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BEE BREAD – PERSPECTIVE SOURCE OF BIOACTIVE COMPOUNDS FOR FUTURE

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ABSTRACT

Bee bread is product with long history used mainly in folk medicine. Nowadays, bee bread is growing in commercial interest due to its high nutritional properties. The objective of this study was to determine biological activity of ethanolic extract of bee bread obtained from selected region of Ukraine – Poltava oblast, Kirovohrad oblast, Vinnica oblast, Kyiv oblast, Dnepropetrovsk oblast. The antioxidant activity was measured with the radical scavenging assays using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as phosphomolybdenum assay. Total polyphenol content was determined with Folin-Ciocalteau reagent and total flavonoid content by aluminium-chloride method. Secondary was also evaluated antimicrobial activity in bee bread samples with disc diffusion method and minimum inhibitory concentrations. Antioxidant activity expressed as mg TEAC per g of dry weight (Trolox equivalent antioxidant capacity) was the highest in bee bread from Poltava oblast in DPPH and also phosphomolybdenum method. Samples of bee bread contained high levels of total polyphenols (12.36 – 18.24 mg GAE – gallic acid equivalent per g of dry weight) and flavonoids (13.56 – 18.24 μg QE – quercetin equivalent per g of dry weight) with the best values of bee bread from Poltava oblast. An elevated level of antioxidant potential in the bee bread determines its biological properties, which conditioned of the biological active substances. The best antibacterial activity of bee bred with disc diffusion method was found against *Bacillus thuringiensis* CCM 19. The antibacterial activity inhibited by the bee bread extract in the present study indicate that best minimal inhibition concentration was against bacteria *Escherichia coli* CCM 3988 and *Salmonella enterica* subs. *enterica* CCM 3807.

Keywords: antioxidant activity; pollen; flavonoids; polyphenols; antimicrobial activity

INTRODUCTION

Bee bread is a product of the hive obtained from pollen collected by bees, to which they added honey and digestive enzymes and subsequently stored in the combs, starting a lactic fermentation which gives it greater power conservation **(Zuluaga et al., 2015)**. This type of lactic acid fermentation is similar to that in yoghurts (and other fermented milk products) and renders the end product more digestible and enriched with new nutrients **(Krell, 1996)**. The process of bee bread formation starts with gathering of pollen, then a bee mixes it with flower nectar or honey and saliva, and carries to the beehive, where non flying bees fill the mixture into honeycomb cells for ¾ of the cell volume. Residual cell volume is filled with honey, thus protecting the pollen mass from oxygen. An anaerobic lactic fermentation process takes place and bee bread is forming. Bee bread differs from pollen by lower pH (3.8 – 4.3), it contains less proteins and fats, but more carbohydrates and lactic acid. Bee bread has a better bioavailability because the walls of pollen, which cannot be destructed by gastrointestinal liquids, have been partly destructed by fermentation and the functionally and energetically rich content of pollen can be assimilated and used easier **(Mizrahi and Lensky, 1997; Fatrcová-Šramková et al., 2010)**. A proper hive management promotes bee-bread collection, aimed at marketing it for human consumption since it can be considered as food supplement due to its content of a wide range of nutrients. One of the contributions to their high nutritional value is the presence of significant amounts of proteins, vitamins and phenolic compounds as natural antioxidants. The potential application of "bee bread" as a food and as a nutraceutical supplement depends in large part on its chemical composition which varies directly with the flora of the region and the time of collection by the bees **(Čeksterytė et al., 2008)**. Bee bread differs from pollen by lower pH (3.8 – 4.3), it contains less proteins and fats, but more carbohydrates and lactic acid. Bee bread has a better bioavailability because the walls of pollen, which cannot be destructed by gastrointestinal liquids, have been partly destructed by fermentation and the functionally and energetically rich content of pollen can be assimilated and used easier **(Mizrahi and Lensky, 1997)**. Bee bread has antimicrobial, antioxidant hepatoprotective, immuno-modulating and antiradiation activity, adaptogenic properties. It stimulates protective forces of a human body, normalizes metabolism, has a positive influence on the liver, nervous and endocrine system functions, and enhances regeneration of tissues, physical and mental persistence of a human body **(Bogdanov, 2015)**.

