Chemical composition of new varietal honeys and they antimicrobial activities

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Article

Keywords: honey, antybacterial, antyfungal, phenolic acids, lysozyme, hydrogen peroxide

Posted Date: August 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1851838/v1

Abstract

Nowadays, there is an urgent need to look for antimicrobial substances due to a widespread occurrence of multidrug resistant bacterial and fungal strains. Honey with its antimicrobial properties is a very promising substance with many valuable properties.

In this study we examined for the first time antimicrobial properties of novel varietal honeys. The experiments were carried out with 11 honey samples, i.e.: Plum, Rapeseed, Lime, Phacelia, Honeydew, Sunflower, Willow, and Multifloral-P (*Prunus spinosa* L.), Multifloral-AP (*Acer negundo* L., *Prunus spinosa* L.), Multifloral-Sa (*Salix* sp.), Multifloral-Br (*Brassica napus* L.). Their antimicrobial activity was tested against bacteria (such as *Escherichia coli*, *Bacillus circulans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*), yeasts (such as *Saccharomyces cerevisiae* and *Candida albicans*) and mold fungi (such as *Aspergillus niger*). In tested honeys, phenolic acids were one of the most important group of compounds with antimicrobial activity. In our study, the highest amount of phenolic acids was found in honeydew honey (808.05 µg GAE/g) with the highest antifungal activity aiming *A. niger*. Among the tested phenolic acids, caffeic acid was in the greatest amount, which was found in the highest amounts in honeys as: phacelia – 356.72 µg/g, multifloral (MSa) and multifloral (MBr) – 318.9 µg/g. The highest bactericidal activity against *S. aures* was found in multifloral honeys MSa and MBr. Additionally, the highest amounts of syringic acid and cinnamic acid were identified in rapeseed honey. Multifloral honey (MAP) showed the highest bactericidal activity against *E. coli*, and multifloral honey (MSa) against *S. aureus*. Additionally, multifloral honey (MBr) was effective against both bacteria: *E. coli* and *S. aureus*.

Novel varietal honeys tested in our study show various levels of antibacterial and antifungal activity and alhough they had some limitations, they can still enhance drug treatment against multidrug resistant bacterial and fungal strains.

1. Introduction

Honey is produced by the honeybee *Apis mellifera* L. from flower nectar and honeydew. It is a valuable raw material in economic, therapeutic, ecological and cosmetic stocks. The first mentions of the healing properties of honey date back to the ancient times. At that time it was used, among others, to accelerate wound healing and to treat bowel diseases. In addition, honey is used in neurological diseases, has antidepressant and anticonvulsant properties [1].

Recently, due to the growing resistance of microorganisms to many antibiotics, attention has been paid to agents of natural origin with antimicrobial effects. Honey can be included among such substances [2]. The main ingredients of honey are simple sugars: fructose and glucose (about 70–80%). In addition, honey contains other sugars, e.g. maltose, sucrose and polysaccharides such as dextrins, the amount of which in honeydew honey exceeds 10%. Other ingredients of honey are proteins (about 3%), primarily enzymes that come from the honey bees body, as well as plant enzymes and proteins found in nectar and pollen. These include: invertase, lactase, α- and β-amylase, glucose oxidase, catalase and phosphatase. In addition, honeys contain water (usually aproxiatelly 18%) and minerals, mainly potassium, small amounts of vitamins (C, H, PP, and group B) and organic acids such as: gluconic, malic, citric, butyric, formic, lactic, succinic, pyroglutamic and others. The chemical composition of honey depends on the species of plants foraged by bees to produce it [1, 3].

The antibacterial and antifungal properties of honey depend on its chemical, physical and biological factors. Physical factors include: low pH of honey and high osmotic pressure, which are the result of a high concentration of sugars, which in turn affects the elimination and inhibition of the growth of most microorganisms. The presence of phenolic compounds (phenolic acids and flavonoids) and hydrogen peroxide produced in the enzymatic reaction are classified as chemical factors. Hydrogen peroxide is formed as a by-product in the oxidation of glucose to gluconolactone catalyzed by glucose oxidase in the presence of atmospheric oxygen. It showed strong activity against Gram-positive bacteria, e.g. *Staphylococcus aureus*, Gram-negative bacteria such as *Pseudomonas aeruginosa* and mold fungi (*Aspergillus niger*) [4, 5]. Examples of phenolic acids found in honey include, among others, caffeic, gallic, syryngic and *p*-coumaric acids. The presence of flavonoids, i.e. apigenin, kaempferol, quercetin or luteolin, has been detected in honeys originating in Poland [6]. The biological factors that affect the development of microorganisms in honey include lysozyme and peptide defensin-1 [7, 8, 9]. Lysozyme is a protein with enzymatic activity, whose molecular weight is about 14.4 kDa. Its main function is the lysis of cell walls of Gram-positive bacteria due to the content of *N*-acetylmuramic acid. This enzyme is less active against Gram-negative bacteria [10]. Defensin-1 peptide with a molecular weight of about 5 kDa is secreted by the hypopharyngeal glands of honey bees. It is characterized by the activity against Gram-positive (*Bacillus subtilis*, *S. aureus*) and Gram-negative bacteria (*Escherichia coli*, *Burkholderia cepacia*) [7, 11, 12]

Many authors report that varietal honeys, e.g. Manuka honey, have antimicrobial activity against antibiotic-resistant bacteria, including *P. aeruginosa*, while others, such as Agastache honey, have been shown to be more effective in inhibiting biofilm formation by methicillin-resistant *S. aureus* [13, 14]. In addition to its antimicrobial activity, honey can also provide protection against oxidative stress and skin cancer, endometrial cancer and bladder cancer development [1, 15, 16, 17].

Due to the broad spectrum of honey activity, this research was aimed at examing new varietal honeys and they antifungal and antibacterial properties.

2. Results

2.1. Mellisopalynogical analysis

Table 1 presents the pollen profile of the honeys used in the experiments. On the basis of mellisopalynogical analysis, 7 varietal honeys and 4 multifloral honeys were distinguished. Among the varietal honeys, the following were distinguished:

- Plum honey (P) – dominant pollen – *Prunus* type (46.98%);
- Willow honey (Sa) – dominant pollen – *Salix* sp. (70.25%);
- Rapeseed honey (Br) – dominant pollen – Brassicaceae type (81.70)%;

- Lime honey (Tc) – dominant pollen – *Tilia* sp. (28.99%);
- Phacelia honey (Ph) – dominant pollen – *Phacelia thanacetifolia* (65.62%);
- Honeydew honey (So) – dominant pollen – *Solidago* type (46.48%);
- Sunflower honey (He) – dominant pollen – *Helianthus* type (73.35%).

In the case of multifloral honeys, they were characterized:

- Multifloral-Br (MBr) – with a predominance of pollen from *Brassicaceae* type (33.01%), *Aesculus hippocastanum (*15.53%) and *Poligonum bistorta* (15.53%);
- Multifloral-Sa (MSa) - with a predominance of pollen from *Salix* sp. (21.55%) *Solidago* type (17.24%) and *Tilia* sp. (17.24%);
- Multifloral-AP (MAP) - with a predominance of pollen from *Acer* sp. (37.38%) and *Prunus* type (37.38%);
- Multifloral-P (MP) - with a predominance of pollen from *Prunus* type (29.47%), *Brassicaceae* type (15.79%) and *Salix* sp.(15.26%).

Table 1
Mellisopalynological analysis of different types of honey.

Nectar producing plant taxons	HONEY TYPE															
	P		MAP		MP		MSa		Sa		MBr		Br		Tc	
	Pollen grains															
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Acer **sp.**	1	0.47	200	37.38	6	3.16			1	0.21						
Achillea millefolium															1	0.21
Aesculus hippocastanum	28	13.02	35	6.54			12	10.34	1	0.21	16	15.53			55	11.39
Anthriscus **sp.**											1	0.97			5	1.04
Arctium **sp.**																
Aster **type**											4	3.88				
Brassicaceae **type**	13	6.05			30	15.79					34	33.01	260	81.76	41	8.49
Centaurea cyanus			25	4.67												
Cirsium **sp.**															2	0.41
Convolvulus arvensis	1	0.47														
Echium vulgare											1	0.97				
Fagopyrum esculentum	3	1.40														
Frangula alnus									2	0.41	1	0.97				
Helianthus **type**	26	12.09									7	6.80			6	1.24
Impateins s**p.**																
Lilium **sp.**					5	2.63	1	0.86								
Lotus corniculatus									3	0.62						
Mallus **type**	7	3.26			15	7.89			1	0.21					8	1.66
Melilotus **sp**.																
Phacelia thanacetifolia					5	2.63	18	15.52			2	1.94			3	0.62
Poligonum bistorta			5	0.93	4	2.11					16	15.53			130	26.92
Prunus **type**	101	46.98	200	37.38	56	29.47			130	26.86			20	6.29	23	4.76
Robinia pseudoacacia											1	0.97			7	1.45
Rubus **sp.**					15	7.89	10	8.62			1	0.97	20	6.29	25	5.18
Salix **sp.**	31	14.42			29	15.26	25	21.55	340	70.25			18	5.66	2	0.41
Sedum **sp.**															2	0.41
Solidago **type**	1	0.47					20	17.24							23	4.76
Trifolim repens	2	0.93														
Trifolium pratense							10	8.62	1	0.21	4	3.88			10	2.07
Taraxacum officinale	1	0.47	20	3.74												
Tilia **sp.**			50	9.35	20	10.53	20	17.24	4	0.83	15	14.56			140	28.99
Viola **type**									1	0.21						

Nectar producing plant taxons	HONEY TYPE																
	P		MAP		MP		MSa		Sa		MBr		Br		Tc		
	Pollen grains																
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	
Others																	
SUM/AMOUNT	215	95.98	535	95.54	190	59.38	116	43.61	484	98.57	103	89.74	318	93.53	483	76.86	381
Not nectarative plant taxons																	
Artemisia sp.	2	22.22					60	40			4	28.57			110	74.32	
Bellis perennis	1	11.11			5	2.63											
Betula pendula	1	11.11													1	0.68	
Chenopodiaceae type	2	22.22									2	14.29			2	1.35	
Filipendula sp.			20	80%	130	100	90	60					20	90.91			
Fragaria sp.															1	0.68	
Pinus sp.			5	20					2	28.57			2	9.09			
Plantago sp.											4	28.57			17	11.49	
Poaceae type	2	22.22							5	71.43	4	28.57			12	8.11	
Rumex sp.															5	3.38	
Quercus sp.	1	11.11															
Verbascum sp.																	
SUM/AMOUNT	9	4.02	25	4.46	130	40.63	150	56.39	7	1.43	14	10.26	22	6.47	148	23.14	

2.2. Physico-chemical properties of honey

Table 2 shows the physico-chemical properties of 11 tested honeys (average ± standard deviation). The water content in the tested honey samples was within the range of 14.67 ± 0.47–18.00%.

Another analyzed parameter was the electrical conductivity, which makes it possible to distinguish between nectar and honeydew honeys. The electrical conductivity was within the range of 0.25 (sunflower He) – 0.91 $mS \cdot cm^{-1}$ (honeydew So). The obtained results were within the values applicable for nectar honeys, i.e. 0.2 ÷ 0.8 $mS \cdot cm^{-1}$, and for honeydew, i.e. above 0.8 $mS \cdot cm^{-1}$.

