Scientific Papers. Series D. Animal Science. Vol. LXII, No. 2, 2019
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CORRELATIVE RESEARCH REGARDING THE TOTAL POLYPHENOLIC CONTENT, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY OF THREE TYPES OF ROMANIAN HONEY

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Abstract

Knowledge of biologically active potential of honey has made significant progress in the last decade, due to the diversification and improvement of the analysis methods regarding the content in various functional compounds. The aim of this study was to evaluate the total polyphenolic content and the antioxidant and antibacterial potential of some honey samples, establishing the correlations between the values of the examined parameters. The investigations were carried out on 7 samples of honey and their botanical origin was determined through melissopalynogical analysis. The samples belonged to the following types of honey: multifloral honey (no=4), lime tree honey (no=2), rapeseed honey (no=1). The total polyphenolic content was determined by Folin-Ciocâlteu method and the antioxidant activity by DPPH radical scavenging method. The antibacterial activity was tested on 3 bacterial strains: Staphylococcus aureus, Bacillus cereus and Enterococcus faecalis. The results showed a positive correlation between the investigated parameters. For instance, one sample of multifloral honey recorded the highest total polyphenolic content (274.65±1.85mg GAE/100g honey), correlated with the highest levels of antioxidant (12.30±0.43 mmol Trolox/100 g honey) and strong antibacterial activity.

Key words*: multifloral honey, lime tree honey, rapeseed honey, antioxidant activity, antibacterial effect.*

INTRODUCTION

In the last decades, a special attention has been drawn to antioxidants, which have been associated with multiple benefits for human health.
Nowadays, honey is considered to be one of the last remaining natural products, minimally affected by industrial technologies.
The main constituents present in honey are represented by the carbohydrates, comprising approximately 95% of its dry weight basis (Nagai et al., 2006).
In addition to this, honey contains numerous compounds such as polyphenols, enzymes (e.g., glucose oxidase, catalase), ascorbic acid, carotenoid-like substances, organic acids, Maillard reaction products, amino acids and proteins recognised as valuable antioxidants (Estevinho et al., 2008; Da Silva et al., 2016).
However, available literature suggest that the antioxidant activity of honey is mainly provided by the polyphenols, and then by the other constituents (Gheldof and Engeseth, 2002).
The term 'polyphenol' is usually defined chemically as a substance that possesses an aromatic ring bearing one or more hydroxyl substituents including functional derivatives (esters, methyl esters and glycosides). Some phenolic compounds are exceedingly widespread, while others are present exclusively in certain plant families or in particular development stages (Cheynier, 2012). Moreover, evidences confirm that among all major groups of polyphenols, only flavonoids and phenolic acids can be found in honey and they mainly exert their antioxidant activity by neutralizing free radicals, by donating an electron or hydrogen atom (Rice-Evans, 1996).
A plethora of research demonstrated that honey may be used for the treatment of various pathologies, such as colds, skin wounds and several gastrointestinal diseases and this effect can be attributed to both antibacterial and anti-inflammatory properties of honey, regarding high osmolarity, acidity and content of hydrogen peroxide. In this regard, the antibacterial activity of honey is well known

and documented as well (Weston, 2000; Taomina et al., 2001).
The aim of our research was to determine the total polyphenolic content, the antioxidant and the antibacterial activity of some Romanian honey samples. Additionally, a correlation between the above mentioned parameters was carried out.
Furthermore, we aimed to identify the botanical origin of the honey samples, by performing the melissopalynological analysis.

