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Antioxidant and antimicrobial properties of monofloral bee pollen

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The main aim of this study was to determine antioxidant properties and antibacterial activity of monofloral bee pollen samples to pathogenic bacteria. These samples were collected in different localities in Slovakia. The antioxidant properties of examined plant species were different and decreasing in the following order: *Brassica napus* subsp. *napus* L. > *Papaver somniferum* L. > *Helianthus annuus* L. The antimicrobial effect of the bee product samples were tested by using the agar well diffusion method. The methanol (99.9% and 70%) and the ethanol (96% and 70%) were used for extraction. In this study, five different strains of bacteria were tested: *Listeria monocytogenes* CCM 4699; *Pseudomonas aeruginosa* CCM 1960; *Staphylococcus aureus* CCM 3953; *Salmonella enterica* CCM 4420; and *Escherichia coli* CCM 3988. The most sensitive bacteria of the poppy pollen ethanolic extract was *Staphylococcus aureus* was (70%) The most sensitive bacteria of rape bee pollen methanolic extract (70%) and sunflower ethanolic extract (70%) was *Salmonella enterica*.

Keywords: Monofloral bee pollen, an antioxidant activity, an antimicrobial activity, pathogenic bacteria.

Introduction

Bee pollen is nature's most complete food. There are several new studies dealing with the properties of bee pollen.[1–3] Nowadays bee pollen is known as the apicultural product with many beneficial properties such as antibacterial, antifungicidal, anti-caryogenic and immunomodulatory effects.[4–7] The bee pollen can be characterized as a functional food with varied enhancing effects in human health due to its nutritional properties.[8]

Phytochemicals, such as phenolic compounds, are considered beneficial for human health because they decrease the risk of degenerative diseases by reducing oxidative stress and inhibiting macromolecular oxidation.[9–10] Natural health products have been studied as food supplements and have gained increased attention in recent years.[11–16]

Bee pollen contains some nutritional compounds such as carbohydrates, proteins, amino acids, lipids, vitamins, minerals and traces of micronutrients. Bee pollen contains a significant amount of polyphenolic substances, mainly flavonoids too. On the other hand, the honeybee gastrointestinal tract microflora and pollen are the primary sources for the honey microbial community.[3,17] Free radical-scavenging and metal-chelating activities in addition to their reported anticarcinogenic properties were shown. Bee pollen could be successfully used for the treatment in some cases of benign prostatitis and for oral desensitization of children who have allergies.[18]

The evaluations of the *in vitro* antioxidant activity of ethanolic extracts of pollen of *P. juliflora* suggest that this pollen is a substance with a high free radical scavenging activity which relate to its phenolic composition. The phenolic composition of pollen principally consists of flavonol glycosides and hydroxycinnamic acids. This composition tends to be species-specific.[19]

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Table 1. Antiradical and antioxidant activity of monofloral bee pollen.

	DPPH (% of inhibition)	*RP_{AA} ($\mu g.mL^{-1}$)*	*Biosensor I/I_0*	*Polyphenols ($mg.kg^{-1}$)*
B. napus subsp. napus L.	86.25 ± 0.94	4495.33 ± 4.19	1.93 ± 0.02	1383.67 ± 3.86
H. annuus L.	47.97 ± 0.29	2778.67 ± 3.30	0.37 ± 0.04	691.67 ± 7.76
P. somniferum L.	75.93 ± 0.53	3452.67 ± 4.64	0.95 ± 0.02	817.33 ± 4.11
Mean	70.05	3575.56	1.09	964.22
SD	17.17	749.04	0.68	319.31
Minimum	47.68	2775.0	0.32	682.0
Maximum	87.51	4501.0	1.95	1389.0

Results are expressed as Mean ± SD; DPPH-1,1-diphenyl-2-picrylhydrazyl; RP_{AA}- Reduction power of compounds; polyphenols- total polyphenols content.