The aim of study was to determine biological activity of selected bee bread samples – antioxidant activity, total polyphenols and flavonoids content. Secondary was also to determine antimicrobial characteristic of these samples.

MATERIAL AND METHODOLOGY

Biological material

Bee bread was obtained from selected region of Ukaine (Poltava oblast, Kirovohrad oblast, Vinnica oblast, Kyiv oblast, Dnepropetrovsk oblast), by patent technology developed by research teams Department of beekeeping, National University of Life and Environmental Sciences of Ukraine, Kyiv. Before the measurement samples were crushed to the powder using mortar and store at 4°C in refrigerator.

Chemicals

All chemicals were analytical grade and were purchased from Reachem (Slovakia) and Sigma Aldrich (USA).

Sample preparation

0.1 g of bee bread was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g (Rotofix 32 A, Hettich, Germany) for 10 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids).

Antioxidant activity

Radical scavenging activity

Radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) **(Sánchéz-Moreno et al., 1998)**. The extracts (0.5 mL) were mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL ethanol). Absorbance of the sample extract was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-100 $mg.L^{-1}$; R^2 = 0.988) was used as the standard and the results were expressed in $mg.g^{-1}$ Trolox equivalents.

Reducing power

Reducing power of samples was determined by the phosphomolybdenum method of **Prieto et al., (1999)** with slight modifications. The mixture of sample extract (1 mL), monopotassium phosphate (2.8 mL, 0.1 M), sulfuric acid (6 mL, 1 M), ammonium heptamolybdate (0.4 mL, 0.1 M) and distilled water (0.8 mL) was incubated at 90°C for 120 min, then rapidly cooled and detected by monitoring absorbance at 700 nm using the spectrophotometer Jenway (6405 UV/Vis, England). Trolox (10-1000 $mg.L^{-1}$; R^2=0.998) was used as the standard and the results were expressed in $mg.g^{-1}$Trolox equivalents.

Total polyphenol content

Total polyphenol content of potato extracts was measured by the method of **Singleton and Rossi, (1965)** using Folin-Ciocalteu reagent. 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Gallic acid (25-250 $mg.L^{-1}$; R^2=0.996) was used as the standard and the results were expressed in $mg.g^{-1}$ gallic acid equivalents.

Total flavonoid content

Total flavonoids were determined using the modified method of **(Willett, 2002)**. 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 ml of 1 M sodium acetate and 4.3 mL of distilled water. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer Jenway (6405 UV/Vis, England). Quercetin ($0.01 - 0.5$ $mg.L^{-1}$; R^2 = 0.997) was used as the standard and the results were expressed in $\mu g.g^{-1}$ quercetin equivalents.

Antimicrobial activity

Microbial strains

Four strains of microorganisms were tested in this study, including two Gram-negative bacteria (*Escherichia coli* CCM 3988, *Salmonella enterica* subs. *enterica* CCM 3807, two Gram-positive bacteria (*Bacillus thuringiensis* CCM 19, *Staphylococcus aureus* subs. a*ureus* CCM 4223). All tested strains were collected from the Czech Collection of microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C.