MATERIALS AND METHODS

Seven samples of honey were collected from the Laboratory for the Quality Control of Apiculture Products within the Institute of Life Sciences of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, where they were brought by beekeepers across Romania, in order to be investigated. All the honey samples were stored at room temperature in dark before analysis.
The melissopalynological analysis was conducted in the Cell Analysis Laboratory of the Institute of Advanced Horticultural Research of Transylvania, Cluj-Napoca, by using the method implemented by Louveaux et al. (1978) and Werner von Der Ohe et al., (2004), with microscopic slides. The examination of the microscopic preparations was realized with an optical microscope (Olympus BX 41), using the 40X lens for the identification of the pollen grains. Moreover, the images of the microscopic slides were achieved with an UC30 camera and processed with an Olympus Stream Basic software.
The total polyphenolic content was determined by a spectrophotometric method, called the Folin-Ciocâlteu method, with some modifications (Folin and Ciocâlteu, 1927; Singleton et al., 1999; Kim et al., 2003).Two g of honey were diluted in 70% methanol solution and the resulting mixture was transferred to 20 ml flasks, filled with 70% methanol solution and then, filtered. After 2 hours, 25 µl of the obtained solution, 125 µl of 0.2 N Folin-Ciocâlteu reagent and 100 µl of Na_2CO_3 were pipetted into a 96-well plate. After incubation in dark and at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 760 nm wavelength with a Biotek Synergy HT multidetector spectrophotometer.
The standard curve was produced for gallic acid and the total polyphenolic content was expressed as mg gallic acid equivalents per 100 g of honey sample (mg GAE/100 g honey sample).The results are presented as mean of four determinations ± standard deviation (Microsoft Excel, 2010).
The scavenging activity against 1.1-diphenyl-2-picrylhydrazyl (DPPH) radical of honey was evaluated according to the procedure described by Velazquez et al., (2003) and Molyneaux (2004), with some modifications. The methanolic DPPH solution was prepared extemporaneously at a concentration of 2 mg/100 ml and then, sonicated for 15 minutes. 200 µl of DPPH solution and 40 µl of the honey solution diluted in methanol 70% were added in a 96-well plate and the control test was made with 200 µl of DPPH solution and 40 µl of 70% methanol. The reaction mixture was incubated for 30 minutes at the room temperature in the dark. Absorbance was measured at 517 nm wavelength, by using a multichannel spectrophotometer. The standard curve was produced for 6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid (Trolox). The results are presented as mean of four determinations ± standard deviation (Microsoft Excel, 2010). Antioxidant activity was expressed as a percent of inhibition of DPPH radical and as Trolox miliequivalents/100 g honey sample.
The testing of three reference bacterial strains sensitivity, such as *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 11778 and *Enterococcus faecalis* ATCC 29212 was performed against the seven honey samples. It was determined according to the technique described in the Kirby-Bauer antibiogrammethod, which is currently one of the most frequently requested laboratory method; in order to obtain a conclusive result, the method should be conducted under standard conditions (Schwalbe et al., 2007). The test is based on the property of the antibiotics, respectively the active components contained in the honey samples to diffuse in solid culture media by achieving different concentration gradients, which gradually decrease from the deposition site to the edge of the diffusion zone. For the diffusometric techniques performed in Petri dishes, there is required the

usage of 90 mm diameter Petri dishes, made of glass or plastic, which have perfectly planar, clean and sterilized surfaces. In the case of dishes presenting irregularities of their surfaces, a thin layer of equalizing agar may be poured.

The culture medium used in the present research was Mueller-Hinton agar.

This growth medium must possess constant qualities from one batch to another in other to allow rapid growth of the tested germs, contain no germ-inhibiting substances or antimicrobial substances.

Before sowing the Petri dishes with the culture medium, depending on the number of the samples used, 0.5 mm wells were cut, by using a template.

From the bacterial culture tested for sensitivity to different honey samples, a suspension with a density equal to 0.5 was made by means of an electronic densitometer.1 ml of this suspension was introduced into the Petri dish with Mueller-Hinton agar and the dish was tilted in different directions in order to cover the entire surface of the medium.

The excess suspension was removed, and the Petri dish was held near the gas bulb in order to dry. Then, 20 µl of honey sample was placed in each well, the order of the samples always respecting the clockwise direction.

Micro-tablets of amoxicillin were used as positive control for bactericidal activity. Petri dishes were incubated then, for 24 hours at 37°C.

The following statistics were assessed by using Microsoft Excel, 2010: Mean, Minimum (Min.), Maximum (Max.), Standard Deviation (Std. Dev.), Coefficient of variation %.

Furthermore, Correlation coefficient (r) was calculated by using GraphPad Prism 6.0 Software in order to determine correlations between the investigated parameters (Correlation statistical function). Moreover, all the chemicals and reagents used in the present research were of analytical grade.