In the past people all over the world used pollen for its goodness and medical properties. Many reasons are the same for today as they were in the past. The properties of bee – pollen still have not been refuted.[20] There are some reports about the antimicrobial[21–22] and antioxidant activities of pollen separated into families.[22–24]

The aim of this study was to find out some of the antioxidant properties and antimicrobial activity of aqueous methanol and ethanol extracts of frozen bee pollen samples from three plant species: poppy (*Papaver somniferum* L.), rape (*Brassica napus* subsp. *napus* L.), sunflower (*Helianthus annuus* L.).

Materials and methods

Pollen samples and their preparation

The samples of bee-collected pollen were obtained from beekeepers, respecting qualitative criteria for gathering and storing, according to Bogdanov.[8] The samples were collected from the western part of Slovakia, from the Nitra region. The fresh bee pollen was stored at −18°C, 20% moisture, for six months until it was analyzed.

The ethanolic extracts used for to determine total phenolic compounds content, reduction power, antiradical activity and antioxidant activity were prepared by extracting five grams homogenized bee pollen in 50 mL of ethanol (90%) in a water bath at 70°C, for 30 min. Then the samples were filtered and stored in tubes with a screw-on cap, at 5°C for further analysis.

Antiradical activity

Antiradical activity was determined by modified DPPH method according to Brand-Williams[25] and expressed as the % of DPPH inhibition. DPPH (1,1-diphenyl-2-picrylhydrazyl) is defined as the stable free radical with red color (absorbed at 517 nm). In the case that the free radicals have been scavenged, DPPH will be yellow.

The reduction power of pollen compounds was evaluated spectrophotometrically by the modified method according to Prieto et al.[26] This method is established on reduction of Mo (VI) to Mo (V) with the effect of reduction parts in the presence of phosphor under formation of green phosphomolybdenum complex. Solution absorbance of reducing sample was measured at 705 nm (UV-1601, Shimadzu, Tokyo, Japan) toward black experiment (distilled water).

Table 2. Inhibitory effects of poppy bee pollen extracts against the pathogenic bacteria (inhibition zone diameter in mm).

		Hours	*LM*	*PA*	*SA*	*SE*	*EC*
Extracts	Methanol 99.9%	24	0.67 ± 0.58	1.67 ± 0.57	2.00 ± 1.00	1.67 ± 0.58	2.00 ± 1.00
		48	1.67 ± 1.04	1.67 ± 0.58	1.67 ± 0.58	2.00 ± 0.00	2.33 ± 1.15
	Methanol 70.0%	24	2.67 ± 1.15	1.67 ± 1.53	2.50 ± 0.50	2.67 ± 0.58	2.67 ± 2.08
		48	2.67 ± 1.15	1.00 ± 1.00	2.33 ± 0.58	2.67 ± 1.53	2.67 ± 1.53
	Ethanol 96.0%	24	2.33 ± 0.58	1.67 ± 1.53	2.83 ± 0.29	2.00 ± 1.00	2.00 ± 0.00
		48	2.00 ± 0.87	1.00 ± 1.00	2.17 ± 0.29	2.17 ± 0.76	1.83 ± 0.29
	Ethanol 70.0%	24	2.33 ± 0.58	1.67 ± 0.58	3.67 ± 1.53	2.00 ± 1.00	3.00 ± 1.00
		48	2.33 ± 0.58	1.17 ± 1.26	1.67 ± 1.53	1.67 ± 0.29	3.00 ± 0.00
Control	Chloramphenicol	24	ND	ND	ND	ND	ND
		48	ND	ND	ND	ND	ND

Results are expressed as Mean ± SD; LM: *Listeria monocytogenes* CCM 4699; PA: *Pseudomonas aeruginosa* CCM 1960; SA: *Staphylococcus aureus* CCM 3953; SE: *Salmonella enterica* CCM 4420; EC: *Escherichia coli* CCM 3988; ND: indicates that no inhibitory concentration was detected in the range tested.