Disc diffusion method

Antimicrobial activity of each bee bred extract was determined by a disc diffusion method. Briefly, 100 µL of the test bacteria were grown in 10 mL of fresh media until they reached a count of approximately 10^5 $cells.mL^{-1}$. Then 100 µL of the microbial suspension was spread onto Mueller Hinton agar plates. The extracts were tested using 6 mm sterilized filter paper discs. The diameters of the inhibition zones were measured in millimeters. All measurements were to the closest whole millimeter. Each antimicrobial assay was performed in at least triplicate. Filter discs impregnated with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentrations (MICs)

MICs were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation **(CLSI, 2014)** in Mueller Hinton broth (Biolife, Italy). Briefly, the DMSO plant extracts solutions were prepared as serial two-fold dilutions obtaining a final concentration ranging between 0.5-2048 $\mu g.mL^{-1}$. After that each well was inoculated with microbial suspension at the final density of 0.5 McFarland. After 24 h of incubation at 37 °C, the inhibition of microbial growth was evaluated by measuring the well absorbance at 450 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 microwell plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values of absorbance. Wells without plant extracts were used as negative controls of growth. Pure DMSO was used as negative control. This experiment was done in eight-replicates for a higher accuracy of the MICs of used medical plant extracts.

Statistical analysis

The basic statistical analyzes were realized in SAS programming packages (THE SAS SYSTEM V 9.2.). Correlation coefficients were calculated by CORR analysis **(SAS, 2009)**.

RESULTS AND DISCUSSION

Antioxidant activity

In the DPPH radical-scavenging method, a compound with high antioxidant potential effectively traps the radical, thereby preventing its propagation and the resultant chain reaction **(Brand-Williams et al., 1995)**. DPPH is a stable free radical that is dissolved in ethanol and its purple color shows a characteristic absorption at 515 nm. Antioxidant molecules scavenge the radical by hydrogen donation and the colour from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance **(Silva et al., 2012)**. As shown Fig. 1 all tested samples had effect to trap DPPH radical, with the best value in bee bred from Poltava oblast (15.78 mg TEAC.g^{-1}) and Vinnica oblast (14.62 mg TEAC.g^{-1}). High antioxidant activity also reported **(Zuluaga et al., 2015),** which evaluated polyfloral Colombian bee bread with ABTS method; values from their study range from 46.1 to 76.3 μmol Trolox/g. In spite of the relevance of bee bread as an antioxidant substance, there is not enough systematic information about the antioxidant activity and profile of bioactive compounds of bee bread.

Phosphomolybdenum method is used to measure the reductive ability of antioxidant, and it is evaluated by the transformation of Mo(VI) to Mo(V) where, the ability of samples to reduce Mo may be attributed from hydrogen donation from phenolic compounds which is also related to presence of reducing agent **(Huda-Faujan et al., 2009)**. The reducing ability of the bee breads (Fig. 1) was in the order: bee bread from Poltava oblast > bee bread from Kyiv oblast > bee bread from Vinnica oblast > bee bread from Kirovohrad oblast > Dnepropetrovs oblast. Similar like DPPH method, the best values were determined in sample from Poltava region. **Barros et al., (2007)** demonstrated that the reducing properties are generally associated with the presence of reductones, which had been shown to exert antioxidant action by breaking the free radical chain by donating the bread hydrogen atom. Higher level of polyphenols in bee bread could act as reductone where these compounds could react with free radicals by converting them to more stable products and terminating the radical chain reaction **(Oh et al., 2013)**. **Siddiqui et al., (2012)** claimed antioxidants chelate and disengage transition metals, thereby preventing such metals from participating in the initiation of lipid peroxidation and oxidative stress through metal catalyzed reaction.

On the basis of the above findings, bee bread seems to be attractive as an important source of antioxidants for the food and pharmaceutical industries. The differences observed between the antioxidant activities of the tested samples may be attributed to the presence of natural antioxidants, mainly phenolic compounds that differed depending on the region where they were collected **(Sati et al., 2013; Tlili et al., 2014)**.

