Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

BEE POLLEN METHANOLIC EXTRACTS: TOTAL POLYPHENOLS CONTENT AND ANTIBACTERIAL ACTIVITY

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Abstract

The aim of this work was to study the total phenolics content and antibacterial activity of bee pollen samples from different sources. Total polyphenols content was quantified according to the Folin-Ciocâlteu spectrophotometric method using GA as standard and the results were expressed in terms of mg GAE/g pollen. Furthermore, the antibacterial activity of bee pollen samples from different sources were collected and investigated against multiple bacterial strains, as follows: five gram-positive Staphylococcus aureus ATCC 6538P, Bacillus cereus ATCC14579, Bacillus laterosporus 6932, Paenibacillus larvae 9820 and Paenibacillus alvei 13253, and four gram-negative Escherichia coli ATCC 10536, Pseudomonas aeruginosa ATCC 27853, Salmonella enteritidis ATCC 13076, Salmonella typhi ATCC 14028 and the yeast Candida albicans ATCC 90028. The samples with predominant pollen in Brassicaceae and Rosaceae possessed high levels of polyphenols content, whereas the samples predominant in Fabaceae showed lower levels of polyphenols. Regarding the antibacterial activity, our results revealed that most of the strains were inhibited by the ½ and ¼ dilutions. The gram-negative bacteria and the yeast Candida albicans proved to be resistant to all bee pollen methanolic extracts. Our results showed that bee pollen samples have strong antibacterial activity against gram-positive bacteria.

Key words*: antibacterial activity, bee pollen, methanolic extracts, palynology, total polyphenols.*

INTRODUCTION

Nowadays, there is an increasing interest in the consumption of honey bee products (bee-collected pollen, beebread, honey, royal jelly, propolis etc.) along with their implementation as functional foods and alternative medicines (Gardana et al., 2018; Margaoan et al., 2019; Daoud et al., 2019; Rzepecka-Stojko et al., 2018).

Bee collected pollen (BCP) contains all the necessary nutrients and phytochemicals which makes it an important source of protein, lipids, polyphenols, macro and microelements, as well as amino and fatty acids (Margaoan et al., 2014; Rzepecka-Stojko et al., 2015). Multiple studies have demonstrated that an enriched diet in polyphenols may fight against multiple diseases, such as cancer (Markiewicz-Żukowska et al., 2013; Sobral et al., 2017; Maric et al., 2020; Al-Yousef et al., 2020), diabetes (Laaroussi et al., 2020; Mohamed et al., 2018), cardiovascular diseases (Rzepecka-Stojko et al., 2015), atherosclerosis (Rzepecka-Stojko et al., 2017) and recently for environmental cleanup (Maric et al., 2020). Furthermore, the chemical composition is strongly correlated to botanical and geographic origin, climate conditions during collection, as well as bee's plant preferences in terms of pollen gathering (Campos et al., 2010).

In a recent study, Kostic and his collaborators determined the phenolic profile and antioxidant properties of methanolic and ethanolic extracts of monofloral bee-collected sunflower pollen from Serbia. Their results showed that the methanolic extract had a higher phenolic content compared to the ethanolic extract. Comparatively, the concentration of quantified phenolic compounds proved to be higher in the ethanolic extract (244.44 mg/kg DW) than in

methanolic (200.58 mg/kg DW). According to their results the methanolic extract showed a higher scavenging activity against ABTS (95.5% inhibition) compared to ethanolic (75% inhibition). The same was noticed for ferric reducing ability. This may be due to the different composition (i.e. proteins) in the ethanolic extract which may reduce the antioxidant capacity (Campos et al., 2003).

Bakour et al. (2019) evaluated the potential antioxidant activity, total phenolics, flavones, and flavonol content of hydro-ethanolic extracts of pollen from fourteen plants. Their result showed that the total phenolic contents varied from 9.20 ± 0.12 mg GAE/g in the *Malva sylvestris* pollen samples, to 71.20 ± 0.72 mg GAE/g in the *Mentha spicata* pollen samples.