The reduction power of compounds (RP_{AA}) which is expressed as quantity of ascorbic acid necessary to achieve the same effect in $\mu g.mL^{-1}$ was calculated using the equation: $RP_{AA} = (A705\ nm - 0.0011) / 0.00236$.

Antioxidant activity

The antioxidant activity was further evaluated by DNA-based biosensor using a voltametric procedure based on the protective effect of antioxidants against the oxidative DNA damage.[27]

The method was employed using a disposable DNA biosensor fabricated as a screen-printed electrode chemically modified by calf thymus double stranded (ds) DNA.

DNA damaging or antioxidant effect is expressed by relative signal I/I_0 value, where "I" is the indicator flow on electrode after solution influence with fissile mixture and antioxidant, and "I_0" is indicator of flow on electrode before fission.

The normalized (relative) signal value I/I_0 which represents the survived part of the original DNA, was obtained. This normalized signal value can be used for to compensate the differences in the properties of the individual strips of the DNA – biosensor.

The Folin-Ciocalteau method was used to quantify the total polyphenols content.[28] This method uses tannin as a reference standard.

Antibacterial activity

The pollen (10 g) was extracted in 80 mL of solvent. Four different solvents were used: 99.9 and 70% (v/v) aqueous methanol (MEh and MEl, respectively), and 96 and 70% (v/v) aqueous ethanol (Eh and El, respectively). MEl and El were acidified with hydrochloric acid to pH 1.5 and 2, respectively. The samples were extracted at 80°C under reflux for 1 hour.

The mixture was centrifuged and the solvent of the supernatant was evaporated under reduced pressure at 40–45°C after chilling. The residue was dissolved in 160 mL of pure ethylacetate and shaken for 30 min. The organic (ethylacetate) phase was separated, the solvent was evaporated and the residue was dissolved in 10 mL 99.9% methanol.

The bacterial strains were purchased from the Czech Collection of Microorganisms (CCM). The antimicrobial effects of the extracts were tested by using the agar well diffusion method in Mueller-Hinton agar (MHA). The agar plates were inoculated with 200 μL of microorganism suspension at a density of 10^7 $CFU.mL^{-1}$ in saline solution and spread on the surface after 30 minutes of the initial drying. Subsequently, four equidistant wells, 9 mm in diameter each, were punched into the inoculated medium with sterile glass. Bacteria were incubated at the temperature 37°C. The inhibition zones (mm) around the disks were measured after 24 and 48 h of cultivation. The chloramphenicol was used as a positive control for bacteria. Five different strains of bacteria were tested: two Gram-positive strains (*Listeria monocytogenes* CCM 4699*; Staphylococcus aureus* CCM 3953) and three Gram-negative strains (*Pseudomonas aeruginosa* CCM 1960*; Salmonella enterica* CCM 4420*; Escherichia coli* CCM 3988) in sets of plates, which were simultaneously processed for each strain. All the experiments were repeated twice, including control with chloramphenicol. After incubation the zones of growth inhibition of the bacteria around the disks were measured. The mean values of three trials and standard deviations were calculated.

Statistical analysis

We calculated the basic variation-statistical values using statistical program Statgraphics 5 for the obtained data. The results were expressed as means ± standard deviation (SD). Tests were carried out in triplicates for all determination methods.

Results and discussion

In the present study, the antioxidant activity, in terms of the scavenging of the radical DPPH, reduction power and antioxidant activity of the ethanolic extracts of various pollen was determined and compared. Results are summarized in Table 1.

The DPPH radical is one of the few stable organic nitrogen free radicals; it has been widely used to determine the free radical scavenging ability of the various samples. The free radical-scavenging activity of the extracts is attributed to their hydrogen-donating ability.[20]

Many investigators have reported that pollen possesses antioxidative activities.[18, 19, 20, 22, 23, 30] They expected that flavonoids, such as quercetin, flavones, isoflavones, flavonones, anthocyanins, catechin and isocatechin can contribute to the antioxidative activity which they observed. Different flavonoid compositions and other factors could be involved in the free radical-scavenging activity.