RESULTS AND DISCUSSIONS

The identification of the floral species of the pollen grains that are present in the composition of the analyzed honey samples was conducted by means of microscopic slides.

The images corresponding to the honey samples (Figure 1) were interpreted on the basis of photos from recent literature. Therefore, a series of features were monitored, namely the morphology and dimensions of the pollen grains, the structure of the tegument, the shape and number of germinating pores (Palmieri et al., 2017).

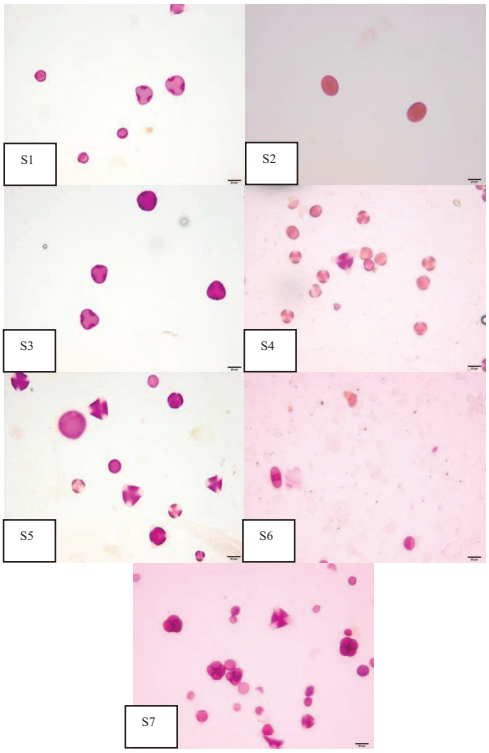

Figure 1. The microscopic image of pollen grains corresponding to honey samples S1, S2, S3, S4, S5, S6, S7 (40X; original photo)

In Table 1 are outlined the botanical families and species of the honey samples and they were classified into four groups, specifically, predominant pollen (>45%), secondary pollen (16-45%), important minor pollen (3-15%) and minor pollen (<3%).

According to the melissopalynological analysis, predominant pollen (> 45%) came from two botanical families, namely: *Brassicaceae (Brassica* ssp.*)* and *Tiliaceae (Tilia* ssp.*)*.

Secondary pollen (16-45%) originated from 7 plant families such as: *Hypericaceae, Fabaceae, Polygonaceae, Rosaceae, Salicaceae, Brassicaceae, Ericaceae* and the

important minor pollen (3-15%) and minor pollen (<3%) belonged to more than 10 plant families.

Table 1. The melissopalynological analysis of the honey samples S1-S7

Sample code (S1-S7)	Predominant pollen (>45%)	Secondary pollen (16-45%)	Important minor pollen (3-15%)	Minor pollen (<3%)
	Family-Species	**Family**-Species	**Family**-Species	**Family**-Species
S1	***Tiliaceae-*** *Tilia* ssp.	***Hypericaceae-*** *Hypericum* ssp.	***Fabaceae-*** *Trifolium* ssp. *Robinia pseudoacacia* ***Rosaceae-*** *Fragaria* ssp.	***Rosaceae-*** *Filipendula ulmaria* ***Fabaceae*** ***Asteraceae*** ***Gramineae***
S2		***Polygonaceae-*** *Fagopyrum esculentum* ***Fabaceae-*** *Trifolium* ssp	***Asteraceae-*** *Achillea* ssp. *Cirsium* ssp. *Ambrosia* ssp ***Boraginaceae-*** *Symphytum* ssp. ***Fabaceae***	***Asteraceae-*** *Taraxacum officinale* *Centaurea* ssp. *Helianthus annuus* ***Rosaceae*** ***Plantaginaceae*** *Plantago* ssp. ***Apiaceae*** ***Boraginaceae-*** *Phacelia tanacetifolia*
S3	***Tiliaceae-*** *Tilia* ssp.		***Fabaceae***	***Rosaceae*** ***Gramineae***
S4	***Brassicaceae-*** *Brassica* ssp.			***Fabaceae-*** *Vicia* ssp. *Trifolium* ssp. ***Salicaceae-*** *Salix* ssp. ***Rosaceae-*** *Prunus* ssp.
S5		***Rosaceae*** ***Salicaceae-*** *Salix* ssp. ***Brassicaceae-*** *Brassica* ssp.	***Fabaceae-*** *Robinia pseudoacacia* ***Asteraceae-*** *Taraxacum officinale*	***Fabaceae-*** *Trifolium* ssp ***Betulaceae-*** *Betula* ssp. ***Fagaceae-*** *Quercus* ssp. ***Lamiaceae***
S6		***Fabaceae-*** *Trifolium* ssp.	***Gramineae-*** *Zea mays* ***Apiaceae*** ***Asteraceae-*** *Cirsium* ssp. *Achillea* ssp. ***Plantaginaceae*** *Plantago ssp.* ***Polygonaceae-*** *Rumex* ssp. ***Gramineae*** ***Rosaceae-*** *Rubus* ssp. ***Lamiaceae*** ***Fabaceae*** ***Fagaceae-*** *Castanea sativa*	***Asteraceae-*** *Centaurea* ssp. ***Fabaceae-*** *Vicia* ssp. *Robinia pseudoacacia*
S7		***Ericaceae***	***Salicaceae-*** *Salix* ssp. ***Asteraceae-*** *Taraxacum officinale* *Centaurea* ssp. ***Onagraceae-*** *Epilobium* ssp. ***Rosaceae*** ***Fabaceae***	***Caryophyllaceae-*** *Silene* ssp. ***Fagaceae-*** *Fagus* ssp. ***Rhamnaceae-*** *Rhamnus* ssp. ***Rosaceae-*** *Rubus* ssp. ***Asteraceae*** ***Tiliaceae-*** *Tilia* ssp. ***Gramineae*** ***Apiaceae***