The phenolic compounds present in honey come from honeydew or pollen. It was observed that the content of phenolic compounds in dark honeys, e.g. honeydew (So) 808.05 ± 7.20 µg GAEs/g, is higher than the content of these compounds in light honeys, e.g. multifloral (MBr) 404.74 ± 9.12 µg GAEs/g and sunflower (He) 431.27 ± 5.45 µg GAEs/g. Rapeseed honey (Br) 378.27 ± 7.3 µg GAEs/g was characterized by the lowest content of phenolic compounds.

Among the tested honeys, the following honeys had the highest protein content above 100 mg/ml: multifloral-AP (116.80 ± 0.57 mg/ml), plum P (112.40 ± 2.47 mg/ml), willow Sa (107.60 ± 0.57 mg/ml) and multifloral-P (103.60 ± 0.57 mg/ml). The honeys: sunflower He (41.20 ± 2.47 mg/ml) and rapeseed Br (49.20 ± 3.40 mg/ml) had the lowest protein content below 50 mg/ml.

Table 2
Physico-chemical characteristics of tested honey types.

	Physico-chemical parameters of honey types (average ± SD, N = 3)				
Honey type	Water content (%)	pH	Electrical conductivity (mS/cm)	Total phenol (µg GAEs/g)	Proteins (mg/ml)
P	16.33 ± 0.47	4.43 ± 0.005	0.29 ± 0.008	670.20 ± 18.96	112.40 ± 2.47
MAP	18.00 ± 0.00	4.62 ± 0.012	0.35 ± 0.009	776.54 ± 9.12	116.80 ± 0.57
MP	14.67 ± 0.47	4.79 ± 0.009	0.29 ± 0.005	700.47 ± 18.18	103.60 ± 0.57
MSa	15.67 ± 0.47	4.68 ± 0.017	0.46 ± 0.014	606.54 ± 27.66	96.80 ± 2.27
Sa	15.00 ± 0.00	4.96 ± 0.029	0.29 ± 0.005	667.14 ± 4.79	107.60 ± 0.57
MBr	18.00 ± 0.00	4.67 ± 0.014	0.36 ± 0.012	404.74 ± 9.12	95.20 ± 2.27
Br	17.67 ± 0.47	4.22 ± 0.012	0.27 ± 0.005	378.27 ± 7.3	49.20 ± 3.40
Tc	18.00 ± 0.00	4.08 ± 0.017	0.42 ± 0.012	624.40 ± 15.43	85.60 ± 3.00
Ph	17.67 ± 0.47	4.62 ± 0.005	0.4 ± 0.005	524.40 ± 18.58	90.80 ± 0.57
So	16.33 ± 0.47	4.85 ± 0.009	0.91 ± 0.008	808.05 ± 7.20	85.20 ± 0.00
He	15.67 ± 0.47	4.35 ± 0.012	0.25 ± 0.005	431.27 ± 5.45	41.20 ± 2.47

The Pfund scale includes 7 classes of honey colours. Among the tested honeys, the following colours were distinguished: extra light amber (18.18%), light amber (45.45%) and amber (36.36%) (Table 3).

Table 3
Colour of honey.

Honey type	Sample result (Absorbance) (average ± SD, N = 3)	Color
P	0,83 ± 0,01	Light Amber
MAP	2,94 ± 0,01	Amber
MP	1,36 ± 0	Light Amber
MSa	1,05 ± 0,01	Light Amber
Sa	0,42 ± 0,01	Extra Light Amber
MBr	0,74 ± 0,01	Light Amber
Br	1,76 ± 0,01	Amber
Tc	0,97 ± 0,01	Light Amber
Ph	1,85 ± 1,12	Amber
So	2,23 ± 0,01	Amber
He	0,36 ± 0,01	Extra Light Amber

The Principal Component Analysis (PCA) of the physico-chemical properties of tested honeys synthetically showed their differentiation and dependence on honey types. The eigenvalues of the first two axes were 2.47 and 1.13. The first axis explained over 49% and second axis over 22% of the variability of the analyzed data/physico-chemical properties of studied honeys, and all four axes over 98%. This proves the major role of the axis 1 and 2 in ordering the variables and determining the factor responsible for the distribution of the honey types in ordination diagram (Fig. 1). All the variables analysed, except for proteins content for axis 2, were statistically significant at a level of $p < 0.05$. The ordination diagram showed two main trends of the variation in the physico-chemical properties of the tested honey (Fig. 1). The first one was related to the first axis and positively correlated with all variables tested, except the water content. The strongest correlation with this axis was shown by total phenolics, pH and proteins content. This axis determined the gradient of the content of the analysed properties in the honey types. Group I of the study honeys (right side of ordination diagram) represents an increasing content of total phenolics, pH, and proteins content, starting from the honeydew (So), multifloral (MAP), multifloral (MP), plum (P) and willow (Sa) honey. Group II (left side of PCA diagram) was negatively correlated with the first axis and characterized by high water content and lower content of the phenolics, proteins and pH values. This group of honeys includes rapeseed (Br), sunflower (He), lime (Tc), multifloral (MBr) and phacelia (Ph). The second axis of the PCA ordination diagram was strongly and positively correlated with electrical conductivity and water content, and second axis determined the gradient of replaced variables in the studied honeys (Group III). The water content increased from the multifloral (MP) (14.67%) and willow (Sa), multifloral (MSa) and sunflower (He) (located under the 2nd axis and

negatively correlated with it) to multifloral (MAP), multifloral (MBr), and lime (Tc), positively correlated with the discussed axis, where the highest content of water was recorded. The highest conductivity honey are: honeydew (So), multifloral (MSa), lime (Tc) and phacelia (Ph) (Fig. 1).

The main components of honey are sugars. Simple sugars, i.e. glucose and fructose, were identified in the highest amount in all the tested honey samples (Table 4).

Table 4
Major sugar components in tested honey samples as determined by HPLC.

Sugars content (g/100 g), average ± SD, N = 3							Fructose/Glucose (Ratio)
Sugar type Honey type	Glucose	Fructose	Sucrose	Rhamnose	Erlose	Fucose	
P	38.80 ± 0.42	39.28 ± 0.12	3.26 ± 0.07	1.18 ± 0.04	3.02 ± 0.10	0.00 ± 0.00	1.01
MAP	44.77 ± 0.1	43.57 ± 0.28	2.42 ± 0.08	1.79 ± 0.07	0.66 ± 0.02	0.23 ± 0.01	0.97
MP	38.80 ± 0.17	38.71 ± 0.23	2.79 ± 0.08	1.24 ± 0.07	2.00 ± 0.07	0.00 ± 0.00	1.00
MSa	40.75 ± 0.21	42.74 ± 0.39	3.08 ± 0.09	1.58 ± 0.04	1.74 ± 0.10	0.00 ± 0.00	1.05
Sa	37.51 ± 0.18	39.24 ± 0.15	3.46 ± 0.07	1.71 ± 0.03	2.92 ± 0.10	0.00 ± 0.00	1.05
MBr	39.56 ± 0.17	42.1 ± 0.58	2.45 ± 0.07	1.01 ± 0.08	1.57 ± 0.06	0.00 ± 0.00	1.06
Br	39.05 ± 0.35	38.53 ± 0.47	4.96 ± 0.08	1.46 ± 0.05	8.26 ± 0.31	0.00 ± 0.00	0.99
Tc	45.10 ± 0.27	38.23 ± 0.28	1.31 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.85
Ph	52.53 ± 0.46	37.17 ± 0.34	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.71
So	39.51 ± 0.35	40.36 ± 0.47	2.89 ± 0.02	1.20 ± 0.05	1.77 ± 0.13	0.00 ± 0.00	1.02
He	37.27 ± 0.29	42.61 ± 0.2	2.91 ± 0.08	1.55 ± 0.02	0.69 ± 0.02	0.35 ± 0.02	1.14

The PCA ordination analysis shows relationships between the honey type and diversity of sugars and their content (Fig. 2). All analysed sugar types were statistically significant at a level of $p < 0.05$ for two first axis of ordination PCA diagram, except glucose for axis 2. The first axis explaining ca. 53% (eigenvalue 3.18), and second axis ca. 32% (eigenvalue 1.9) of the data variability. All four axes explain over 97% of data variability.

Axis 1 is positively correlated with all variables tested, except glucose content. The first axis determined the falling share of glucose in the honey types form right side of PCA diagram for sunflower (He), willow (Sa), multifloral (MP) and plum (P) to multifloral (MAP) in the middle, and lime (Tc), phacelia (Ph) in left side. In relation to the content of other sugars, axis 1 is positively correlated with them. The strongest correlation with sucrose and rhamnose is observed and discussed axis shows rising gradient of this sugars from rapeseed (Br), willow (Sa) and multifloral (MSa) to lime (Tc) and phacelia (Ph).

Axis 2 of the diagram is strongly, positively correlated with erlose content and strong negatively correlated with fructose and fucose content. The erlose content decrease form rapeseed Br (8.26 g/100 g), plum P (3.02 g/100 g) and willow Sa (2.92 g/100 g) by honeydew So, multifloral MSa, multifloral MBr and multifloral MAP honey where erlose content ranges from 1.77 g /100 g to 0.66 g /100 g appropriately to lime (Tc) and phacelia (Ph) honey in which no erlose was found. The high fucose content in sunflower (He) and multifloral (MAP) honey is positively correlated with fructose.

The ratio of fructose to glucose was typical for honey. The more glucose a honey has, the faster it tends to crystallize. In honey, the ratio of fructose to glucose ideally should range from 0.9 to 1.35. A fructose to glucose ratio below 1.0 leads to faster honey crystallization whereas crystallization become slower when this ratio is more than 1.0 [18, 19, 20]. In the present study, the average ratio of fructose to glucose was around 1. However, two tested honeys (Tc and Ph) had ratio well below 1.0 (0.85 and 0.71, respectively) which indicates the greater chances for honey crystallization (Table 4).

2.3. Antimicrobial activity of honey

The antimicrobial activity of the honey samples expressed by inhibition of the growth of the tested bacteria around the wells on the agar medium was varied (Fig. 3). The Gram-positive bacteria *B. circulans* proved to be the most sensitive to the activity of the honeys. The inhibition zones of bacterial growth were observed in all concentrations (62.5–500 mg/ml) in 7 honey samples: plum (P), rapeseed (Br), lime (Tc) and multifloral (MBr, MAP, MP, MSa). In the case of 3 honeys: willow (Sa), phacelia (Ph) and sunflower (He) no activity against *B. circulans* waqs found at concentrations of 125 and 62.5 mg/ml. On the other hand, honeydew honey (So) didn`t inhibit bacterial growth only in the concentration of 62.5 mg/ml. Taking into account the antibacterial activity observed after the use of the lowest concentration of honeys (62.5 mg/ml), it should be stated that the most effective *B. circulans* were honeys: multifloral (MSa), plum (P) and rapeseed (Br) (zones of growth inhibition 15.22, 14.17, 13.55 mm respectively).