The antioxidant properties were different in particular plant species. The highest values of all antioxidant parameters were found in the ethanolic extract of *B. napus* pollen. The highest total phenolic content was determined for this extract, too (Table 1). All examined antioxidant properties decreased in the following order: *B. napus* > *P. somniferum* > *H. annuus*.

There was a distinct correlation between the total phenolic content and the antioxidant characteristics in the ethanolic extracts of bee pollen from different plant species in this study. The correlation coefficient (r) between the total phenolic content and the antiradical activity (DPPH) was 0.6624. The correlation between the total phenolic content and reducing power and between the total phenolic content and antioxidant activity was similar: 0.9482 and 0.9581, respectively.

Total phenolic phytochemical concentration was measured in twelve honeybee-collected pollens of selected

Table 3. Inhibitory effects of rape bee pollen extracts against the pathogenic bacteria (inhibition zone diameter in mm).

		Hours	*LM*	*PA*	*SA*	*SE*	*EC*
Extracts	Methanol 99.9%	24	2.33 ± 0.58	1.67 ± 0.58	2.67 ± 1.53	2.00 ± 0.00	1.67 ± 1.53
		48	3.00 ± 1.00	1.33 ± 1.15	2.33 ± 1.53	2.33 ± 2.52	2.33 ± 2.52
	Methanol 70.0%	24	2.67 ± 0.58	2.00 ± 1.73	2.00 ± 1.00	3.33 ± 1.58	2.33 ± 2.08
		48	3.00 ± 1.00	2.66 ± 0.58	3.67 ± 1.53	3.83 ± 1.26	3.00 ± 2.65
	Ethanol 96.0%	24	2.33 ± 1.15	2.00 ± 2.00	2.33 ± 2.31	2.00 ± 0.00	2.67 ± 1.15
		48	3.33 ± 0.58	1.67 ± 2.89	2.67 ± 2.08	2.50 ± 2.50	3.67 ± 2.08
	Ethanol 70.0%	24	3.33 ± 2.08	2.67 ± 2.52	2.33 ± 2.31	2.67 ± 1.53	2.33 ± 1.53
		48	3.67 ± 2.08	3.67 ± 3.52	3.00 ± 2.65	3.00 ± 1.00	3.33 ± 2.08
Control	Chloramphenicol	24	ND	ND	ND	ND	ND
		48	ND	ND	ND	ND	ND

Results are expressed as Mean ± SD; LM: *Listeria monocytogenes* CCM 4699; PA: *Pseudomonas aeruginosa* CCM 1960; SA: *Staphylococcus aureus* CCM 3953; SE: *Salmonella enterica* CCM 4420; EC: *Escherichia coli* CCM 3988; ND: indicates that no inhibitory concentration was detected in the range tested.

floral species as well as their antioxidant capacity with the same analytical methods as in our study.[30] The highest polyphenol concentration was determined in the methanol extracts of bee pollen from *Salix* sp. (16.4 mg/ GAE/g) followed by *T. officinale* Web. bee pollen (16.2 mg/GAE/g), *C. cyanus* L. bee pollen (16.0 mg/GAE/g), *C. monogyna* J. bee pollen and (7.7 mg/GAE/g) *C. bursa pastoris* L. bee pollen (15.2 mg/GAE/g). The lowest level of total polyphenol content was determined in bee pollen from *K. arvensis* (L.) Coult. with value of 4.4 mg/GAE/g and *Pinus* sp. and *Carex* sp. bee pollen with value of Q4 6.4 mg/GAE/g.[30]

The antibacterial activities of the rape, the poppy and the sunflower bee pollen extracts *in vitro* tested against different Gram positive and negative pathogenic bacteria are shown in Tables 2–4.

