was to measure a total flavonoids content, as well as, individually the content of quercetin, luteolin, kaempferol, apigenin in *Papaver somniferum* L. bee pollen, then to analyse a reduction power of bee pollen (dried, frozen, freeze-dried), and also to determine an antibacterial activity of bee pollen extracts, obtained with different concentrations of ethanol. Heavy metals concentrations were analysed in bee gathered (in case of all three treatment) and flower pollen samples. The reduction power of bee pollen compounds was 3592.56 ± 105.29 μg · cm^{-3}. The highest value achieved the freeze-dried pollen. Comparison of the flavonoids content (averaged 262.33 ± 4.42 mg · kg^{-1}) refers on higher values in the frozen bee pollen than in the dried and freeze-dried forms. In the freeze-dried pollen was the highest content of two flavonoids (quercetin and apigenin), but in the case of the other two analysed flavonoids (luteolin and kaempferol) their content was the highest in the dried bee pollen. The obtained results characterize *Papaver somniferum* L. bee pollen ethanolic extract samples as the product with the broad antimicrobial effect. From the heavy metals, in flower and bee pollen, the lead level was 0.64 mg · kg^{-1} and less than 0.1 mg · kg^{-1}, respectively. Consecutively, the contents of mercury were 0.019 mg · kg^{-1} in the flower pollen and ranging from 0.004 to 0.005 mg · kg^{-1} in the frozen, freeze-dried and dried bee pollen. The cadmium concentration in the flower pollen was 0.12 mg · kg^{-1}, and in the bee pollen ranged from 0.22 to 0.26 mg · kg^{-1}.

Keywords: *Papaver somniferum* L. bee pollen, flower pollen, flavonoids, reduction power, antibacterial activity, antifungal activity, heavy metals, contamination

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Introduction

A bee gathered pollen is regarded as valuable special food and is used also in an apitherapy [1, 2]. Bee-collected pollen ("bee pollen") is promoted as a health food with a wide range of nutritional and therapeutic properties [3]. This beehive product also has several useful pharmacological properties, such as antibiotic, antineoplastic, antidiarrhoeatic and as an antioxidant agent [4]. Also bee-pollen, as well as other apicultural products, has gained increased attention for its therapeutic properties, such as antibacterial [5, 6], antifungal [5], anti-caryogenic [7] and immunomodulatory [8] effects.

The antioxidant activity of honeybee-collected pollen has been recognized as a free radical scavenger and as a lipid peroxidation inhibitor [4, 9]. This activity has been associated with the phenolic pollen content [4]. Usually, honeybee-collected pollen is a mixture of pollen pellets from different botanical origins. Each one is an important source of flavonol glycosides [10], and in some species, of hydroxycinnamic acids [4]. These compounds are found in a species-specific profile [4], which suggests that honeybee-collected pollen from different areas or seasons could have different antioxidant activities. In spite of the relevance of honey-bee collected pollen as an antioxidant substance, there is not enough systematic information about the antioxidant activity levels associated to the flavonol content and profile of honeybee-collected pollen from different botanical origins [11]. Bee-collected pollen is an apicultural product, which is composed of nutritionally valuable substances and contains considerable amounts of polyphenol substrates, which may act as potent antioxidants. It was concluded, that pollen and propolis extracts inhibit respiratory burst within cancer cell lines, probably by their antioxidant potentials [12]. The bioactive properties of apicultural pollen extracts can be increased using a solvent suitable for its extraction, improving the activity of free radicals sequestration (antirust activity) [13]. Appropriate extracts of pollen can be used as functional food or alimentary supplement. They had the amount of phenolic composites and their capacity of sequestered free radicals which are responsible for carcinogenesis [14]. Pollen grains have specific characteristics according to the floral species or cultivation methods, but the quality depends on the collection process, cleanness, drying and storage applied by beekeepers with the objective to increase the product shelf-life.

The aim of our study was to measure the total flavonoids content, as well as, individually the content of quercetin, luteolin, kaempferol, apigenin in *Papaver somniferum* L. bee pollen. Then, to analyse the reduction power of bee pollen (dried, frozen, freeze-dried), and also to determine the antibacterial activity of bee pollen extracts, obtained with different concentrations of ethanol. The heavy metals concentrations were analysed in bee gathered (in case of all three treatments) and flower pollen samples of *Papaver somniferum* L.