The synthesis of the data presented above (Table 1) revealed that two samples were classified as lime tree honeys (S1 and S3), one sample as rapeseed honey (S4) and four samples proved to be multifloral honeys, having different types and percentages of pollen (S2, S5, S6 and S7).

The Folin-Ciocâlteu method was used in order to evaluate the total polyphenolic content and the following regression equation of the gallic acid calibration curve was used: $y = 5.3634x + 0.0812$, $R^2 = 0.9991$.

The amounts of polyphenols in the honey samples ranged between 19.49±0.78 mg GAE/100g honey and 274.65±1.85 mg GAE/100g honey (Table 2).

Table 2. The total polyphenolic content of the honey samples S1-S7

Honey sample	Total polyphenolic content (mg GAE/100g honey)
S1	23.50±1.32
S2	274.65±1.85
S3	21.40±1.32
S4	19.49±0.78
S5	20.01±0.78
S6	49.93±3.87
S7	75.01±1.40

The highest content of total polyphenols was identified in honey sample S2 (multifloral honey), while S4 (rapeseed honey) emphasized the smallest amounts of total polyphenols. Large amounts of total polyphenols were also recorded in honey samples S6 (multifloral honey) and S7 (multifloral honey), while honey samples S1 (lime tree honey), S3 (lime tree honey) and S5 (multifloral honey) revealed decreased levels of total polyphenols.

Honey samples belonging to the same assortment highlighted very varied values regarding the total polyphenolic content. For instance, honey sample S2 (multifloral honey) recorded the highest value (274.65 ± 1.85 mg GAE/100 g honey), while S5, also a multifloral honey presented low amounts of total polyphenols (20.01±0.78 mg GAE/100 g honey).

The properties and composition of honey depend on several factors, such as the floral source, climatic conditions, processing, storing and handling technologies (Kaskoniene and Venskutonis, 2010; Khalil et al., 2011).

The free radical scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was evaluated by a spectrophotometric method.

Moreover, for determining the antioxidant activity, the regression equation of the calibration curve % Inhibition/Trolox concentration was used: $y = 743.88x - 13.306$, $R^2 = 0.9988$.

Thereby, antiradical activity was expressed as an Inhibition percent and Milliequivalents Trolox/100 g honey sample (Table 3).