The antibacterial activity of monolforal and polyfloral BCP has been extensively evaluated in numerous studies. Recently, Velasquez et al. (2017) demonstrated the positive correlation between the chemical properties and botanical origin of several BCP samples from Chile. Their study showed that the samples predominant in *Brassica* sp. and *Galega officinali*s an abundant source of antioxidants and antibacterial compounds. The same was noticed in the case of *Corylus avellana* BCP collected from different locations in Slovakia (Nikolaieva et al., 2019).

MATERIALS AND METHODS

Palynological analysis

For the palynologycal analysis the methods described by Louveaux et al. (1978) and Almeida-Muradian et al. (2005) were adapted for the bee collected pollen samples. A sample of 2 g, corresponding to approximately 200 pollen pellets, was considered to be represent-tative for botanical origin determination. From each sample, one microscopic slide was prepared without acetolysis, by dissolving and washing the pollen in diluted H_2SO_4 (0.5%) and colored using a mixture of glycerine - gelatin - fuxine for permanent preparation (Louveaux et al., 1978). Slide examination was performed using a Nikon Eclipse 50i optic microscope at 1000× magnification (for identification) and 400× magnification for counting. Five hundred pollen grains were counted from every slide, and percentages of different botanical species were calculated.

Total phenolic content

The total polyphenol content of the bee polyfloral pollen samples taken in the study was determined by the Folin-Ciocâlteu spectrophotometric method using gallic acid as a reference standard (Singlenton et al., 1999; Meda et al., 2005).

The method was adapted for the Biotek SynergyHT multidetection spectrophotometer, with 96-well plate, the volumes used respecting the stoichiometry of the original method. A volume of 25 µl of methanolic pollen extract was mixed for 5min with 125 µl of 0.2N Folin-Ciocâlteau reagent, then 100 µl of Na_2CO_3 solution (75 g/L) was added. The obtained mixture was incubated for 2 hours at room temperature in the dark. The absorbance was read at 760 nm compared to a control (80% methanol).

Antibacterial activity

The antibacterial activity of methanolic bee pollen extract was tested on 10 bacterial strains grouped as follows: 5 gram-positive bacterial strains: *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Bacillus laterosporus* 6932, *Paenibacillus larvae* 9820 and *Paenibacillus alvei* 13253, 4 strains of gram-negative bacteria *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* ATCC 13076, *Salmonella typhi* ATCC 14028 and a yeast *Candida albicans* ATCC 90028. The strains used in this study came from the Universities' strain collection of the Microbiology laboratory.

Serial dilution method (MIC- minimum inhibitory concentration)

The minimum inhibitory concentration (MIC) of methanolic bee pollen extracts was achieved in 96-well ELISA-type microplates.

100µl of sterile Muller-Hinton nutrient broth was introduced into each well. From bee pollen extracts of 15% concentration successive dilutions were made in the range 1/2-1/32. 100 ml of these dilutions were introduced into each well over the Muller-Hinton medium. This was followed by the addition of 10 µl of bacterial suspension with a density of 0.5 on the McFarland scale. The antibiotic Streptomycin was used as positive control (100 µl) and as

negative control the solvent used to extract the active ingredients from pollen (MeOH 70%). Afterwards, the plates were thermostated with the lid closed at 37ºC for 24 hours.

Statistical analysis

Three different replicates of each BCP samples were assayed. Statistical differences between samples were estimated using ANOVA (one-way analysis of variance; Tukey's multiple-comparison test; GraphPad Prism version 4.0, Graph Pad Software Inc., San Diego, CA, USA). A probability value of $p<0.05$ was considered to be statistically significant. We also used principal components analysis (PCA) to verify the relationships between the analysed variables and BCP samples using XLSTAT software (Addinsoft, New York, NY) and PAST software package (version 2.17, Oslo).

RESULTS AND DISCUSSIONS

Palynological analysis

The palynological analysis of the bee pollen samples used in this study was previously determined (Margaoan et al., 2014).