Material and methods

Samples preparation

Samples of bee-collected pollen were obtained from beekeepers, which respected qualitative criteria for gathering, drying and storing as proposed by Bogdanov [2]. The

samples were collected during the spring season 2007 from different regions of western Slovakia. The flower pollen samples were obtained in the year 2007. A poppy is a self-pollinated plant species and anthers are opened inside of a flower bud before it opens. For this reason the pollen was collected from plants of different genotypes closely before flowering. The samples of flower pollen were stored in dry place at room temperature until analysed. The fresh bee pollen was stored at –18 ^{o}C, 20 % moisture, approximately six months until analysed. The dried pollen samples were dried (9–11 % humidity) approximately 8 hours at maximum temperature 35 ^{o}C. The moisture was tested by thermogravimetric analyzer WPS 50SX/1 by RADWAG. The lyophilized samples of bee pollen were dried in the table laboratory lyophilizator LYOVAC GT 2 by Amsco/Finn-Aqua, 80 hours without heating, until 2 % moisture. The drying process was realized without heating so the nutritive compounds of the pollen were not changed.

Total flavonoids and selected flavonoids analysis

HPLC determination of flavonoids. Chromatographic separation were performed on a Purospher Star RP-18e (Merck) column (250 × 4 mm I.D., 5 μm), protected by a Merck Purospher Star (4 × 4 mm, 5 μm) guard column. The HPLC system consisted of Shimadzu LC 10ADvp series pumping system, SPD 10AV/VP UV/VIS detector set at 360 nmand C-R6A chromatography data station software. Two solvents were used with constant flow rate 1 cm^3/min.

The injection volume was 20 mm^3. Solvent A consisted 0.05 % of TFA/methanol (95:5, V/V), solvent B included methanol/0.05 % TFA (95:5, V/V). For the elution program, the following proportions of solvents B were used: 0–15 min, 40 % B; 15–30 min, 40–55 % B; 30–35 min 55–70 % B. The ethanolic extracts were injected under this conditions as well as a mixture of authentic samples of quercetin, luteolin, kaempferol and apigenin.

The correlation between content of flavonoids and antioxidant activity (expressed as RP_{AA}) was analysed using SAS 9.1.3 software.

Reduction power. The reduction power of pollen compounds was evaluated spectrophotometrically by the modified method Prieto [15]. This method is established on reduction of Mo(VI) to Mo(V) with an effect of reduction parts in the presence of phosphorus under formation of green phosphomolybdenum complex. Solution absorbance of reducing sample was measured at λ = 705 nm (UV-1601, Shimadzu, Tokyo Japan) toward blank experiment (distilled water). The reduction power of compounds (RP_{AA}) expressed as quantity of ascorbic acid necessary to achieve the same effect in $\mu g \cdot cm^{-3}$ was calculated using the equation: $RP_{AA} = (A_{705\ nm} - 0.0011) / 0.00236$.

Microbiological analysis

Preparation of bee pollen ethanolic extracts (BPEE). The bee pollen samples (2 g) were milled, homogenized and extracted individually using 15 cm^3 of the ethanol solution as the extraction solvent in different concentrations (BPEE 50, BPEE 70, and

BPEE 90 %) at temperature of 70 ^{o}C for 30 min with constant agitation. The supernatant was separated and the solid residue was re-extracted. Then, the ethanol extracts of pollen were combined and stored at 5 ^{o}C for further analysis. All samples were extracted in duplicate [16].

Antimicrobial activity of bee pollen ethanolic extracts (BPEE). The disk diffusion assay described in detail by Bauer et al [17] was used in this study, with some modifications. The stock of bacterial cultures (*Staphylococcus* sp., family *Enterobacteriaceae*) and fungal (*Penicillium citrininum*, *P. crustosum*, *P. expansum*, *P. brevicompactum* and *P. chrysogenium*) were grown in the nutrient agar at 26–27 ^{o}C for 24 h and the Malt agar at 25 ^{o}C for 48 h in a shaker. The aliquots of 40 mm^3 of bee pollen ethanolic extract were applied in a paper disk and placed in plates containing the nutrient agar that was previously inoculated with active cultures of these microorganisms with sterile swabs. Antimicrobial activity was assessed by measuring the diameter of the inhibition zone around each disk after 24 hours of inoculation at 37 ^{o}C. The control (40 mm^3 of 96 % ethanol) used in all the plates and extracts analysed in duplicate. Chloramphenicol (40 mm^3) was used as the positive control for bacteria.