The analysis of the results of the significance of differences test showed that the factors influencing the antibacterial activity of honeys against *B. circulans* are the type of honey and its concentration. The highest activity, expressed by the size of the inhibition zone, was observed for rapeseed honey (Br), this result is significantly different from plum (P) and multifloral (MAP) honey, which show similar activity, and willow (Sa), phacelia (Ph) and sunflower (He) (Figs. 4A, B). At a lower concentration (Fig. 4C) rapeseed (Br), lime (Tc), multifloral (MSa), plum (P) and multifloral (MBr) are less efficient, but retain their antibacterial

properties, which significantly differs from willow honeys (Sa), phacelia (Ph) and sunflower (He) which show no activity. At the lowest concentration, rapeseed (Br), multifloral (MSa), plum (P) and smaller multifloral (MBr) honeys show high activity, which significantly differs from the others, which have lost their properties (Fig. 4D).

For all tested honey types, inhibition of bacterial growth in all tested microorganisms at the highest concentration of 500 mg/ml was visible (Figs. 4A, 5).

It should be noted that the tested honey varieties showed significantly lower activity against other Gram-positive bacteria used in the experiments, i.e. *S. aureus*. In this case, the zones of inhibition of bacterial growth were observed only after the application of 50% honey concentrations. The growth of *S. aureus* was most strongly inhibited by the honeys: multifloral MBr (8.42 mm) and multifloral MSa (9.97 mm) (Fig. 5).

Similarly, only at a concentration of 500 mg/ml, the tested honeys inhibited the growth of Gram-negative bacteria *E. coli*. The largest zones of growth inhibition (9.6–11.9 mm), and thus the highest activity, was recorded for the following honeys: multifloral MBR and MAP. The exception was sunflower honey (He), which showed no activity against this bacteria (Fig. 5).

At a lower concentration (250, 125, 62.5 mg/ml), all tested honeys did not cause a decrease in *E. coli* and *Staphylococcus aureus* growth. A broader effect was evident when testing was done with honey at lower concentrations in relation to *B. circulans* and *A. niger* (Figs. 4B, C, D; Figs. 9B, C, D). In addition, based on the results obtained by the diffusion method, it was found that *P. aeruginosa* bacteria, both standard and clinical strain, was the microorganism completely insensitive to the tested honey varieties was.

The analyzed honeys show inhibitory activity against *E. coli* and *S. aureus* only in the highest concentration (Figs. 6, 7). Honeys: multifloral MAP, MP, MBr have the highest activity against *E. coli* and statistically significantly differ in this respect from honeys: multifloral (MSa) and plum (P) (Fig. 6). However, the activity of honeys: multifloral (MSa) and (MBr) against *S. aureus* is statistically significantly different from the properties of multifloral honeys: MAP and MP and lime (Tc) (Fig. 7).

2.4. Antifungal activity of honey

The antifungal activity of the honey samples used at concentrations ranging from 62.5 to 500 mg/ml was tested against *A. niger, C. albicans* and *S. cerevisiae* using the radial diffusion method. On the basis of the obtained results, it was found that *C. albicans* and *S. cerevisiae* showed resistance to the tested honey samples at all concentrations.

On the other hand, the tested honey varieties effectively inhibited the growth of *A. niger* (Fig. 8). The maximum antifungal activity was found in all honey samples at a concentration of 500 mg/ml in the range from 62 to 99.25 µg/ml based on the activity of amphotericin B (µg/ml). At this concentration, the most active honeys were: multifloral (MSa), plum (P) and honeydew (So), which differs significantly from multifloral (MBr) and phacelia (Ph) honeys (Fig. 9A). At a lower concentration (Fig. 9B) the properties of multifloral (MP), multifloral (MSa) and willow (Sa) honeys are comparable and significantly different from phacelia (Ph). At the next concentration, i.e. 125 mg/ml (Fig. 9C), multifloral (MSa) and honeydew (So) honeys retained antifungal properties, significantly different from rapeseed (Br) and phacelia (Ph). At the lowest concentration (Fig. 9D) willow (Sa) and multifloral (MBr) honeys are the most active, differing from plum (P) and rapeseed (Br), which show the lowest antifungal activity.

2.5. Catalase

All the honey samples with catalase addition had the same or similar growth inhibition zones compared to the control i.e. honey without catalase. The tested honeys remained active against *B. circulans, E. coli* and *S. aureus*, which proves that the activity was related to other factors and that hydrogen peroxide didn`t affect the antimicrobial activity of these honeys (Figs. 10–12).

2.6. Lysozyme activity of honey

In subsequent experiments, lysozyme activity was checked by applying the tested honey samples to plates containing *M. lysodeikticus* according to the procedure of Mohrig and Messner [10]. Lysozyme activity was found in all the tested honeys. The highest lysozyme activity corresponding to the activity of 447.26 ug/ml and 159.74 ug/ml EWL measured in multifloral honeys: MAP and MP. The other varietal honeys have low lysozyme activity. Comparable values were obtained for the following honeys: multifloral (MSa), willow (Sa), multifloral (MBr), sunflower (He) and plum (P), rapeseed (Br), lime (Tc), phacelia (Ph), honeydew (So) which are statistically significantly different from other samples (Fig. 13).

2.7. HPLC analysis of phenolic compounds in honey samples

The obtained results are presented in Table 5. The presence of caffeic and syringic acid in various amounts was found in all tested honeys. Some honeys have identified coumaric acid (in 45% of samples) and cinnamic acid (in 73% of samples). The highest content of caffeic acid was observed in honeys: phacelia (Ph) – 356.72 µg/g, multifloral Sa (MSa) and multifloral Br (MBr) – 318.9 µg/g, and cinnamic acid in willow honey (Sa) – 11.9 µg/g. The content of coumaric and syringic acid in the honey samples didn`t exceed 10 µg/g.

Table 5
Selected phenolic acids content.

Honey type	Phenolic acids (µg/g)			
	Coumaric	Caffeic	Cinnamic	Syringic
P	8.2	7.2	0.15	3.96
MAP	–	210.78	–	1.6
MP	–	221.6	0.15	1.99
MSa	–	318.9	0.15	2.6
Sa	5.0	0.36	0.33	1.1
MBr	–	318.9	0.3	2.7
Br	4.4	3.0	1.6	4.7
Tc	–	286.45	0.3	2.6
Ph	–	356.72	–	3.2
So	4.4	10.8	0.15	0.99
He	0.65	0.7	–	2.7

3. Discussion

Nowadays, there is an urgent need to look for antimicrobial substances due to a widespread occurrence of multidrug resistant (MDR) bacterial and fungal strains. Especially, a very high percentage of postoperative complications are the nosocomial infections, which are very difficult to treat. Therefore, honey with its antimicrobial properties is a very promising substance with many valuable properties [21]. In the honeys tested in our study, similarly as in earlier publications [22, 23, 24, 25, 26], several substances with antimicrobial properties were identified. However, honey has some limitations and it cannot be used as a drug still it can enhance drug treatment against MDR bacterial and fungal strains.

In honey, phenolic acids are one of the important group of compounds with antimicrobial activity. Phenolic acids and flavonoids were recognized in the 1990s as important antibacterial substances. In studies of various honeys from Burkina Faso, it was found that honeydew honeys had the highest content of phenolic compounds 113.05 ± 1.10-114.75 ± 1.30 mg GAE/100 g [27]. Moreover, the level of phenolic acids depends on the botanical and geographic origin of the honey [25, 28]. In our study, the highest amount of phenolic acids was found in the honeydew honey (808.05 µg GAE/g, Table 2) with the highest antifungal activity aiming *A. niger* (Figs. 8–9). Among the tested phenolic acids, caffeic acid was the most abundant, which was found in the highest amounts in the following honeys: phacelia (Ph) − 356.72 µg/g, multifloral (MSa) and multifloral (MBr) − 318.9 µg/g (Table 2). The highest bactericidal activity against *S. aures* was found in multifloral honeys MSa and MBr. Moreover, multifloral MSa honey at all concentrations showed high antifungal activity (*A. niger*). Additionally, the highest amounts of syringic acid and cinnamic acid were identified in rapeseed honey (Br) (Table 5). In a study by Chong et al. [29], it has been shown that caffeic and syringic acid have antibacterial and antifungal activity. In addition, caffeic acid was bactericidal against *S. aureus* [30]. On the other hand, cinnamic acid shows antifungal properties against *A. niger, C. albicans* and antibacterial, among others against *Mycobacterium tuberculosis, E. coli* [14, 31]. The above-mentioned compounds are connected with the antimicrobial effect of the most effective honeys tested in our study. At the highest concentration (500 mg/ml) multifloral honey (MAP) showed the highest bactericidal activity against *E. coli* (inhibition zone: 11.9 mm), and multifloral honey (MSa) against *S. aureus* (inhibition zone: 9.9 mm). Additionally, multifloral honey (MBr) is effective against both bacteria: *E. coli* (inhibition zone: 9.6 mm) and *S. aureus* (inhibition zone: 8.4 mm) (Figs. 5–7). The antimicrobial is active against bacteria and fungi if the inhibition of zone is greater than 6 mm [32, 33]. The honeys tested in our study also show antifungal activity, e.g. on *A. niger*. However there was no fungicidal activity against *C. albicans* and *S. cerevisiae*. The highest activity against *A. niger* was observed in multiflower (MSa) and honeydew (So) honey (Figs. 8–9). Most likely, due to the high content of phenolic compounds, multiflorous honeys had this high antifungal activity [34].

The activity against various bacteria, including *Bacillus cereus, S. aureus* and *E. coli*, was tested in a 75% and 50% solution of multifloral honey from Turkey [35]. The results indicated that at a higher concentration, multifloral honey showed bactericidal activity against *S. aureus* (inhibition zone: 0–7 mm) and *B. cereus* (inhibition zone: 0–6 mm). No activity was demonstrated in both concentrations against *E. coli* [36]. In multifloral honeys from Spain, the activity against *S. aureus* was checked by the method of agar well diffusion in a 75% honey solution. Osés et al. [35] found that the tested honeys show an inhibitory effect on *S. aureus* in the form of zones of inhibition of bacterial growth 14.05 ± 2.31 mm. An experiment by Alvarez-Suarez et al. [37] tested, the activity against *S. aureus* of multiflower honey from Cuba, produced by 2 different species of bees: *Melipona beecheii* and *Apis mellifera*. The authors found that honey produced by *M. beeicheii* showed about 7 times higher activity against this bacteria than honey produced by *Apis mellifera* [37, 38]. Honey of various concentrations (10, 20, 30 and 100%) from Pakistan showed a different degree activity against *A. niger* and *Penicillum chrysogenum* [34]. Moussa et al. [39] showed no activity against *C. albicans* honey from Algeria. By contrast, Irish et al. [40] found that different honeys inhibit clinical isolates of *C. albicans, C. glabrata* and *C. dubliniensis*. Hence, honey is important in combating fungal infections that arise in immunocompromised patients, which may lead to the development of opportunistic infections [5].

Osmosis is an important physical phenomenon connected with antimicrobial properties of the honey. High sugar content exerts osmotic pressure on bacterial cells which results in water loss in bacterial cells. Dehydrated cells are unable to grow and develop in hypertonic sugar solution [21, 25, 41]. Furthermore, osmotic pressure can affect the ability of bacteria to form biofilms [42]. The presence of sugars in honey can also interfere with bacterial quorum sensing [21].