Total polyphenol and flavonoid content

Phenolic compounds are considered among the largest contributors to the antioxidant potential of natural food products. Total polyphenol content (Table 1) in bee-bread ranged from 12.36 to 25.4 mg GAE.g^{-1}. The highest value was observed in sample from Poltava region. **Nagai et al., (2004)** also determined high level of total polyphenols in bee bread and also reported that bee bread can be applied more as health food and medicine. **Zuluaga et al., (2015)** determined in Colombian bee bread values from 2.1 to 13.7 mg GAE.g^{-1} of polyphenols. The information about spectrum of polyphenol compounds in bee bread is missing, but we can expect, that bee bread contains similar polyphenols like bee pollen. It is also potential, that and bee bread can contain new type of polyphenols. According to **Fanali et al., (2013)** in bee pollen, polyphenolic compounds are commonly glycosylated, esterified, present in free forms or combined with other pollen components. **Bonvehi et al., (2001)** reported that bee pollen is rich for gallic acid, vanillic, protocatechuic, *p*-coumaric acid, hesperidin, rutin, luteolin, apigenin, kaempferol, quercetin and isorhamnetin.

Total flavonoid content (Table 1) in observed samples of bee bread ranged from 13.56 to 18.24 μg QE.g^{-1}. The highest value, similarly like polyphenol content was observed in sample from Poltava region. Flavonoids are the secondary components of most importance in bee bread and influence the visual appearance of the grain (pigmentation) and flavour (astringency and bitterness) **(DeGrandi-Hoffman et al., 2013)**. In pollen grains, most of flavonoids exist as glycosides, known as aglycones, being quercetin the major compound. Although there is not a recommended daily ingest for flavonoids, it is suggested an intake of about 200 – 100 mg per day. **Zuluaga et al., (2015)** determined total flavonoid content in Colombian bee bread from 1.9 to 4.5 mg QE.g^{-1}. It is very difficult determine average total flavonoid content in bee bread generally. **Zuluaga et al., (2014)** reported that bee pollen contains higher content of total flavonoids with compare to bee bread due to possible differences in botanical origin of pollen and also the fact that a degradation of the outer layer of the grain makes more available bioactive compounds to degrade by environmental conditions. These authors also published that in Colombian region was established average content of flavonoids in 5.16 mg.g^{-1} (QE) of bee pollen. The separation of the individual polyphenols and flavonoids and detection of the other antioxidants will be necessary for evaluate of biological activity of bee bread in future.

Antimicrobial activity

Bee bread samples showed a potential activity against the growth of both gram positive and gram negative bacteria which was resistant to antibiotics. This would be a very interesting approach to control more dangerous species of micro-organism in medical sciences. Because of the development of resistance by the microorganisms to common antibiotics, it has become necessary to search for an alternative approach dealing with this situation. It had been suggested that natural products are preferable to synthetic ones **(Abouda et al., 2011)**.

Results of antibacterial testing with disc diffusion method (Figure 2) showed that higher antibacterial activity was

found against *Bacillus thuringiensis* in sample from Vinnica oblast, Kyiv oblast and Dnepropetrovsk oblast. The higher inhibition zone was found in sample from Kirovohrad oblast against bacteria *Escherichia coli*. The higher antimicrobial activity against *Salmonella enterica* subs. *enterica* was found in sample from Kyiv oblast. Samples of natural bee-bread from different aromatic and medicinal plant**s** were studied for their antimicrobial activities on antibio-resistant bacterial strains isolated from human pathology. Four samples of bee-bread were collected from different regions in Morocco. Dilutions of bee-bread from 1/2, 1/4, 1/8 and 1/16 were tested by the agar well diffusion method on various strains of bacteria including *Escherichia coli*, *Staphylococcus aureus***,** *Bacillus cereus* and *Pseudomonas aeruginosa*. Results revealed that most of strains were inhibited by the dilution 1/2 and 1/4. The gram positive bacteria were more sensitive to bee-bread and bee-pollen than gram negative bacteria. All the samples showed strong antimicrobial activities on the bacterial strains, which were first tested for their resistance to antibiotics **(Abouda et al., 2011)**. The best antimicrobial activity (Tab. 2) MIC50 was found in sample from Poltava region where minimal inhibition concentration (6.40 μg.mL^{-1}) against gram negative bacteria; very good antibacterial activity were also found in same sample against bacteria in MIC90 (6.40 μg.mL^{-1}). In generally all tested samples against all tested bacteria had antibacterial influence.