Heavy metals analysis

Heavy metals in flower and bee pollen were analysed by standard methods in the accredited analytic laboratory BEL/NOVAMANN International Ltd. Nové Zámky. The samples of pollen were homogenized, and then they were further processed according to determined chemical compounds.

An Advanced Mercury Analyzer (AMA 254) was assessed for determination of mercury in analysed samples of pollen. The method is based on sample catalytic combustion, preconcentration by gold amalgamation, thermal desorption, and atomic absorption spectrometry.

In the case of cadmium and lead determination was used electrothermal atomic absorption spectrometry (ETA-AAS).

Results and discussion

Total flavonoids and selected flavonoids analysis

The reduction power of bee pollen compounds was 3592.56 ± 105.29 μg · cm^{-3}. In the present submission we have compared analysed results of dried, frozen and freeze-dried bee pollen. The highest value achieved the freeze-dried pollen (Table 1).

An antioxidant ability of pollen seems to be due to phenolic compounds [18]. High levels of phenolic constituents are often accompanied by a high antioxidative capacity of pollen; however, according to the reports of Campos et al [3] and Campos et al [19], no direct correlation between flavonoids and a radical-scavenging activity was found. A gradual decrease of RSA in pollen stored for 4 years was not accompanied by a parallel reduction of flavonoids [3] and some pollen with high levels of phenolics did not present significant antiradical activity [19]. Pollen, containing more than 6 % of

water will ferment upon storage. Storage for one year or longer will reduce the free radical scavenging capacity of pollen [3].

Table 1

The reduction power (RP_{AA}) of bee pollen compounds [$\mu g \cdot cm^{-3}$]

Pollen	RP_{AA} [$\mu g \cdot cm^{-3}$]
Frozen	3452.67 ± 4.64
Dried	3653.67 ± 4.64
Freeze-dried	3671.33 ± 3.40

Comparison of flavonoids content (averaged 262.33 ± 4.42 mg · kg^{-1}) refers on higher values in the frozen bee pollen than in the dried and freeze-dried forms (Table 2). In the freeze-dried pollen was the highest content of two flavonoids (quercetin and apigenin), but in the case of the other two analysed flavonoids (luteolin and kaempferol) their content was the highest in the dried bee pollen. For any from four investigated flavonoids the highest content was not determined in the frozen bee pollen. Sum of particular four selected flavonoids decreased in the order: dried > freeze-dried > frozen bee pollen.

Table 2

The content of flavonoids in bee pollen [mg · kg^{-1}]

Flavonoids [mg · kg^{-1}]	Pollen		
	frozen	dried	freeze-dried
Quercetin	2.05 ± 0.14	3.99 ± 0.06	5.19 ± 0.10
Luteolin	1105.80 ± 0.57	1390.67 ± 0.35	1340.58 ± 0.59
Kaempferol	12.96 ± 0.75	22.40 ± 0.77	23.61 ± 0.45
Apigenin	4.60 ± 0.46	6.56 ± 0.29	15.65 ± 0.67
Total flavonoids	266.00 ± 3.74	258.67 ± 1.70	262.33 ± 2.87

In investigations by Leja et al [20] great variability regarding content of total phenols, phenylpropanoids, flavonols and antioxidant capacity in 12 examined pollens was found. In some of them (*P. tanacetifolia* and *S. alba*) a very high antioxidant activity, expressed as a radical-scavenging activity, inhibition of a lipid peroxidation and a hydroxyl radical scavenging activity, corresponded to high levels of total phenols, phenylpropanoids and flavonols. A variability of total antioxidant activity in investigated species seems to correspond to their phenylpropanoid contents, being manifested by the significant positive correlation coefficient [20].

There were observed the statistically significant strong dependence between antioxidant activity and individual flavonoids, namely quercetin, luteolin, kaempferol ($p < 0.001$), and apigenin ($p < 0.05$), but not between total flavonoids (Table 3).