Table 3. The antioxidant activity of honey samples (S1-S7) by DPPH method

Honey sample	Inhibition %	Mmols Trolox/100 g honey sample
S1	11.96±5.28	3.40±0.71
S2	78.19±3.19	12.30±0.43
S3	15.6±4.01	3.89±0.54
S4	8.33±1.93	2.91±0.26
S5	8.83±4.84	2.98±0.65
S6	20.59±1.62	4.56±0.22
S7	27.86±5.99	5.53±0.81

The highest antioxidant activity, expressed in both ways was recorded by a multifloral honey sample (S2), while S4, a rapeseed honey sample presented the lowest antioxidant activity. An increased antioxidant activity was also revealed by honey samples S6 and S7, both of them being multifloral honeys.

In the present study we showed that the honey samples that recorded a strong antioxidant activity also revealed an increased content of total polyphenols.

These results were in agreement with the findings of other authors. Ferreira et al. (2009) and Kaškonienė et al. (2009) have also demonstrated that polyphenol-rich honey samples have higher antioxidant activity. Therefore, it can be stated that there is a strong relationship between these two parameters (Hołderna-Kędzia and Kędzia, 2006).

The hierarchy of the honey samples was almost identical for all the seven samples from the point of view of the total polyphenolic content and antioxidant activity (Table 4).

Table 4. Total polyphenolic content and radical scavenging activity (antioxidant activity) of the analysed honey samples

Investigated Parameters		Honey Samples						
		S1	S2	S3	S4	S5	S6	S7
Total Polyphenolic Content (mg GAE/100 g honey sample)	Min-Max range	22.34-25.13	272.0-276.3	19.54-22.34	18.42-20.29	19.17-20.84	44.71-54.03	73.80-76.78
	Mean	23.50	274.6	21.40	19.5	20.01	49.93	75.01
	St. Dev.	1.32	1.85	1.32	0.78	0.78	3.87	1.40
	Coefficient of variation %	5.61	0.68	6.17	4.02	3.9	7.75	1.86
Inhibition %	Min-Max range	7.04-19.30	74.62-81.92	11.89-21.30	5.62-10.18	4.19-15.03	19.59-23.01	21.87-36.16
	Mean	11.96	78.19	15.6	8.33	8.83	20.59	27.86
	St. Dev.	5.28	3.19	4.01	1.93	4.84	1.62	5.99
	Coefficient of variation %	44.14	4.08	25.72	23.13	54.88	7.88	21.51
Mmols Trolox/100 g honey sample	Min-Max range	2.74-4.38	11.82-12.80	3.39-4.65	2.54-3.16	2.35-3.81	4.42-4.88	4.73-6.65
	Mean	3.40	12.30	3.89	2.91	2.98	4.56	5.53
	St. Dev.	0.71	0.43	0.54	0.26	0.65	0.22	0.81
	Coefficient of variation %	20.8	3.49	13.84	9.02	21.94	4.77	14.57

For instance, the most valuable honey sample was represented by multifloral honey sample (S2), which reported the highest polyphenolic content and antioxidant potential.

Honey sample S4 (rapeseed honey), on the other hand, emphasized the lowest values regarding the investigated parameters.

The correlation between the total polyphenolic content and the antioxidant activity (Inhibition % and Milliequivalents Trolox) was performed by statistical analysis, which underlined that there was a strong positive correlation between the analyzed parameters (r=0.9828702 for Total Polyphenolic Content/Inhibition %; r=0.9828702 for Total Polyphenolic Content/Trolox; r=0.9999996 for Inhibition %/Trolox; $p<0.05$) (Table 5).

Table 5. Correlation between total polyphenolic content and radical scavenging activity of the analysed honey samples (correlation coefficients (r) value)

Parameters	Total Polyphenolic Content	Inhibition %	Trolox
Total Polyphenolic Content	-	0.9828702	0.9829022
Inhibition%	0.9828702	-	0.9999996
Trolox	0.9829022	0.9999996	-

Regarding the bactericidal activity of the honey samples S1-S7, the interpretation of the results was made on the basis of the diameters of the lysis zones, expressed in mm (Table 6).

Table 6. The bactericidal activity of honey samples (S1-S7)

Samples	Bacterial strains- diameters of lysis zones, expressed in mm		
	Staphylococcus aureus	*Bacillus cereus*	*Enterococcus faecalis*
S1	13.04	11.90	0
S2	20.98	12.85	14.37
S3	10.51	9.70	9.09
S4	0	7.43	7.65 PI
S5	0	0	0
S6	12.53	9.67	6.34
S7	15.64	10.46	12.36 PI
Positive control (Amoxicillin)	26.49 RC	R	18.05

RC= resistant colonies; R= resistant strain; PI= partial inhibition.