Wahdan et al. [28] showed that fungi are more tolerant to osmosis compared to bacteria and the sugar solution didn`t inhibit the growth of *C. albicans* [39]. Low water content inhibits yeast fermentation and bacterial growth [26]. The composition of the honeys tested in our study consists mainly of sugars and water, and also in smaller amounts phenolic compounds and proteins. Water content in the tested honeys was within the normal ranges accepted for honeys according to International Honey Commission [43], i.e., from 14.6 to 18.0% (Table 2, Fig. 1). The highest content of glucose was recorded in phacelia honey (Ph) – 53 ± 0.46 g/100g, while fructose in multifloral honey (MAP) – 43.57 ± 0.28 g/100g (Table 4). Moreover, rhamnose sugar was detected in the highest amount in multifloral honey (MAP), which showed the greatest activity against *E. coli.* A characteristic sugar found in this honeys was erlose, which is formed by the action of invertase on sucrose. The presence of erlose in honey was first confirmed by White and Maher in 1953 [44]. Erlose is an intermediate trisaccharide in the metabolism of nectar sugars by honeybees [45]. The highest erlose content (8.26 g/100 g, Table 4) was characterized in rapeseed honey (Br), which showed the highest activity against *B. circulans*. Additionally, this honey had the highest sucrose content (4.96 g/100 g, Table 4) among the tested honeys. Also, in rapeseed honey from various regions of Poland, the presence of sucrose was identified: 0.5–2.4 g/100 g [46]. On the other hand, in rapeseed honey from Germany, no sucrose or erlose was detected [47].

Another important physical factor that affects the antimicrobial activity of honey is pH. Low pH ranging from 4.08 (lime honey - Tc) to 4.96 (willow honey - Sa) was observed in our study (Table 2). The low pH in honey is due to the presence of organic acids in honey, which include gluconic acid with antimicrobial activity formed by the oxidation reaction of glucose by glucose oxidase [22, 48].

In our study, we showed that the best honey against *E. coli* bacteria is multifloral honey (MAP), which is also characterized by the highest content of proteins (116.80 mg/ml, Table 2) and lysozyme (447.26 µg/ml EWL, Fig. 13) among all tested honeys. Lysozyme is active against Gram-positive bacteria by acting on peptidoglycan. Gram-negative bacteria, e.g. *E. coli* are not susceptible to the action of lysozyme due to the presence of the outer membrane. Based on the morphological and immunocytochemical studies by Wild et al. [49], it has been illustrated that lysozyme doesn't act on membranes but on *E. coli* cytoplasm leading to its degradation. In order to clarify the action of lysozyme on *E. coli*, Wild et al. [49] additionally used cryotechnics. They found that lysozyme can bind to the outer membrane and penetrate the periplasmic space, possibly reaching the inner cell membrane. Moreover, Wild et al. [49] conducted antimicrobial tests which showed that lysozyme is bactericidal against *E. coli* but doesn't completely break down the bacteria. Two years later, Pellegrini et al. [50] showed that lysozyme inhibits DNA and RNA synthesis. In addition, it has been found that lysozyme causes damage to the outer cell membrane and permeabilization of the inner membrane, which results in the death of *E. coli* bacteria. In contrast, ultrastructural studies showed no effect of lysozyme on bacterial morphology [50]. The mechanism of the bactericidal activity of lysozyme on Gram-negative bacteria requires further research.

In conclusion, novel varietal honeys tested in our study show broad spectrum of antibacterial and antifungal activities. This may suggest that the studied honeys may act as natural products that could reduce the effects of fungal and bacterial infections. Compounds in honeys, such as lysozyme and phenolic acids i.e. coumaric, caffeic, cinnamic and syringic acids played the key role in the health benefit properties of honeys tested in our study.

4. Materials And Methods

4.1. Honey samples collection and classification

The experiments were carried out with 11 honey samples originating in Poland, collected in 2018 and grouped in Table 1. The honeys were classified according to the standard methods recommended by the European Union [51]. Then, the honeys were grouped in terms of the dominant pollen or most common pollen in the honey sample (Table 6). The flowering periods of plants from which the pollens originated, were given after the Biolflor Database (Trait Database of the German Flora: http://www.ufz.de/biolflor) and the possibility to collect given variety of honey [52].

Table 6
Type of honey depending on the dominant pollen or most common pollen.

No.	Honey sample	The acronym of the name of honey in article	The most common plant pollen/pollens in a given honey	The flowering period of the plant	The place of origin of the honey
1	Plum	P	*Prunus spinosa* L.	April-May	Lublin city, Urban Artistic Apiary, 51°14'53"N 22°34'13"E
2	Multifloral-AP	MAP	*Acer negundo* L., *Prunus spinosa* L.	April-May	Lublin city, Urban Artistic Apiary, 51°14'53"N 22°34'13"E
3	Multifloral-P	MP	*Prunus spinosa* L.	April-May	suburban areas, Nowy Gaj 51°15'43"N 22°13'46"E
4	Multifloral-Sa	MSa	*Salix* sp.	April-June	suburban areas, Czerniejów 51°07'16,5"N 22°35'59,5"E
5	Willow	Sa	*Salix* sp.	April-June	ecologically clean areas of Podkarpackie voivodeship 49°47'40"N, 21°56'54"E
6	Multifloral-Br	MBr	*Brassica napus* L.	May-June	Lublin city, Urban Artistic Apiary, 51°14'53"N 22°34'13"E
7	Rapeseed	Br	*Brassica napus* L.	May-June	suburban areas, Nowy Gaj 51°15'43"N 22°13'46"E
8	Lime	Tc	*Tilia cordata* Mill.	June-July	Lublin city, Urban Artistic Apiary, 51°14'53"N 22°34'13"E
9	Phacelia	Ph	*Phacelia tanacetifolia* Benth.	June-October	ecologically clean areas of Podkarpackie voivodeship 49°47'40"N, 21°56'54"E
10	Honeydew	So	*Solidago virgaurea* L.	August-October	ecologically clean areas of Podkarpackie voivodeship 49°47'40"N, 21°56'54"E
11	Sunflower	He	*Helianthus* sp.	August-November	ecologically clean areas of Lubelskie voivodeship (around the Polesie National Park), 51°27'19"N 23°10'24"E

4.2. Honey samples classification using pollen analysis

Ten rams were weighed from each honey sample, 20 ml of distilled water was poured into them and then heated on a water bath until the honey samples completely dissolved. The obtained solution was subjected to centrifugation in an MPW 341 centrifuge with a horizontal rotor at a speed of 3000 rpm (MPW Med. Instruments, Warsaw, Poland). Next, the liquid was decanted, but about 5 ml of suspension was left. The solution was poured into smaller test tubes and centrifuged again, maintaining the previous parameters. The liquid was then decanted again, leaving 2 ml of suspension above the sediment of pollen grains. Fifty microliters of the suspension were taken and applied to microscope slides. Two preparations were made of each honey sample. The microscopic analysis was carried out with the Olympus CX21 microscope (600x) (Olympus, Shinjuku, Tokyo, Japan). An average of 300 pollen grains of nectariferous plants were counted and classified to the lowest possible taxon.

Pollen grains were classified into: dominant pollen ≥ 45%, accompanying pollen between 16–45%, single pollen between 3–16%, and occasional pollen ≤ 3%. If the share of the leading taxons was more than or equal to 45%, such honey was classified as nectar-varietal honey.

4.3. Honey samples preparation

Two grams of each honey sample were weighed in sterile beakers and were dissolved in 2 ml of sterile water. Samples prepared in such a manner were incubated at 37°C for about 3 hours in the incubator, stirring several times until the honey was dissolved completely. Immediately before use, honey samples were twice diluted with sterile water to obtain the following dilutions: 1:2 (500 mg/ml), 1:4 (250 mg/ml) 1:8 (125 mg/ml) 1:16 (62.5 mg/ml) which were used in further analyses.

4.4. Physico-chemical properties of honeys

4.4.1. Water content

The water content of honey was checked with the PAL-22S refractometer (Conbest, Cracov, Poland). Each honey sample was thoroughly mixed and a drop of liquid honey was transferred to the prism of a refractometer according to the manufacturer instruction. Each honey sample was checked in triplicate.

4.4.2. Electrical conductivity

The electrical conductivity in honey was measured with the CC-105 electrical conductivity meter (Elmetron, Zabrze, Poland) at 20^{o}C. Twenty grams of honey were dissolved in 100 ml of distilled water and in such solution the electrical conductivity of honey sample was measured. Each honey sample was checked in triplicate [43].

4.4.3. pH

The pH of honey sample was measured in a 10% honey solution using an analogue pH-meter (HANNA Instruments, Olsztyn, Poland). Each honey sample was checked in triplicate.

4.4.4. Colour intensity

Honey colour was determined using the Pfund scale according to the USDA (United States Department of Agriculture, United States Standards for Grades of Extracted Honey) classification [53]. Pure honey samples were heated at 60^{o}C in a water bath until their complete dissolution. Next, samples were placed in 10 mm cuvettes and the absorbance (λ = 560 nm) was measured, using deionized water as a blank. The absorbance results were multiplied by a 3.15 factor. The obtained results were compared to the values in Table 7 and the honey color was determined.

Table 7
Color designations of honey.

USDA color standard designation	Color range Pfund scale (mm)	Sample result range
Water White	≤ 8	0- 0.094
Extra White	> 8 and ≤ 17	0.0094–0.189
White	> 17 and ≤ 34	0.189–0.378
Extra Light Amber	> 34 and ≤ 50	0.378–0.595
Light Amber	> 50 and ≤ 85	0.595–1.389
Amber	> 85 and ≤ 114	1.389–3.008
Dark Amber	> 114	> 3.008

4.4.5. Total phenolic content

The content of phenolic compounds was determined with a spectrophotometric method using the Folin-Ciocalteu reagent (Sigma-Aldrich, Saint Louis, MO, USA) [54, 55]. Onevgram of honey sample was dissolved in 20 ml of distilled water. Five ml of 0.2N Folin-Ciocalteu reagent was added to 1 ml of honey solution. Then, after a 5 minute incubation, 4 ml of 75% w / v aqueous sodium carbonate solution was added to the solution and incubated for 2 h at room temperature. After this time, the absorbance was measured (λ = 765nm), using a distilled water as a blank. The total phenolic content was calculated on the basis of a standard curve prepared for known concentrations of gallic acid (5-100 µg/ml) (Sigma, EC 3.2.1.17) and was expressed in µg of gallic acid equivalent (GAE) per g of honey.

4.4.6. Sugar analysis in honey samples

Sugar profiles of 11 honey samples were analysed by HPLC using the Shimadzu chromatographic system (Kyoto, Japan) with the RID-10A refractive index detector. The mobile phase (Milli-Q water obtained using the Elix® Essential 3 Water Purification System with Synergy® UV Water Purification System, Merck Millipore, Darmstadt, Germany) was run at a flow rate of 0.6 ml/min at 75°C through the REZEX RPM-Monosaccharide Pb^{2+} column (300 x 7.8 mm, Phenomenex, Torrence, USA). The column was calibrated using sixteen carbohydrate standards. Standard solutions of mono-, di- and trisaccharides: glucose, fructose, galactose, rhamnose, xylose, mannose, sucrose, turanose, maltose, celobiose, fucose, trehalose, melibiose, erlose, melezitose and raffinose (Sigma-Aldrich, Saint Louis, MO, USA) were used for interpretation and quantification of sugars in the honey samples. Sugar concentrations were expressed in g per 100 g honey.

