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Effect of storage conditions on antioxidant activity of bee pollen extracts

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Summary

Bee pollen is a product of rich and varied chemical composition, and its biological activities are diverse. Many of these activities are related to the antioxidant effect of bee pollen. The aim of this study was to determine the effect of storage conditions on the antioxidant activity of bee pollen extracts. The study was conducted on three types of bee pollen extracts, namely, ethanol and pepsin extracts of bee pollen, as well as on ethanol extracts of pepsin-digested bee pollen. Antioxidant activity was determined by a DPPH method, directly after obtaining the extracts and after storing them for twelve months under various conditions, i.e. at –18°C in the dark, at 4-8°C in the dark, at room temperature in the dark, and at room temperature in the light. It was concluded that the 12-month storage of bee pollen extracts caused a decrease in the antioxidant activity of all extracts examined, and the decrease depended on storage conditions. The highest decrease in antioxidant activity was observed in all types of extracts stored at room temperature in the light. The lowest decrease in antioxidant activity was found in ethanol extracts of pepsin-digested bee pollen.

Keywords: bee pollen, antioxidant activity, free radicals, DPPH, storage

Bee pollen (grains of plant pollen collected by bees), also known as bee pollen pellets or pollen loads, is a natural product well recognized in apitherapy. It consists of bee-collected pollen grains, as well as honey, nectar and bee salivary gland secretions. Bees collect pollen to transform it into propolis, which is their main source of protein. The appearance of pollen depends on the plant species from which it originates. Its grains have different shapes, size, color and weight. A palynologic analysis of grains is important for identifying not only the origin of bee pollen, but also other bee products, mainly honeys. Dried bee pollen is used as material in the pharmaceutical and cosmetic industry or as a food product. Powdered bee pollen can be used in capsules, tablets, granules or extracts (3, 10).

Its extremely rich chemical composition accounts for the valuable properties of bee pollen. It contains 250 various chemical substances, 70% of which have biologic activity. The kind and amount of individual compounds in the total mass of pollen is widely diverse, depending on the plant species and its climatic zone of origin, as well as the season of the year when pollen was collected (8, 24).

Total protein content in bee pollen is approximately 23.9%, whereas exogenic amino acids constitute 8.6% of pollen dry mass. Therefore, bee pollen is used as a dietary supplement high in protein. Among proteins, various enzymes have been identified, and their number, according to different authors, ranges from 40 to over 100 (15, 25).

Lipids constitute approximately 5.41% of bee pollen dry mass, and their amount differs greatly, depending on the pollen origin. Lipids present in pollen include fatty acids, triglycerides, phospholipids and phytosterols. There are more unsaturated fatty acids (including essential unsaturated fatty acids, EUFA) than saturated ones, and the ratio is 1.5 to 2.67, which is worth noting. Unsaturated fatty acids, phospholipids and phytosterols are responsible for the antiatherogenic effect of bee pollen (10, 23).

Carbohydrates are the most abundant in bee pollen. Assimilable polysacharydes, digestible by humans, constitute approximately 30.8% of pollen, which is crucial for the nutritive value of bee pollen pellets. Fructose and glucose prevail among simple sugars. Cellulose content, which is indigestible by humans,

amounts to 22.4%. Cellulose is a constituent of fiber, and its importance is related to the dietary use of bee pollen (10, 26).

Moreover, bee pollen is a rich source of polyphenols, especially of flavonoids and phenolic acids. Depending on the origin, their content may vary significantly. Flavonoids present in bee pollen include quercetin, apigenin, luteolin, genistein, and isorhamnetin, which are usually glycosides (1, 2, 11).

Vitamin content in bee pollen is also widely diverse. Vitamin C is the most plentiful of water-soluble vitamins, whereas vitamin E and beta-carotene are the most abundant of all fat-soluble vitamins. These compounds belong to antioxidants of low molecular weight, which protect the body from reactive oxygen species (ROS) (10, 12). Bee pollen pellets are also a valuable source of macro- and microelements, especially iron, and are therefore used in the prophylaxis of iron deficiency anemia (22).

Bee pollen has various biological activities and medicinal uses, which result from the cumulative action of its constituent substances. Owing to its special nutritional properties, pollen is used as a dietary supplement in undernutrition and during convalescence (13, 14, 16).