According to the palynological analysis, all pollen samples proved to be polyfloral, with six plant families found to be predominant. In summary, the Rosaceae family was predominant in P1-4, P6 and P11, Fabaceae in P5, P10 and P16, wehreas Asteraceae was predominant in P7 and P8. Sample P13 had Brassicaceae (*Brassica* sp.) as dominant plant family, whereas P14 had Ericaceae (*Calluna vulgaris*) and P15 Salicaceae (*Salix* sp.).

Total phenolic content

The total polyphenols in the studied samples have an average concentration of 5.92 ± 0.12 mg GAE/g sample and vary between a minimum of 2.46 ± 0.04 mg GAE/g (sample P11) and a maximum of 8.87 ± 0.03 mg GAE/g (sample P6) (Figure 1). It should be noted that both samples (P6 and P11) have pollen predominantly from the Rosaceae Family. The major difference is the dominant pollen species: P6 - *Prunus* sp. (83%) and in P11 - *Rosa canina* (48%). Pollen samples P1, P4, P6 and P13 showed a higher content of total polyphenols (>7 mg GAE/g) explained by the presence of *Brassica* sp. pollen. The dominant pollen of Fabaceae family from samples P5, P10 and P16 determined a low content of total polyphenols reaching a maximum of 5.66 mg GAE/g.

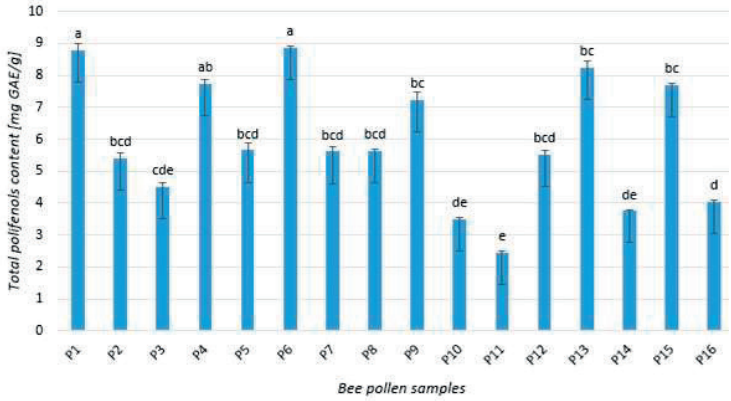

Figure 1. Total polyphenol content of bee pollen samples. Results are given as the mean ± SD (n = 3). Values with different letters (a - e) are significantly different ($p < 0.05$), using ANOVA Tukey's multiple-comparison test. P1−P16, samples of multifloral pollen

Similar results were reported by Stanciu et al. (2012), which showed that the higher TPC was noticed in samples with dominant pollen of *Calluna vulgaris* (4.36 mg GAE/g), *Salix* sp. (8.12 mg GAE/g), *Fillipendula ulmaria* (7.44 mg GAE/g), *Brassica* sp. (8.44 mg GAE/g). Kroyer and Hegeduș (2001) reported the TPC in the range of 7.4-9.7 mg GAE/g sample, whereas Mărghitaș et al. (2009) determined values between 4.4-16.4mg GAE/g sample. Bee pollen samples from SE Brazil studied by Negri et al. (2011) showed a TPC between 1.6

± 0.03 and 2.3 ± 0.03% GAE/g dry pollen, whereas Morais et al. (2011) reported that Portuguese bee pollen samples had a TPC between 10.5-16.8 mg GAE/g extract. Very high values for the TPC published by Freire et al. (2012) with limits between 41.5 ± 0.2 and 213.2 ± 1.1 mg GAE/g, by using etanolic pollen extracts. High values for these compounds were also reported by Menezes et al. (2010), 14.31 ± 0.1 - 132.38 ± 0.7 mg GAE/g sample. Bonvehi et al. (2001) using as extraction solvent of 70% methanol obtained an average of 1.24 ± 0.2 mg GAE/g sample value lower than the TPC determined in the present study. Comparatively, Pascoal et al. (2014), determined high values for this parameter 18.55-32.15 mg GAE/g pollen, using commercial pollen from Portugal and Spain.