4.4.7. Protein content

The protein content was determined by the Bradford method [56] in 50% (w/v) honey samples solutions. Twenty microliters of such a solution was added to 1 ml of Bradford's reagent (Bio-Rad, Hercules, CA, USA) (Coomassie Brilliant Blue G-250), using deionized water as a control. After 5 minutes of incubation, absorbance was measured at 595 nm using bovine serum albumin in deionized water as a standard (0.1–0.9 mg/1 ml).

4.5. Microorganisms used in the antimicrobial assays

The antimicrobial activity of honey samples was tested against the following bacteria:

1. *Escherichia coli* D31 (CGSC 5165; Genetic Stock Centre, New Haven, CT, USA)
2. *Bacillus circulans* strain ATCC 61;
3. *Staphylococcus aureus*, clinical strain 1-KI, obtained from The Department of Immunobiology, Maria Curie-Sklodowska Uniwersity in Lublin, Poland.
4. *Pseudomonas aeruginosa* strain ATCC 27853;
5. *Pseudomonas aeruginosa* clinical strain 02/18, obtained from The Department of Microbiology and Epidemiology, Millitary Institute of Hygiene and Epidemiology in Warsaw, Poland.

The bacteria were cultured in Luria-Bertani broth (LB; Biocorp, Warszawa, Poland) at 37°C for 24 hours.

The following fungi strains were obtained from the fungal collection of the Department of Immunobiology, UMCS Lublin (Poland):

1. *Aspergillus niger* 71, grown in PDA broth (5% potato extract; 0,5% dextrose; 1,7% agar);
2. *Saccharomyces cerevisiae*, subcultured in sterile Sabouraud broth (1% pepton; 4% glucose; 1,5% agar);
3. *Candida albicans*, grown in YPD broth (1% yeast extract; 2% pepton; 2% glucose; 1,6% agar).

4.6. Antibacterial activity assay

The presence of antibacterial activity in honey samples was detected by the radial diffusion assay on solid agar plates containing appropriate bacterium (150 µl) in the amount of 1.5-4.2x10^6. Each well on the Petri plates was filled with 5 µl of honey samples (1:2 – 1:16), next the agar plates were incubated for 24 hours at 37°C. The diameters of bacteria growth inhibition zones were measured with digital caliper (Pro, Bielsko-Biała, Poland) and expressed in milimeters. The experiment was repeated four times.

4.7. Antifungal activity assay

Antifungal activity was detected by a diffusion well assay against *A. niger* using PDA plates (8 ml) containing about 1.6 x 10^6 spores/ml of the medium. Each well on the Petri plates was filled with 5 µl dilutions of honey (1:2 – 1:16). Agar plates with PDA medium were incubated for 24 hours at 28°C, next the diameters of *A. niger* growth inhibition zones were measured with a digital caliper (Pro, Bielsko-Biała, Poland). The obtained results in millimeters were calculated on equivalent of amphotericin B (µl/ml).

In the case of *C. albicans* and *S. cerevisiae* 24-hour fungi culture was standardized to 0.5 McFarland. After incubation, the reaction mixture was sown in the amount of 100 µl on agar plates (1.6%) with Sabouraud medium (10 ml). The appropriate dilutions of honey (1:2 – 1:16) were added to the wells in the medium. The plates were incubated at 37°C for 24 hours. After incubation the diameters of growth inhibition zones in millimeters were measured with digital caliper (Pro, Bielsko-Biała, Poland).

4.8. Lysozyme activity of honey samples

Lysozyme activity of honey samples was checked using agarose plates containing freeze-dried *Micrococcus lysodeikticus* (Sigma-Aldrich, Saint Louis, MO, USA) [10]. Each well on the Petri plates was filled with 5 µl samples containing appropriate honey dilutions, followed by the incubation of the plates at 28°C for 24 hours. After this time, peptidoglycan digestion zones were measured with digital caliper (Pro, Bielsko-Biała, Poland) and the activity was defined as an equivalent of EWL activity (egg white lysozyme, Sigma-Aldrich, Saint Louis, MO, USA) The level of lysozyme activity was calculated on the basis of standard curve prepared for known concentrations of lysozyme EWL (µg/ml) (Sigma-Aldrich, Saint Louis, MO, USA).

4.9. Antimicrobial activity connected with hydrogen peroxide in honey samples

Agar plates (0.7%) with the LB medium (10 ml) (LB; Biocorp, Warszawa, Poland) containing appropriate bacterium (150 µl) in the amount of 1.5-4.2x10^6 were used to detect the antimicrobial activity connected with hydrogen peroxide in honey samples. Each well on the Petri plates was filled with 5 µl samples containing appropriate honey dilutions as a control and 5 µl samples containing appropriate honey dilutions with catalase (Sigma-Aldrich, Saint Louis, MO, USA), as a test samples. Next, plates were incubated at 37°C for 24 hours and the diameters of bacterial growth inhibition zones were measured with digital caliper (Pro, Bielsko-Biała, Poland).

4.10. Solid phase extraction of honey samples

Honey samples (5 g) were mixed with 20 ml of deionised water adjusted to pH 2 with HCl and stirred in a magnetic stirrer for 15 min. The samples were then filtered to remove the solid particles. Extraction of phenolic compounds was performed with the Visiprep™ SPE Vacuum Manifold (Sigma-Aldrich, Saint Louis, MO, USA). The SPE cartridges used were Strata-X (500 mg) obtained from Phenomenex (Warsaw, Poland). They were conditioned by washing with 15 ml of methanol, and 20 ml acidified water. Afterwards the filtrated honey sample was passed through an cartridge, which was then washed with 20 ml of deionised water to remove all sugars and other polar constituents of honey. The adsorbed compounds were eluted with 5 ml methanol [57].

4.11. HPLC analysis of phenolic compounds in honey samples

The concentration of phenolic compounds was quantified by high performance liquid chromatography (HPLC, Agilent Infinity 1260 equipped with DAD detector) (Agilent Technologies, Santa Clara, USA). The HPLC system fitted with Zorbax Eclipse Plus C18 column (100 mm x 4.6 mm x 3.5µm, Agilent Technologies, Santa Clara, USA) was operated at 40°C and the flow rate of 1 ml/min^{-1}. Each 1 µl sample was injected using an autosampler. The mobile phase consisted of 50 mM formate buffer adjusted to pH 4.1 using 1 M NaOH (eluent A) and methanol (eluent B). The elution included an isocratic step with 20% v/v of eluent B for 1 minute after injection of the sample; afterwards, a gradient step of elution (10 minute) was applied in the range of 20–90% of eluent B. The separation was ended within 3 minutes of isocratic elution with 90% of eluent B. The total run time of each analysis was 14 minutes. After each analysis, a 4 minutes post run was conducted with 20% of eluent B to restore the start conditions of the analysis.

The components peaks were identified by comparison of retention times of the commercial available standards of the following phenolic acids: p-coumaric, caffeic, syringic, vanillic and cinnamic acids. Detection were performed at 280 nm. Agilent OpenLAB CDS ChemStation LC and Ce Drivers (A.02.10 (026) version) software was used for data processing and reporting.

4.12. Statistical analysis

Normal distribution of variables was tested with Shapiro-Wilk tests and, given that all continuous variables were not normally distributed, Kruscal-Wallis H tests (one-way ANOVA on ranks) were performed to compare the mean and standard deviations according to the inhibition abilities of various honey types in four concentrations against selected bacteria and fungi. The results were considered significant at $p < 0.05$. Statistical differences are marked with different letters and their significance at $p \leq 0.001$ – with capital letters, $p \leq 0.05$ – with lower case letters. Statistical analyses were performed using the Statistica 13.2 PL package. To analyze the relationships between species richness inhibition honey activity and physicochemical parameters of honey samples and them sugars content, we used multivariate ordination methods in CANOCO version 5.0 package [58, 59]. According to the length of the gradient from a preliminary Detrended Canonical Analysis (DCA), a linear model, the Principal Component Analysis (PCA) was used. In the PCA, honey samples were entered as cases and physicochemical parameters and sugars content as dependent variables.

Declarations

Data availability

All data generated or analysed during this study are included in this published article. Pollen collections and honey samples are available stationary at the Department of Immunobiology Maria Curie-Skłodowska University in Lublin, Poland.

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Figures

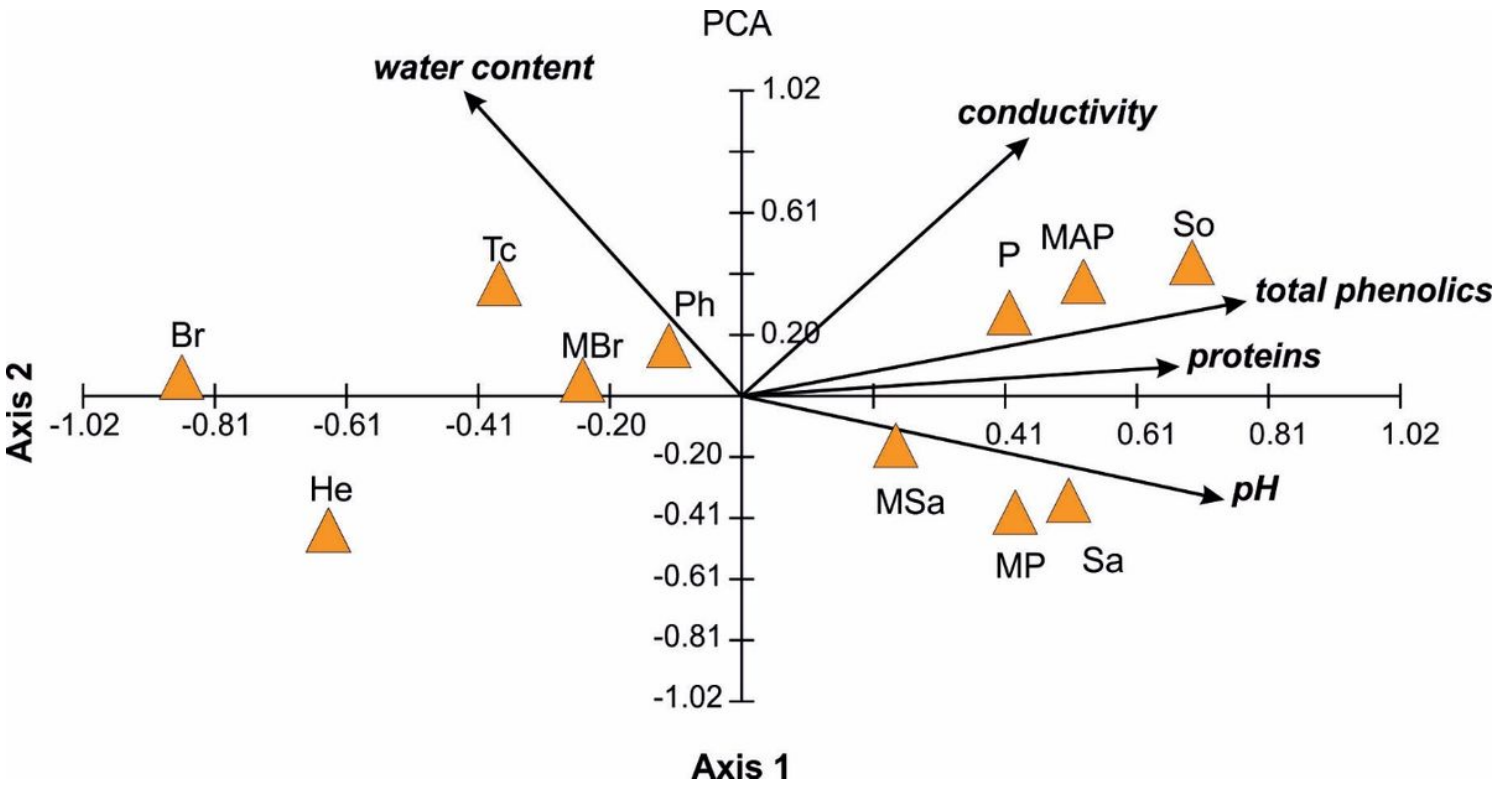

Figure 1

Principal component analysis (PCA) ordination diagram illustrating physico-chemical differences among the 11 study honeys based on 5 variables (solid line vectors). The variables: total phenolics and pH determined the gradient of Axis 1, while the water content of Axis 2.