Table 3

Correlation matrix for antioxidant activity and content of flavonoids

	AA (RP_{AA})	Flavonoids	Quercetin	Luteolin	Kaempferol	Apigenin
AA (RP_{AA})	1	$P \geq 0.05$ NS	$P < 0.001$ $r = 0.94759$	$P < 0.001$ $r = 0.97081$	$P < 0.001$ $r = 0.99064$	$P < 0.05$ $r = 0.68926$
Flavonoids		1	$P \geq 0.05$ NS	$P < 0.05$ $r = –0.67444$	$P \geq 0.05$ NS	$P \geq 0.05$ NS
Quercetin			1	$P < 0.01$ $r = 0.84820$	$P < 0.001$ $r = 0.94193$	$P < 0.01$ $r = 0.87972$
Luteolin				1	$P < 0.001$ $r = 0.95397$	$P \geq 0.05$ NS
Kaempferol					1	$P < 0.05$ $r = 0.70517$
Apigenin						1

AA – antioxidant activity.

Microbiological analysis

The antimicrobial activity of *P. somniferum* L. bee pollen extracts was analysed according to the disk diffusion assay, and the results are shown in Table 4. The obtained results characterize *P. somniferum* L. bee pollen ethanolic extract samples as the product with the broad antimicrobial effect. The strongest antibacterial effect was shown by *P. somniferum* L. bee pollen ethanolic extract of 90 % concentration against *Staphylococcus* sp. and family *Enterobacteriaceae.* The strongest antifungal effect was shown by *P. somniferum* L. bee pollen ethanolic extract samples of 90 % concentration against *Penicillium citrininum* and *Penicillium crustosum* strains. The least antifungal effect was shown by *P. somniferum* L. bee pollen ethanolic extract samples of 50 % concentration against to *Penicillium chyrysogenium* strains. Chloramphenicol the antibiotic was used for comparison, showing different ways of action to each microorganism. The control proved that the ethanol used in the extractions did not have inhibiting action.

Table 4

Antimicrobial activity [mm] of *Papaver somniferum* L. bee pollen ethanolic extracts (BPEE)

BPEE	*Enterobacteriaceae*	*Staphylococcus* sp.	*Penicillum citrininum*	*Penicillium crustosum*	*Penicillium expansum*	*Penicillium brevicompactum*	*Penicillium chrysogenium*
50 %	6.0	5.0	4.0	4.5	4.0	2.0	0.0
70 %	6.5	5.5	4.0	6.0	3.5	3.0	0.0
90 %	7.0	6.0	5.0	9.0	1.5	1.0	0.0
Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0
A	9.0	11.0	6.0	4.0	5.0	0.0	5.0

Pollen ethanolic extracts in Carpes et al. [16] study of all pollen at 60 % showed the same inhibition degree against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella* sp., *Bacillus cereus* and *Staphylococcus aureus* bacteria. In Carpes et al [16] study *Bacillus subtilis* bacteria were inhibited for Parana pollen in a pollen ethanolic extracts at 40 %, 50 %, 60 % and 70 % and for Alagoas pollen in PEE at 50 %, 70 % and 90 %. In this study, the pollen ethanolic extracts of Parana pollen at 90 % showed the biggest clear zones around each disk (7.0 mm) against *Klebsiella* sp. Nevertheless, *Klebsiella* was also inhibited by the pollen from Alagoas in extracts of 60 and 70 %. The Alagoas extract pollen at 70 %, contained the highest antibacterial activity. *Pseudomonas aeruginosa* bacteria were inhibited by 80 % and 90 %, and *Staphylococcus aureus* bacteria were inhibited by extracts of pollen at 50 %, 60 %, 70 % and 80 % of ethanol solution.

Heavy metals analysis

From the heavy metals in flower and bee pollen the lead level was 0.64 mg · kg^{-1} and less than 0.1 mg · kg^{-1}, respectively (Table 5). Consecutively, the contents of mercury were 0.019 mg · kg^{-1} for the flower pollen and ranging from 0.004 to 0.005 mg · kg^{-1} for frozen, freeze-dried and dried bee pollen. The cadmium concentration in the flower pollen was 0.12 mg · kg^{-1} and in the bee pollen ranged from 0.22 to 0.26 mg · kg^{-1}. The content of cadmium was lower in the flower pollen than in the bee pollen, but other heavy metals like the lead and mercury had higher levels in the flower pollen.