The 16 pollen samples analyzed in terms of TPC had values between 2.46 mg GAE/g sample and 8.87 mg GAE/g sample (Figure 1). Comparing the results, it can be seen that the pollen from the Rosaceae family had the highest values P6 (8.87 mg GAE/g sample), P1 (8.80 mg GAE/g sample), P4 (7.74 mg GAE/g sample). These were followed by samples predominant in Brasicaceae and Salicaceae, respectively.

Antibacterial activity

The results of the antibacterial activity of alcoholic bee pollen extract on the Gram-positive bacterium *S. aureus* ATCC 6538P are presented in Figure 2. The diameter of inhibition was between 5.33 mm and 12.33 mm. The negative control used showed no antibacterial activity, and the positive control Streptomycin had an inhibitory diameter of 18 mm. Comparatively, *B. laterosporus* showed a higher susceptibility to the treatment with pollen extracts, the inhibition diamater zone having values between 6.67 and 12.00 mm and the bacterium not being so sensitive to the positive control (18 mm inhibition diameter). The gram-positive bacterium *P. alvei* was the most sensitive to the action of pollen extracts, the diameter of the inhibition zones having values between 8.67 and 21.33 mm. Furthermore, this bacterium proved to be resistant to the positive control (Streptomycin) The Gram-positive bacteria *P. larvae* 9820, as well as the 4 strains of Gram-negative bacteria: *E. coli* ATCC 10536, *P. aeruginosa* ATCC 27853, *S. enteritidis* ATCC 13076, *S. typhi* ATCC 14028 and the yeast *C. albicans* ATCC 90028, proved to be resistant to all methanolic bee pollen extracts. The antibiotic used in the experiment, Streptomycin, was active for the bacteria *E. coli, P. aeruginosa, S. enteridis* and *S. typhi* (data not shown).

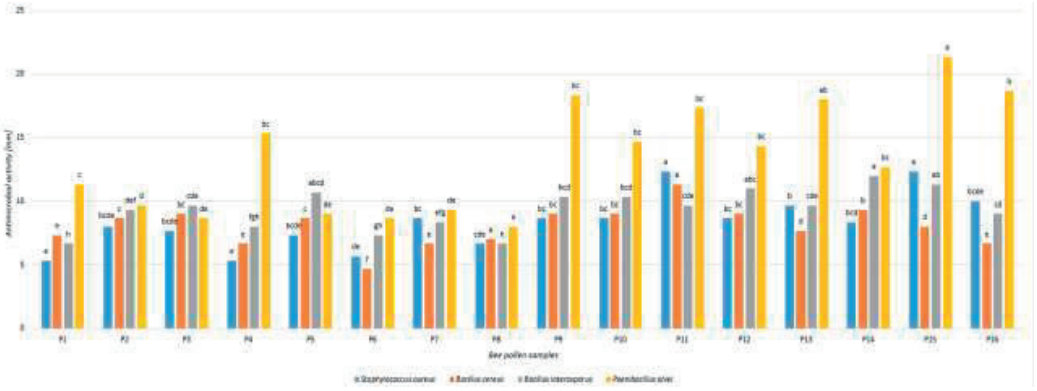

Figure 2. Antimicrobial activity of bee collected pollen samples. Results are given as the mean ± SD (n = 3). Values with different letters (a - h) are significantly different ($p < 0.05$), using ANOVA Tukey's multiple-comparison test. P1 - P16, samples of multifloral pollen

The diameter of the inhibition zones of the *S. aureus* strain falls within the limits of 5.33 ± 0.58 mm for samples P1 and P4 (both samples belonging to the family Rosaceae: P1 - *Crataegus monogyna*, P4 - *Malus domestica*) and 12.33 ± 0.58 mm determined for samples P11 (Rosaceae - *Rosa canina*) and P15 (Fabaceae - *Salix* sp.). The weakest action on *B.*

cereus strain was sample P6 sample (Rosaceae - *Prunus* sp.) with 4.67 ± 0.58 mm and the most intense action was noticed in sample P11 (Rosaceae - *Rosa canina*), 11.33 ± 0.58mm.