Similar results were achieved by Carpes et al.[22] who tested *Pseudomonas aeruginosa*. These bacteria were inhibited by extracts of pollen at 80 and 90% ethanol solution whereas; *Staphylococcus aureus* bacteria were inhibited at 50, 60, 70 and 80% ethanol solution. Different results were reported by Almeida-Muradian et al.[30] We found very good antibacterial effect of ethanolic pollen extracts of *Pseudomonas aeroginosa* similar to results of Carpes et al.[22] and Abouda et al.[31] In all extraction conditions applied in our experiment, there was found the same non-inhibitory effect of bee pollen extracts to *Staphylococcus aureus* bacteria as for Parana pollen.[22]

The best antibacterial effect of poppy bee pollen extracts was found at methanolic extracts 70% to *Listeria monocytogenes, Salmonella enterica* and *Escherichia coli*. There was found a very good inhibitory effect of *Staphylococcus aureus* on ethanolic extract 70%. The inhibitory effect of *Pseudomonas aeroginosa* was similar for all extracts. The best inhibitory properties of rape bee pollen extracts were found on ethanolic extracts 70% to *Listeria monocytogenes* and *Pseudomonas aeroginosa*. Very good inhibitory effect of sunflower bee pollen was found at ethanolic extracts 70% to *Salmonella enterica*. All sunflower bee pollen extracts had similar antibacterial effect to *Listeria monocytogenes*.

Table 4. Inhibitory effects of sunflower bee pollen extracts against the pathogenic bacteria (inhibition zone diameter in mm).

		Hours	*LM*	*PA*	*SA*	*SE*	*EC*
Extraxts	Methanol 99.9%	24	2.33 ± 0.58	2.33 ± 0.58	2.00 ± 1.00	2.67 ± 0.58	1.33 ± 1.15
		48	2.67 ± 0.58	2.67 ± 1.53	2.00 ± 0.00	2.67 ± 0.58	1.50 ± 1.32
	Methanol 70.0%	24	2.33 ± 0.58	2.00 ± 1.00	1.67 ± 1.53	3.33 ± 0.58	2.00 ± 1.73
		48	3.67 ± 2.31	2.33 ± 2.08	1.67 ± 1.53	2.67 ± 0.58	2.67 ± 2.52
	Ethanol 96.0%	24	2.33 ± 2.89	1.00 ± 1.73	2.67 ± 1.15	3.00 ± 1.00	2.33 ± 0.58
		48	2.67 ± 0.57	1.67 ± 1.53	1.67 ± 2.08	2.17 ± 1.89	1.67 ± 1.53
	Ethanol 70.0%	24	2.33 ± 3.79	2.00 ± 1.00	2.67 ± 1.15	3.67 ± 1.15	2.33 ± 0.58
		48	2.43 ± 3.79	2.67 ± 2.31	1.00 ± 1.00	3.67 ± 0.58	2.67 ± 0.58
Control	Chloramphenicol	24	ND	ND	ND	ND	ND
		48	ND	ND	ND	ND	ND

Results are expressed as Mean ± SD; LM: *Listeria monocytogenes* CCM 4699; PA:*Pseudomonas aeruginosa* CCM 1960; SA: *Staphylococcus aureus* CCM 3953; SE: *Salmonella enterica* CCM 4420; EC: *Escherichia coli* CCM 3988; ND: indicates that no inhibitory concentration was detected in the range tested.

Conclusions

Poppy and rape bee pollen had higher content of polyphenols than the sunflower bee pollen. These polyphenols have an important influence on antioxidant activity. The highest antibacterial activity of poppy bee pollen was found against Gram positive bacteria *Staphyloccoccus aureus* in 70% ethanolic extract after 24 hour. The high sensitivity of rape bee pollen extracts was found in 70% methanolic extract against *Salmonella enterica* after 48 hours. The best antimicrobial activity of sunflower bee pollen was found in 70% methanolic extract after 48 hour against *Listeria monocytogenes* and in 70% ethanolic extract after 24 and 48 hours against *Salmonella enterica*. We verified that the Gram positive bacteria were more sensitive to bee pollen than the Gram negative bacteria. We can confirm that the bee pollen has some interesting biological properties and could be considered as a functional food.

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