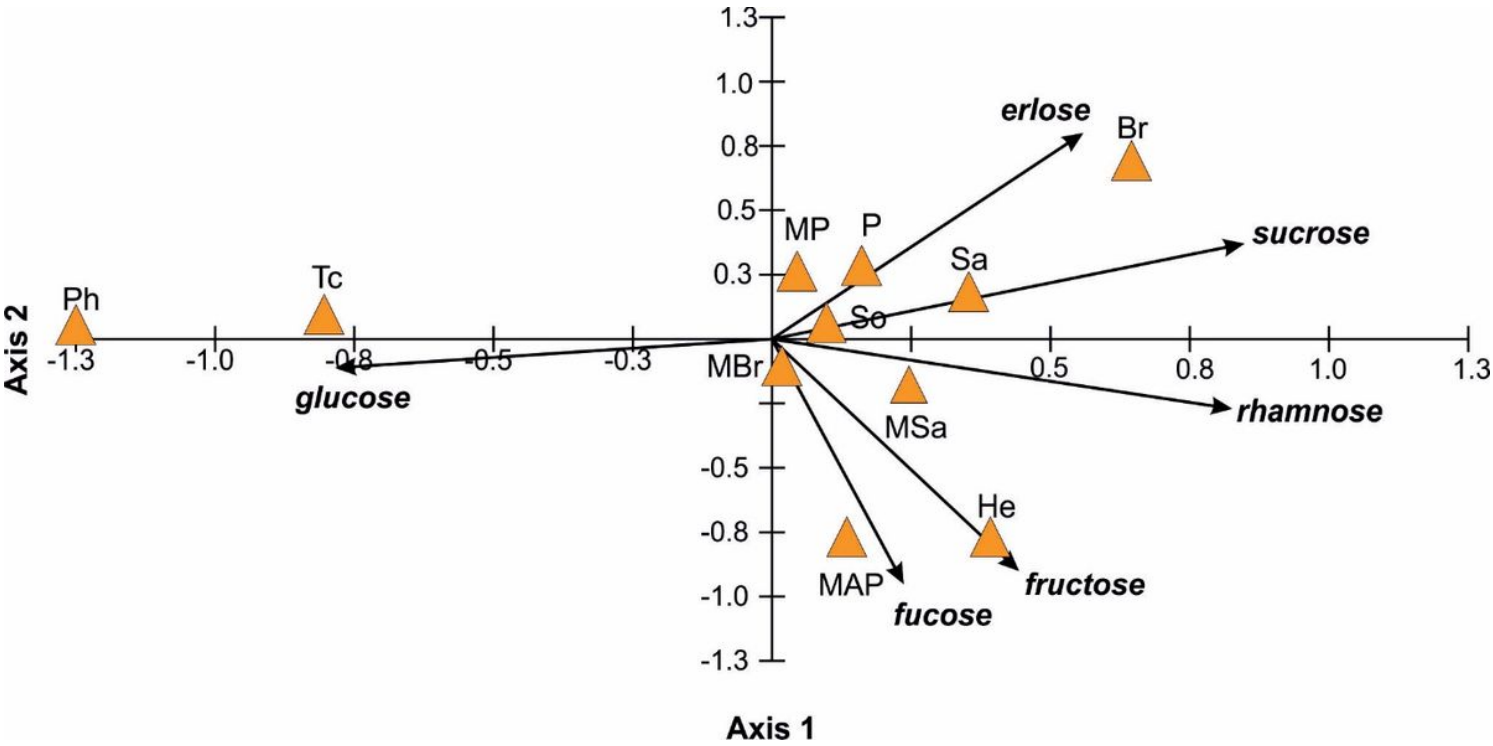

Figure 2

Principal component analysis (PCA) ordination diagram illustrating differences in sugars content among the 11 study honeys (solid line vectors). Content of sucrose determined the positive gradient and glucose content negative gradient of the Axis 1. In the case of the Axis 2 the positive gradient was determined by the erlose content and negative by fructose.

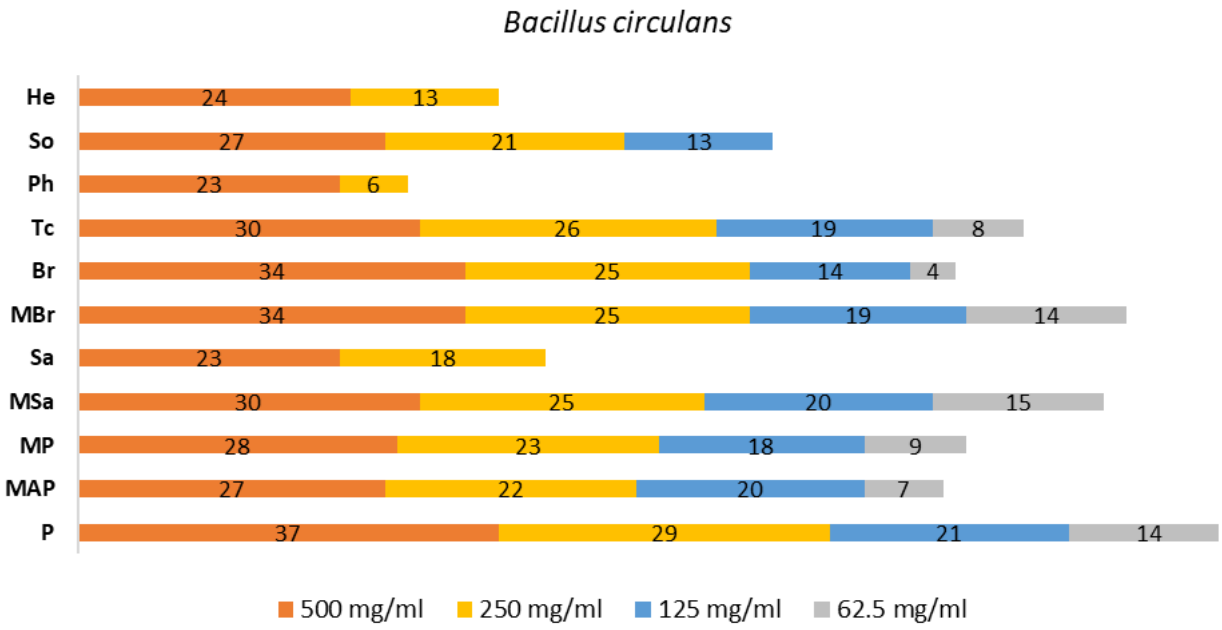

Figure 3

Differentiation of inhibitory activity various concentrations of honeys.

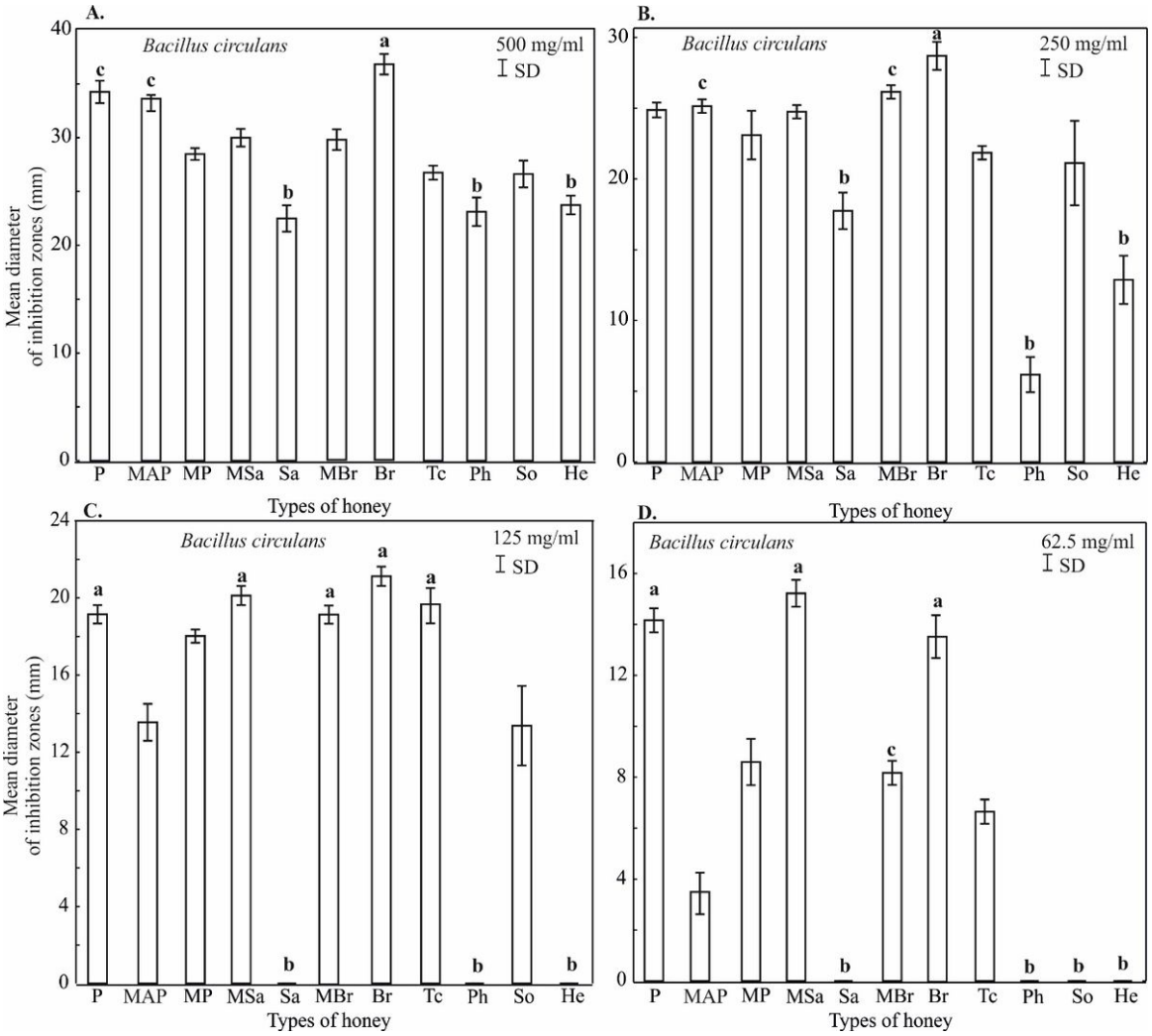

Figure 4

Results of the Kruscal-Wallis H test for inhibition activity of all tested honey types and their concentrations against *B. circulans*. Statistical differences ($p \leq 0.05$) are marked with different letters.