The *B. laterosporus* strain showed a minimum inhibition of 6.67 ± 0.58 mm determined by extracts from samples P1 (*Crataegus monogyna*) and P8 (*Taraxacum officinale*) and the maximum inhibition was determined by sample P14 (*Calluna vulgaris*) followed by P15 (*Salix* sp.).

Regarding *P. alvei* strain, the greatest action was noticed for sample P8 (*Taraxacum officinale*) and P15 (*Salix* sp.) with and an inhibition zone of 8.00 ± 0.00 mm and 21.33 ± 0.58 mm, respectively.

Carpes et al. (2007) used ethanolic extract from two pollen samples from Parana and Alagoas, with alcohol concentrations between 40-90%. The antibacterial activity differs both in terms of the concentration of the solvent and the sample used. Parana pollen showed no activity on *B. cereus* and *S. aureus* strains, but showed a 2.5 mm inhibition zone on the *P. aeruginosa* strain. Comparatively, the pollen from Alagoas showed an activity of 3 mm on *Stafilococcus aureus*, 0.5 mm on *B. cereus* and 1.00 mm on *P. aeruginosa*.

Abouda et al., 2011, studied various pollen samples from Morocco using extracts in DMSO. In their study, 14 pathogenic Gram-positive and Gram-negative bacterial strains showed resistance to 5 different types of antibiotic, but were sensitive to pollen extracts. This could be an interesting approach to control multiple microorganisms in the medical field as possible. As microorganisms, such as gram positive bacteria tend to develop resistance to certain antibiotics, our study showed that BCP proves to be an alternative product in this aspect. Furthermore, Velásquez et al. (2017) showed that there is a strong correlation between the chemical properties of different bee pollens and plant families.

Serial dilution method

The method of successive dilutions was tested on bacterial strains of *S. aureus*, *B. cereus*, *B. laterosporus*, and the obtained results are presented in Table 1. The presence of the microorganism in the well can be observed in samples P1, P4 - P6, P8 starting with the 1/8 dilution for the *S. aureus* strain, which means that starting from this dilution the methanolic extract no longer has an antimicrobial effect.

Regarding the *B. cereus* strain, the lack of antibacterial effect also starts from the 1/8 dilution for samples P1, P4, P6-8 and P16.

The same was noticed for *B. laterosporus* strain, with the exception of methanolic extracts from samples P1, P6 and P8.

Table 1. Minimum inhibitory concentration (MIC) of bee pollen methanolic extract

Sample	Strains tested														
	Staphylococcus aureus					*Bacillus cereus*					*Bacillus laterosporus*				
	Extract concentration tested														
	1/2	**1/4**	**1/8**	**1/16**	**1/32**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**
P1	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
P2	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
P3	-	-	-	+	+	-	-	-	+	+	-	-	-		+
P4	-	-	+	+	+	-	-	+	+	+	-	-	-	+	+
P5	-	-	+	+	+	-	-	-	+	+	-	-	-	-	+
P6	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
P7	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+
P8	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
P9	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
P10	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
P11	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
P12	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
P13	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+
P14	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
P15	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+
P16	-	-	-	-	+	-	-	+	+	+	-	-	-	+	+

+ well in which the tested microorganism was present; - well in which the tested microorganism was absent

Studies conducted by Abouda et al. (2011), Graikou et al. (2011), Pascoal et al. (2014) on antibacterial activity and implicitly the minimum inhibitory concentration of different types of bee pollen (Morocco, Greece, Portugal) in different solvents (methanol, ethanol, DMSO) had different results regarding MICs. This means that an important role in the control of the biological action of pollen extracts is played by both the botanical and geographical origin of the sample and the solvents used in the extractions.

Research has also shown that reference strains are much more sensitive than those isolated from biological fluids (Pascoal et al., 2014).