Table 5

The content of heavy metals in flower and bee pollen [mg · kg^{-1}]

Heavy metals [mg · kg^{-1}]	Flower pollen (year 2007)	Bee pollen (year 2007)	
Lead (Pb)	0.64	Dried	< 0.1
		Frozen	< 0.1
		Freeze-dried	< 0.1
Mercury (Hg)	0.019	Dried	0.005
		Frozen	0.004
		Freeze-dried	0.004
Cadmium (Cd)	0.12	Dried	0.25
		Frozen	0.22
		Freeze-dried	0.26

Chlebo and Čermáková [20] investigated; that a bee pollen contamination can comes from two sources – by industrial emissions and agricultural pesticides. They determined following content of risk chemical elements in 16 samples of bee pollen, from 4 high industrialized regions of Slovakia (Stredný Spiš, Ružomberok, Horná Nitra, and Bratislava): a lead (Pb) 1.70 mg · kg^{-1} (year 1990) and 0.38 mg · kg^{-1} (year 1999), a mercury (Hg) 0.018 mg · kg^{-1} (year 1990) and 0.003 mg · kg^{-1} (year 1999), a cadmium (Cd) 0.180 mg · kg^{-1} (year 1990) and 0.043 mg · kg^{-1} (year 1999). In

comparison with our results the level of cadmium was higher in bee pollen samples, and the level of mercury was slightly higher in flower and bee pollen samples (Table 5).

Conclusions

Further studies of the antimicrobial and the antioxidant properties and the antioxidant components of bee pollen from different botanical origins are required. There is also important problem with the heavy metals content in bee pollen, which has to be closely investigated; in consequence that bee pollen is regarded as valuable food supplement.

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ZAWARTOŚĆ METALI CIĘŻKICH ORAZ AKTYWNOŚĆ ANTYOKSYDACYJNA I ANTYBAKTERYJNA PYŁKU *Papaver somniferum* L.

Abstrakt: Celem pracy było zmierzenie całkowitej zawartości flawonoidów oraz kwercetyny, luteoliny, kemferolu i apigeniny w pyłku pszczelim pochodzącym z *Papaver somniferum*. Następnie zbadano siłę redukcyjną pyłku pszczelego (suszonego, mrożonego, suszonego i mrożonego) oraz aktywność antybakteryjną ekstraktów pyłku pszczelego uzyskanych przy użyciu etanolu o różnych stężeniach. Zawartość metali ciężkich zbadano w pyłku zebranym przez pszczoły oraz w próbkach pyłku pobranych z kwiatów. Siła redukcyjna pyłku pszczelego wynosiła 3592,56 ±105,29 $\mu g \cdot cm^{-3}$. Największą siłę redukcyjną miał pyłek suszony i zamrożony. Największą zawartość flawonoidów stwierdzono (średnio 262 ± 4.42 $mg \cdot kg^{-1}$) w zamrożonym pyłku pszczelim. W pyłku, który był jednocześnie wysuszony i zamrożony, występowała największa ilość kwercetyny i apigeniny. Pozostałe flawonoidy (luteolina i kemferol) występowały w największej ilości w suszonym pyłku pszczelim. Uzyskane rezultaty wskazują na bardzo szerokie działanie antybakteryjne etanolowych ekstraktów pyłku pszczelego z *Papaver somniferum*. Zawartość ołowiu w pyłku pszczelim oraz próbkach zebranych bezpośrednio z kwiatów wynosiła odpowiednio 0,64 $mg \cdot kg^{-1}$ i mniej niż 0,1 $mg \cdot kg^{-1}$. Zawartość rtęci wynosiła 0,019 $mg \cdot kg^{-1}$ w pyłku zebranym bezpośrednio z kwiatów. Natomiast w pyłku pszczelim mrożonym, suszonym oraz suszonym i mrożonym zawartość rtęci wynosiła od 0,004 do 0,005 $mg \cdot kg^{-1}$. Zawartość kadmu w pyłku zebranym z kwiatów wynosiła 0,12 $mg \cdot kg^{-1}$, a w pyłku pszczelim od 0,22 do 0,26 $mg \cdot kg^{-1}$.

Słowa kluczowe: pyłek pszczeli z *Papaver somniferum* L., pyłek kwiatowy, flawonoidy, siły redukcyjne, aktywność antybakteryjna, aktywność antygrzybiczna, metale ciężkie, zanieczyszczenia