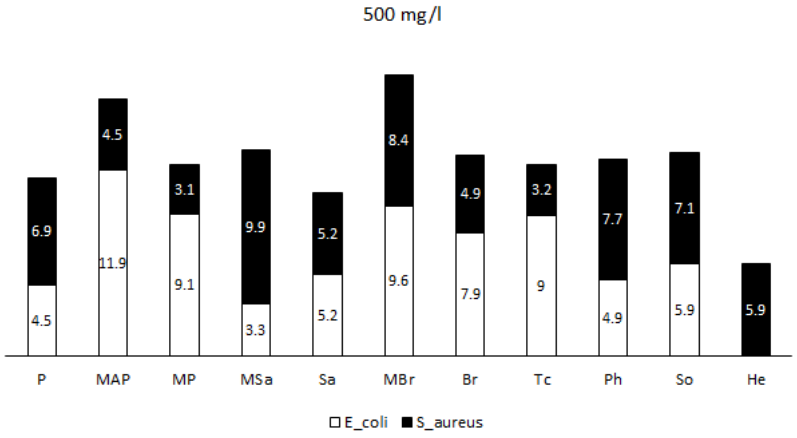

Figure 5

Inhibitory activity of tested honey types relative to *E. coli* and *S. aureus*.

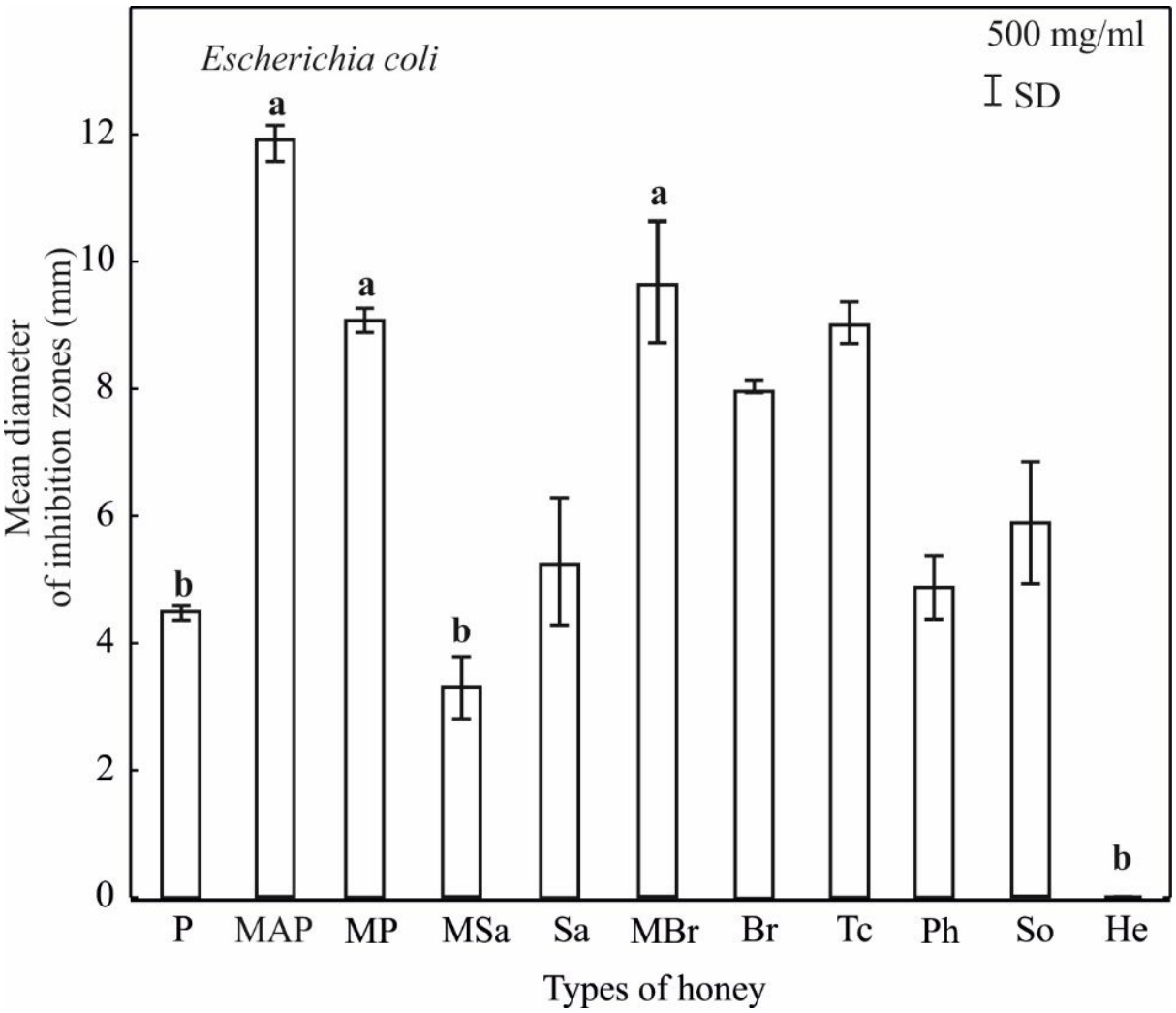

Figure 6

Results of Kruscal-Wallis H test for inhibition activity all tested honey types and their concentrations against *E. coli*. Statistical differences ($p \leq 0.05$) are marked with different letters.

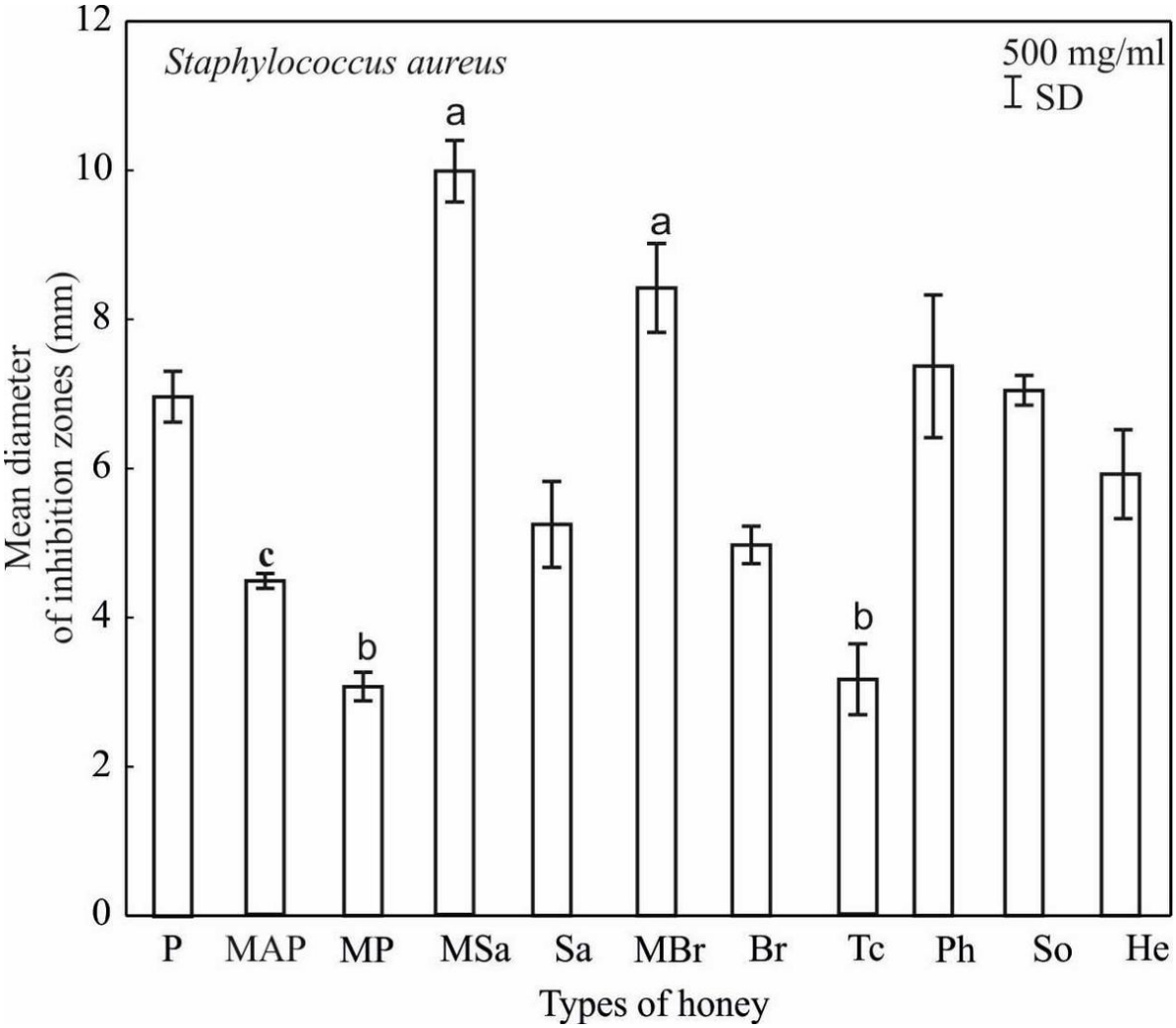

Figure 7

Results of Kruscal-Wallis H test for inhibition activity of all tested honey types and their concentrations against *S. aureus*. Statistical differences ($p \leq 0.05$) are marked with different letters.

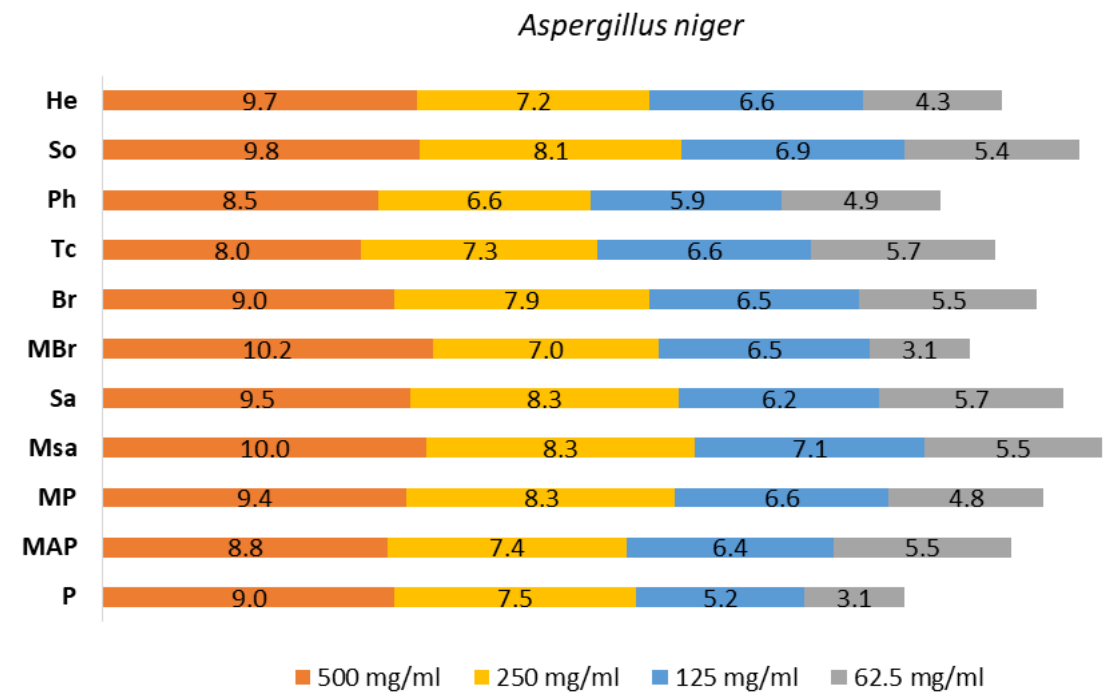

Figure 8

Inhibitory activity of tested honey types relative to *A. niger*.