Statistical analysis

Using Pearson correlation coefficients was verified correlation (Table 3) between antioxidant activity determined by DPPH and phosphomolybdenum method and total polyphenol and flavonoid content. The strong correlation dependence (0.95) was found between antioxidant activity (DPPH) and polyphenol content and also between flavonoid content and antioxidant activity (phosphomolybdenum method) (0.89). Between two different methods for determining the antioxidant activity, was determined the mean linear relationship (0.54). Based on these results, it can be concluded that polyphenols and flavonoids have a strong impact on the antioxidant activity of bee bread.

CONCLUSION

In conclusion, the results of this study demonstrate that bee bread is very good source of bioactive compounds not only with antioxidant but also antimicrobial effect. The best results were observed in most of parameters in sample from Poltava oblast. Bee bread can be use more in future not only in medicine, pharmacy but also in food industry. For confirmation of biologically effect is necessary more and intensive study, *in vivo* test for evaluating bioactive components and digestibility properties; very important is also determining some negative compounds which can

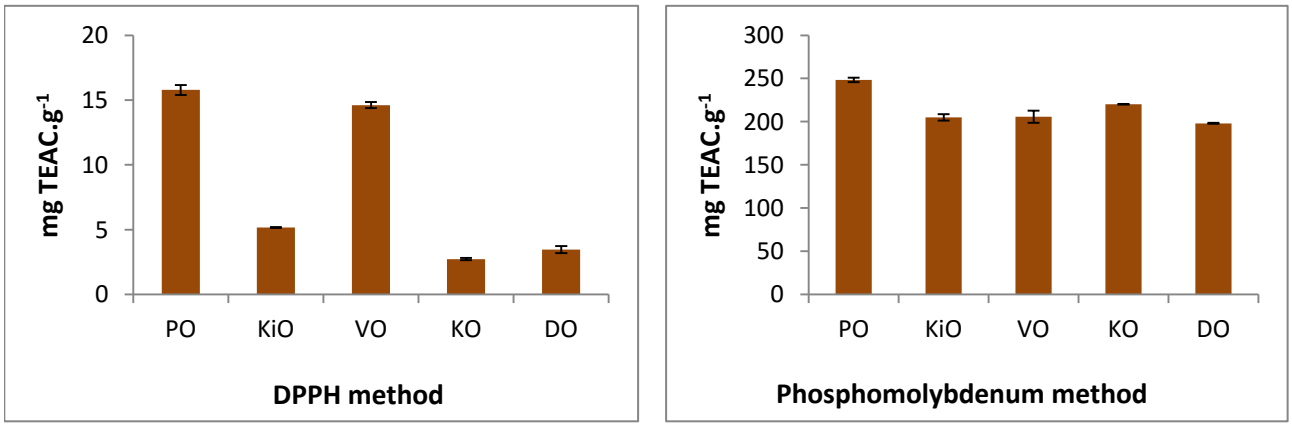

Figure 1 Radical scavenging activity and reducing power of bee bread (TEAC – Trolox equivalent antioxidant capacity PO – Poltava oblast, KiO – Kirovohrad oblast, VO – Vinnica oblast, KO – Kyiv oblast, DO – Dnepropetrovsk oblast.

Table 1 Total polyphenol and flavonoid content in bee bread.

Sample	Total polyphenol content (mg GAE.g^{-1})	Total flavonoid content (μg QE.g^{-1})
Poltava oblast	25.44 ±0,22	18.24 ±0.08
Kirovohrad oblast	19.96 ±0.59	15.25 ±0.04
Vinnica oblast	20.88 ±0.34	13.56 ±0.04
Kyiv oblast	12.36 ±0.34	15.35 ±0.09
Dnepropetrovsk oblast	13.47 ±0.56	14.04 ±0.03

Note: GAE – gallic acid equivalent; QE – quercetin equivalent; ± standard deviation.