Many biological properties of bee pollen are related to its antioxidant effect, which results from the presence of numerous polyphenols, vitamins C and E, and beta-carotene. Antioxidant activities of bee pollen consist in its ability to deactivate free radicals and to protect the body from negative effects of their activity. In recent years, following the discovery of the relationship between the oxidative stress and the etiology of civilization diseases, increasing attention has been paid to the role of antioxidants present in food products. Consequently, bee pollen preparations are used in the prophylaxis and treatment of conditions related to free radical processes, such as chronic inflammations, rheumatoid arthritis, atherosclerosis, hypertension, diabetes, CNS diseases, inflammation and ulceration of alimentary tract, neoplasms, AIDS, autoimmune disorders, mucoviscidosis, kidney diseases and accelerated aging syndromes (5, 7, 9, 11, 12, 16, 21).

Changes in bee pollen properties are related to the storage period. Published data suggest that the storage of bee pollen significantly decreases its antioxidant activity, and in consequence the quality of pollen preparations (7). The aim of our study was to determine the effect of storage conditions on the antioxidant activity of bee pollen extracts (extracts of plant pollen grains collected by bees). For this purpose, three types of bee pollen extracts were prepared, namely, ethanol extracts of bee pollen (EEP), pepsin extracts of bee pollen (PEP) and ethanol extracts of pepsin-digested bee pollen (EEPP), and their antioxidant activity was determined. Then, the extracts were stored for 12 months at various temperatures and under different lighting conditions. After this period, their antioxidant activity was measured again to determine the extent of changes for different types of extracts and storage conditions.

Material and methods

The study material consisted of ground bee pollen collected in 2011 in the apiary "BARC" in Kamianna. Three types of extracts were prepared from the bee pollen: ethanol extracts, enzymatic extracts obtained by pepsin digestion of bee pollen, and ethanol extracts obtained from the substrate from an enzymatic hydrolysis of bee pollen.

Ethanol extracts (EEP) were prepared according to a slightly modified method of Almaraz-Abarca et al. (1). The ethanol extract of bee pollen was prepared by weighing 20 g of ground bee pollen to within an accuracy of 0.01 g. The bee pollen sample was extracted 5 times with a 50% (v/v) ethanol aqueous solution, in 200 cm^3 portions, and shaken each time for 60 min at room temperature to macerate the sample. After each extraction, the sample was filtered under reduced pressure with a water pump. The filtrate was collected, and the substrate was extracted again with another portion of ethanol. The filtrate obtained was centrifuged at 10 000 rpm for 10 min. It was evaporated under reduced pressure in a rotary vacuum evaporator (UNIPAN-PRO 350P). The evaporated extract was dried in a laboratory incubator at 38°C to obtain a solid mass. The dry residue of extract was weighed and dissolved in a 50% (v/v) ethanol aqueous solution to obtain a concentration of 100 mg/cm^3. These extracts are further referred to as EEP.

Pepsin extract of bee pollen (PEP) was prepared according to a slightly modified method described by Nagai et al. (17). An amount of 20 g of bee pollen was weighed. Then the sample was mixed with distilled water acidified with concentrated HCl to pH = 2. The volume of distilled water was 5 times the volume of the sample. Pepsin was added to the sample to obtain a concentration of 1.0%. Next, the sample was incubated at 37°C for 48 h. Hydrolysis was arrested by boiling for 10 min. The enzymatic extract obtained was filtered under reduced pressure with a water pump. The filtrate was centrifuged at 10 000 rpm for 10 min, and the supernatant was evaporated in a rotary vacuum evaporator (UNIPAN-PRO 350P). The extract obtained was dried in a laboratory incubator at 38°C. A dry pepsin extract was weighed and dissolved in acidified distilled water to obtain a concentration of 100 mg/cm^3. This extract was used for further assays and is referred to as PEP.

Ethanol extracts of pepsin-digested bee pollen (EEPP) were obtained in accordance with a method described earlier by Rzepecka-Stojko et al., (18). The supernatant obtained, after pepsin extraction of bee pollen, was extracted with 200 cm^3 of 50% (v/v) ethanol aqueous solution for 60 min at room temperature and frequently shaken. The extract was filtered under reduced pressure, and then the filtrate was centrifuged at 10 000 rpm for 10 min. The supernatant was evaporated under reduced pressure in a rotary vacuum evaporator (UNIPAN-PRO 350P). Next, the extract was dried in a laboratory incubator at 38°C. The dry extract was dissolved in a 50% (v/v) ethanol aqueous solution to obtain a concentration of 50 mg/cm^3. This extract was used for further assays and is referred to as EEPP.