Statistical analysis

On the basis of the PCA a good discrimination of the BCP samples was achieved (the first two principal components explaining 89% of the data variance). In Figure 3 it can be observed that the botanical origin of pollen samples was correlated with the total phenolic content and antimicrobial activity. Samples P6, P11 and P15 had a very distinctive pattern, whereas samples P2, P3, P5 and P7 showed pattern similarities. The same was noticed for samples P10 and P12, respectively.

The variables close to the central axis (zero value) have similar concentrations in all BCP samples.

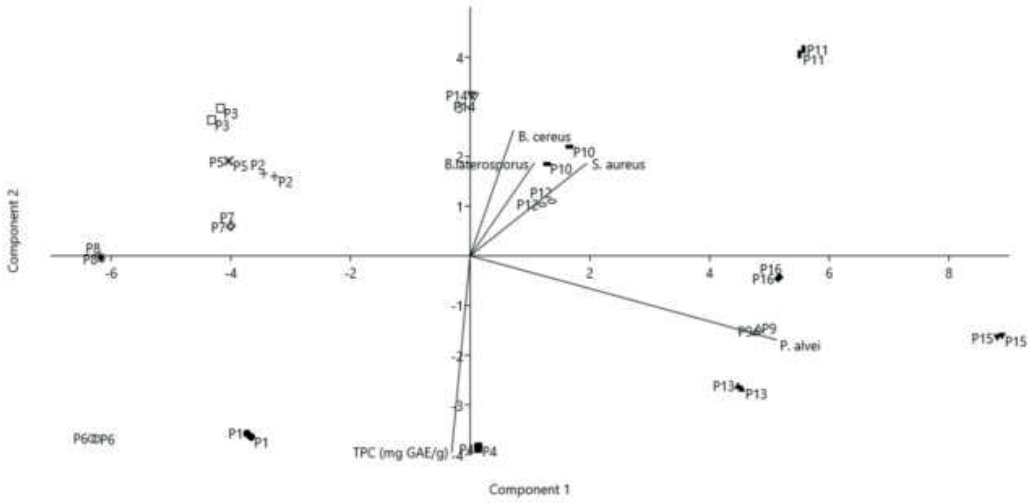

Figure 3. Principal component analysis biplot of the bee collected pollen samples. Two dimensions together explained 89% of the variance data

Thus, the total phenolic content and the antimicrobial activity against *P. alvei* significantly contribute to the discrimination between samples. In Figure 3, from left to right, the first group is formed by samples predominant in Rosaceae (P1, P4, P6 and P9), Brassicaeae (P13) and Fabaceae (P16) displaying significantly higher phenolic content compared to the other samples. In the second quadrant, sample P15 (Salicaceae - *Salix* sp.) possesses the highest antimicrobial activity against *P. alvei*, followed by samples P9, P13 and P16. The third quadrant highlights the antimicrobial activity against *S. aureus*, *B. cereus* and *B. laterosporus*. Samples P11 (Rosaceae - *Rosa canina*) and P15 (Salicaceae - *Salix* sp.) had the strongest antimicrobial activity against *S. aureus* and *B. cereus*, respectively. Sample P14 (Ericaceae - *Calluna vulgaris*) showed the highest inhibition zone against *B. laterosporus*. In the last quadrant, samples P2, P3, P5 and P7 showed pattern similarities, displaying the lowest phenolic content, as well as minimum inhibition zones in *S. aureus*, *B. cereus* and *B. laterosporus.* Comparatively, these samples had a moderate inhibitory activity against *P. alvei*.

CONCLUSIONS

Our study confirms that bee collected pollen is a rich source of phenolics, especially the

samples rich in Rosaceae, Brassicaeae and Salicaceae family, respectively. Furthermore, BCP possesses significant antimicrobial activity against gram positive bacteria, especially *S. aureus*, *B. cereus*, *B. laterosporus* and *P. alvei.* These findings are in accordance with previous reported researches which emphasized the importance of bee collected pollen as a functional food rich in nutrients and bioactive compounds.

ACKNOWLEDGEMENTS

This research work was financed from the project 26.526/07.12.2017.

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