Table 2 The antimicrobial activity of bee bread (MIC, μg.mL^{-1})

	PO		KiO		VO		KO		DO	
	MIC50	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**
Escherichia coli	6.40	6.84	12.81	13.64	12.81	13.64	12.81	13.64	12.81	13.64
Salmonella enterica	6.40	6.84	12.81	13.64	12.81	13.64	12.81	13.64	8.53	9.54
Staphylococcus aureus	12.81	13.64	12.81	13.64	17.07	19.08	25.58	27.20	12.81	13.64
Bacillus thuringiensis	12.81	13.64	12.81	13.64	12.81	13.64	25.58	27.20	17.07	19.08

Note: PO – Poltava oblast, KiO – Kirovohrad oblast, VO – Vinnica oblast, KO – Kyiv oblast, DO – Dnepropetrovsk oblast; ± standard deviation.

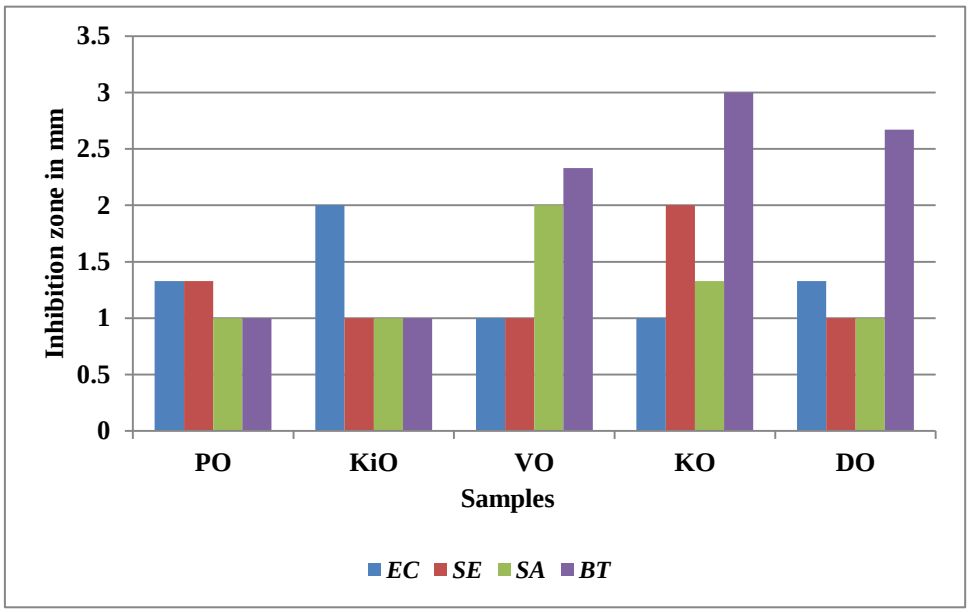

Figure 2 Antimicrobial activity of bee bread against bacteria.
Note: (*EC-Esherichia coli* CCM 3988, *SE-Salmonella enterica* subs. *enterica* CCM 3807, *BT-Bacillus thuringiensis* CCM, *SA- Staphylococcus aureus* subs. a*ureus* CCM 4223); PO – Poltava oblast, KiO – Kirovohrad oblast, VO – Vinnica oblast, KO – Kyiv oblast, DO – Dnepropetrovsk oblast.

Table 3 Results of correlation analysis

Sign/Marker	Phosphomolybdenum method	Polyphenol content	Flavonoids content
DPPH method	0.54*	0.95***	0.35^{-}
Phosphomolybdenum method		0.63*	0.89***
Polyphenol content			0.54*

p ≤0,001 ***; ≤0,05 *; >0,05 $^{-}$

decrease the quality of bee bread (heavy metal, radionuclide, and microbes). Results in this work can be an important tool for recognizing bee bread as being a beneficial source of natural nutrients.

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