Tab. 1. Yield of extraction and antioxidant activity of the extracts of the bee pollen, fresh and stored

Extracts of bee pollen	Yield of extraction (%, w/w)	Antioxidant activity EC_{50} (µg/cm³) (± SEM)				
		Fresh extracts	Extracts stored for 12 months			
			Room temperature in the light	Room temperature in the dark	4-8°C in the dark	–18°C in the dark
EEP (n = 3)	58.49 (± 0.56)	72.05 (± 4.90)	297.90 (± 19.33)	269.90 (± 28.75)	247.11 (± 20.07)	225.32 (± 14.65)
PEP (n = 3)	67.29 (± 0.88)	178.09 (± 39.20)	597.25 (± 48.69)	706.41 (± 38.31)	553.59 (± 43.88)	359.40 (± 15.90)
EEPP (n = 3)	5.73 (± 0.16)	25.82 (± 0.80)	36.40 (± 2.80)	34.85 (± 1.83)	33.11 (± 2.02)	32.08 (± 1.73)

The antioxidant activity was evaluated by a slightly modified method of Brand-Williams et al. (6) using 2,2-diphenyl--1-picrylhydrazyl (DPPH). The method is based on the reduction of a DPPH free radical with antioxidant compounds. The electron transfer from antioxidant present in a sample to DPPH induces the change of the solution color from purple to yellow proportionally to the antioxidant concentration.

The reduction of a reagent is monitored spectrophotometrically, and a decrease in the solution absorbance is recorded at (λ) 517 nm wave-length. To evaluate their antioxidant activity, bee pollen extracts were diluted to a concentration of 1 mg/cm³. Next, 0.1 cm³ of extract was collected and mixed with 3.9 cm³ of the ethanol extract of DPPH at a concentration of 6×10^{-5} M. The mixture was incubated for 30 min in the dark at room temperature. Then, the absorbance of the mixture was measured with a SP-830 Plus Metertech spectrophotometer. Three samples of each type of extract were prepared (n = 3), and the measurement was performed in five replications.

Antioxidant activity was determined for fresh extracts and for those stored for 12 months under the following conditions: at –18°C in the dark, at 4-8°C in the dark, at room temperature in the dark, and at room temperature in the light. The antioxidant effect of all extracts was expressed as EC_{50}, which refers to the antioxidant concentration which induces the reduction of DPPH by 50% of its initial value. The value of this parameter increases as the antioxidant activity of the samples decreases. Ascorbic acid and butylated hydroxytoluene (BHT) at a concentration of 0.2 mg/cm³ and EC_{50} of 4.88 µg/cm³ and 7.85 µg/cm³, respectively, were used as standard antioxidants.

Moreover, for each extract, i.e. EEP, PEP and EEPP, the percentage of antioxidant activity after a 12-month storage under specified conditions was determined in relation to the activity of fresh extracts. The calculations were based on percent inhibition (PI), which determines a decrease in the absorbance of DPPH solution caused by samples of the extracts. The antioxidant activity of fresh extract was assumed as 100%.

For each extract, extraction efficiency was calculated on the basis of the amount of dry mass in relation to the surplus of bee pollen pellets.

Results and discussion

The method of evaluating antioxidant effect based on the reduction of DPPH is especially useful for testing samples rich in polyphenols, such as bee pollen extracts. Therefore, it is one of the most frequently used methods for the evaluation of antioxidant activity of bee products (4, 7, 21).

Among the three types of bee pollen extracts examined, namely, ethanol extracts (EEP), pepsin extracts of bee pollen (PEP) and ethanol extracts of pepsin-digested bee pollen (EEPP), the highest antioxidant activity of fresh extracts was recorded for EEPP. For these extracts, an average value of EC_{50} was 25.82 µg/cm³ (Tab. 1). A weaker antioxidant effect was recorded for fresh EEP, for which EC_{50} was 72.05 µg/cm³ (Tab. 1). According to published data, the antioxidant activity of such extracts was 104.5 µg/cm³ (20), i.e. lower than the one observed in our study. The lowest antioxidant activity of all fresh extracts was recorded for PEP, since their EC_{50} was the highest, i.e. 178.09 µg/cm³ (Tab. 1). The values of antioxidant activity observed in this study are in agreement with previous study results obtained by the electron paramagnetic resonance (EPR) method (19).

The analysis of the effect of storage conditions on the antioxidant activity of bee pollen extracts revealed a decrease in the antioxidant activity of all extracts after a 12-month storage, which was reflected by an increase in the value of EC_{50} (Tab. 1).