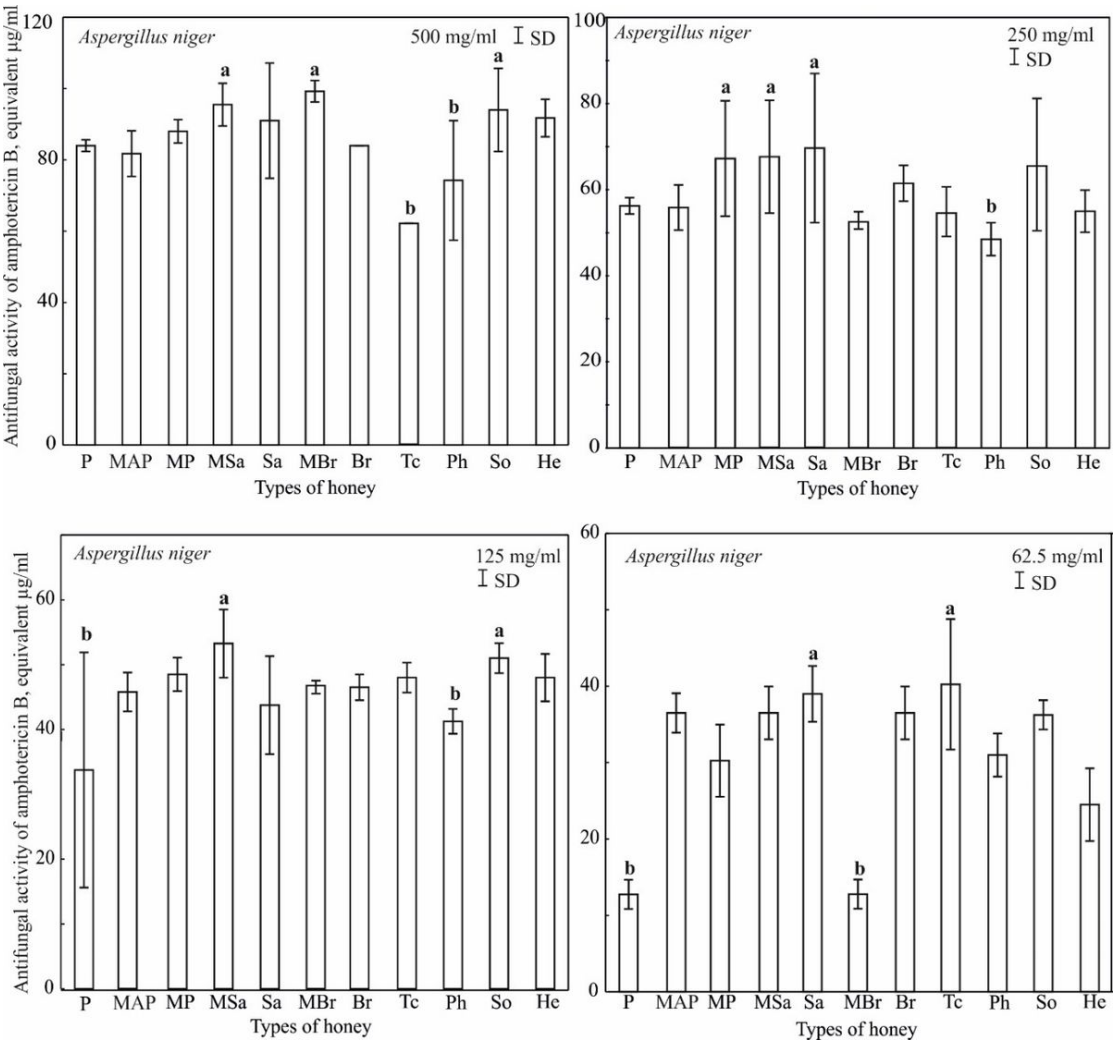

Figure 9

Results of Kruscal-Wallis H test for inhibition activity of all tested honey types and their concentrations against *A. niger*. Statistical differences ($p \leq 0.05$) are marked with different letters.

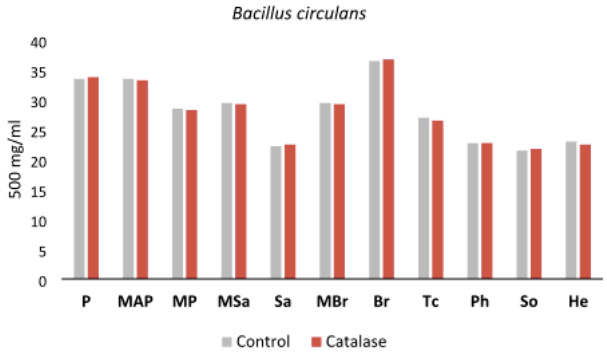

Figure 10

Inhibitory activity of tested honey to *B. circulans*. The control sample only contains honey. The test sample contains honey with catalase at a final concentration of 0.2% (w/v).

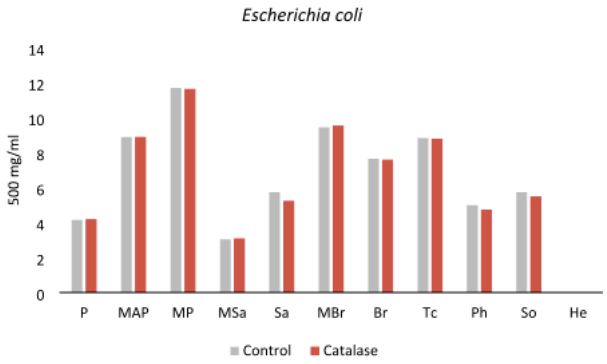

Figure 11

Inhibitory activity of tested honey to *E. coli*. The control sample only contains honey. The test sample contains honey with catalase at a final concentration of 0.2% (w/v).

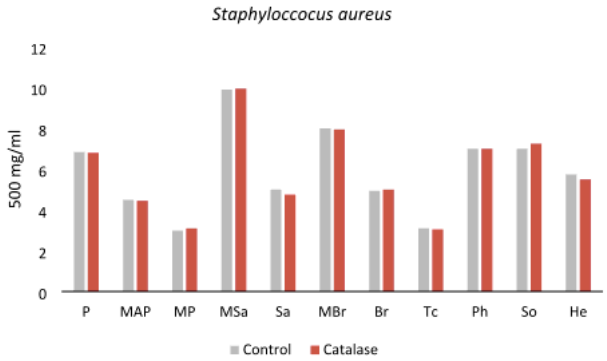

Figure 12

Inhibitory activity of tested honey to *S. aureus*. The control sample only contains honey. The test sample contains honey with catalase at a final concentration of 0.2% (w/v).

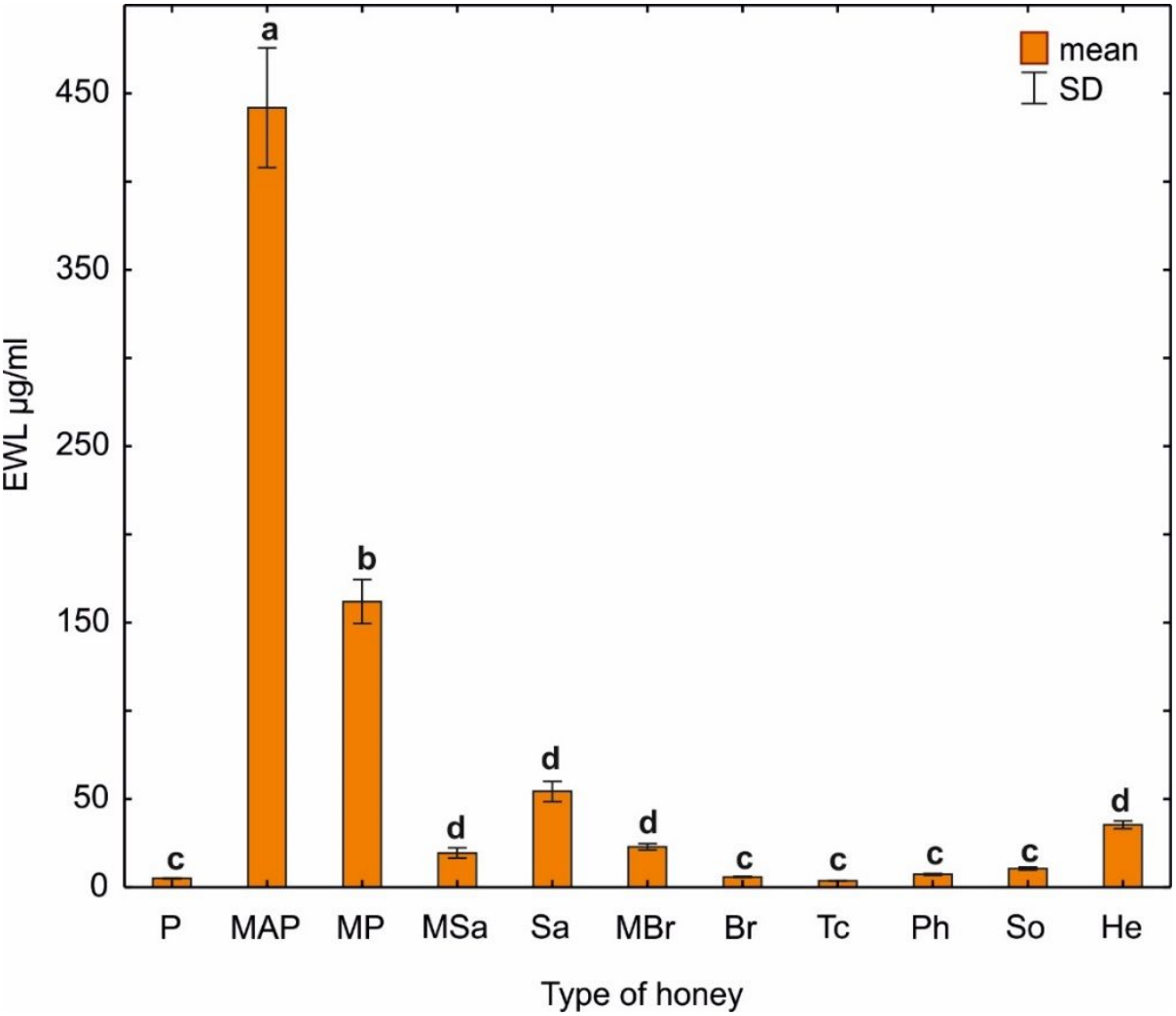

Figure 13

Lysozyme activity was determined by the radial diffusion assay and presented as an equivalent of EWL activity (µg/ml).