The most intense bactericidal activity on the *Staphylococcus aureus* strain (Figure 2) was observed in honey sample S2 (multifloral), the

diameter of the lysis zone approaching that of the positive control (amoxicillin).

It should be noted that the secondary pollen (16-45%) of the honey sample S2 belongs to the species *Fagopyrum esculentum* (Watanabe et al., 1997) and the genus *Trifolium* (Jerković et al., 2016), recognized in the literature for their strong antibacterial and antioxidant effects.

In the multifloral honey sample S5, the bactericidal activity was absent, while in honey samplesS6and S7 (both multifloral), the bactericidal activity was poor towards intermediate. Figure 2 also highlighted the presence of a synergic effect between lime honey and multifloral honey.

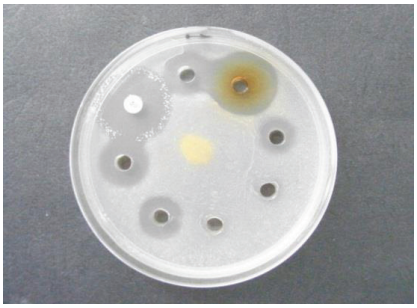

Figure 2. The bactericidal activity of honey samples (S1-S7) on the *Staphylococcus aureus* strain

The bactericidal activity against the strain of *Bacillus cereus* was absent in the honey sample S5 and decreased in the others, the lysis zone ranging from 9.67 mm (S6) to 12.85 mm (S2).

However, the examined honey samples (excepting honey sample S5) showed better bactericidal activity than the positive control (Figure 3).

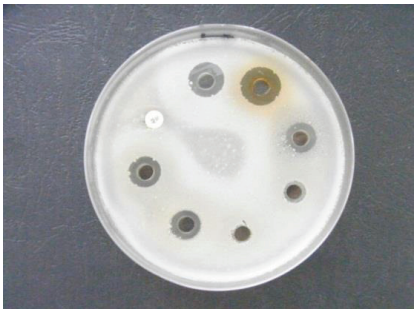

Figure 3. The bactericidal activity of honey samples (S1-S7) on *Bacillus cereus* strain

Only two of the fourmultifloral honey samples presented an increased bactericidal activity on the *Enterococcus faecalis* strain, namely honey samples S2 and S7 (Figure 4).

The two lime tree honey samples and the rapeseed honey sample indicated a low bactericidal activity on the three international reference strains used in the present research.

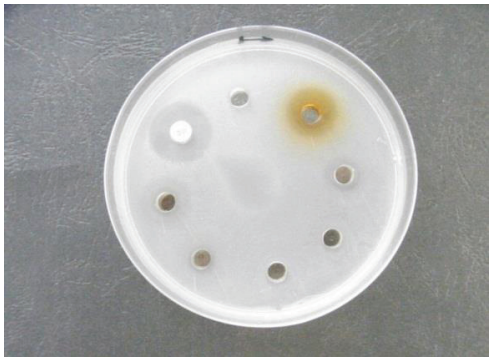

Figure 4. The bactericidal activity of honey samples (S1-S7) on *Enterococcus faecalis* strain

CONCLUSIONS

The melissopalynological analysis allowed to highlight the botanical origin of the honey samples, with the predominant plant species and the secondary species, many of the samples not being in conformity with the beekeeper's statement. According to the results obtained in the melissopalynological analysis, three of the seven honey samples were found to be monofloral and four were multifloral honeys, with different types and percentages of pollen. From the category of multifloral honey,two samples were lime tree honeys and one sample was a rapeseed honey.

We have shown a close positive correlation between the total polyphenolic content, antioxidant and antibacterial activity. In addition to this, the multifloral honey sample S2 recorded the highest content of total polyphenols, the strongest antioxidant activity and presented an extremely effective bactericidal activity against all three strains tested. Moreover, the honey samples S4 (rapeseed honey) and S5 (multifloral honey) have obtained the lowest values regarding the investigated parameters.

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