Of the three types of extracts, the smallest change in antioxidant activity was recorded for ethanol extracts of pepsin-digested bee pollen (EEPP). Depending on temperature and lighting conditions, the highest decrease in antioxidant activity was observed for EEPP stored at room temperature and exposed to sunlight. The average EC_{50} value was 36.40 µg/cm³ (Tab. 1), i.e. 70.79% of the antioxidant activity of a fresh extract (Fig. 1). The smallest changes were recorded for the extracts stored at –18°C in the dark. In their case, EC_{50} was 32.08 µg/cm³ (Tab. 1), i.e. 80.47% of the antioxidant activity of fresh extracts (Fig. 1).

The analysis of the antioxidant activity of ethanol extracts of bee pollen (EEP) after a 12-month storage under various conditions, shows that the decrease in their antioxidant effect was the highest, compared with the other extracts. For EEP stored at room temperature in the light, EC_{50} was 297.90 µg/cm³ (Tab. 1), i.e. 24.37% of the antioxidant activity of fresh extracts (Fig. 1). The antioxidant activity of EEP stored at room temperature in the dark was 27.01% of the activity of

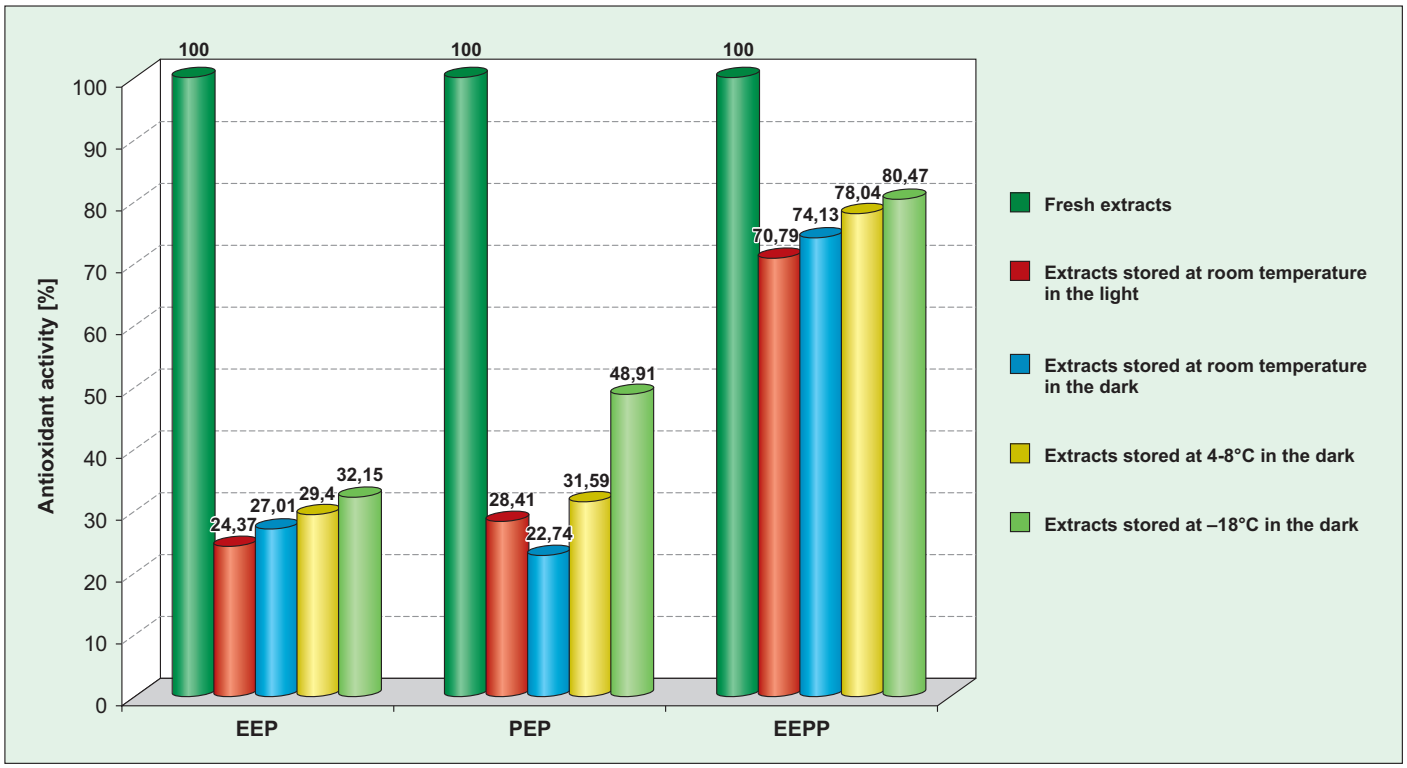

Fig. 1. Effect of storage conditions on the antioxidant activity of ethanol extract of bee pollen (EEP), pepsin extract of bee pollen (PEP), and ethanol extract of pepsin-digested bee pollen (EEPP). The antioxidant activity in fresh extracts was assumed to be 100%

fresh extracts (Fig. 1), whereas EEP stored at 4-8°C in the dark retained 29.40% of the antioxidant activity of fresh extracts (Fig. 1). The lowest decrease in antioxidant activity was observed for EEP stored at –18°C in the dark. EEP retained on average 32.15% of the antioxidant activity of fresh extracts (Fig. 1), and EC_{50} was 225.32 µg/cm^3 (Tab. 1).

The antioxidant activity of the pepsin extract of bee pollen (PEP) after a 12-month storage was retained almost completely at –18°C in the dark, and it amounted to 48.91% of fresh extract activity (Fig. 1), with EC_{50} at 359.40 µg/cm^3 (Tab. 1). The highest decrease in antioxidant activity was recorded for the extracts stored at room temperature. However, a regular decrease in EC_{50} values accompanying the reduction of lighting was not observed, unlike in the case of EEPP and EEP. This might be explained by the fact that the samples of PEP stored at room temperature in the light had become turbid. At the same time, it should be stressed that such storage conditions are unsuitable for pepsin extracts of bee pollen (PEP).

The changes in the antioxidant activity of bee pollen extracts stored under different conditions presented in this paper closely correspond to the results of our previous study on the effects of storing bee pollen extracts on their polyphenol content, which determines to a large extent their antioxidant activities. Polyphenol content was the highest in fresh ethanol extracts of pepsin-digested bee pollen, whereas the lowest polyphenol content was found in a pepsin extract of bee pollen. The storage of bee pollen extracts caused a decrease in polyphenol concentration, and these changes depended on storage conditions. The highest decrease in polyphenol concentration was noted in all types of extracts stored at room temperature in the light, whereas polyphenol content was the most stable during the storage of ethanol extracts of pepsin-digested bee pollen (20). It should be stressed that the decrease in the antioxidant activity of stored extracts was much higher than the decrease in polyphenol content, which suggests that polyphenols are not the only components with antioxidant effect. According to published reports, similar observations were made in studies on the effect of storage on the antioxidant activity of bee pollen. Bee pollen stored for 1 year retains 50% of its antioxidant activity in comparison to fresh pollen, although the decrease in polyphenol content is much lower (7).

On basis of the present study, we also analyzed the efficiency of the extracts obtained by three different extraction methods. The highest efficiency of an extract, i.e. 67.29%, was obtained for the pepsin extraction of pollen (PEP) (Tab. 1). The efficiency of this method is discussed in a paper by Nagai et al. (17), who put it at 45%, i.e. 22.29 percentage points less than our result. Moreover, the average efficiency of extracts obtained by this method in our previous study was 70.70% (18), i.e. higher by 3.41 percentage points than the one reported in the present study. A slightly lower efficiency, i.e. 58.49%, was obtained for the extraction with 50% ethanol (EEP) (Tab. 1). According to published data (13), the efficiency of ethanol extraction of bee pollen is 31.10%, whereas in our previous study it amounted

to 49.57% (18). Thus, the average efficiency of EEP obtained is the present study is higher by 27.39 percentage points and by 8.92 percentage points, respectively. The efficiency of ethanol extraction of pepsin-digested bee pollen increased by 5.73 percentage points (Tab. 1).

The results obtained in the present study suggest the following conclusions. The storage of bee pollen extracts for 12 months reduces antioxidant activity in all three types of extracts examined, and these changes depend on storage conditions. The best conditions for preserving the quality of all types of extracts are no sun exposure and a temperature of –18°C. The highest decrease in antioxidant activity was recorded for all extracts stored in the light at room temperature. It should be emphasized that such storage conditions are particularly unsuitable for the pepsin extract of bee pollen (PEP). The smallest changes in antioxidant activity were observed for ethanol extracts of pepsin-digested bee pollen EEPP under all storage conditions studied. Moreover, the antioxidant activity of bee pollen extracts is closely related to the extraction method. Among the three methods employed in our study, the ethanol extraction of pepsin-digested bee pollen (EEPP) was the most effective in obtaining extracts of high antioxidant content. However, it is the least efficient of all methods of bee pollen extraction described here.

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