ORIGINAL ARTICLE

ACTIVITY OF SELECTED HONEY TYPES AGAINST *STAPHYLOCOCCUS AUREUS* METHICILLIN SUSCEPTIBLE (MSSA) AND METHICILLIN RESISTANT (MRSA) BACTERIA AND ITS CORRELATION WITH HYDROGEN PEROXIDE, PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY

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Abstract

The development of bacterial resistance against antibiotics makes necessary the search of new substances with antimicrobial properties. Nowadays, infections with *S. aureus* strains, especially those caused by methicillin resistant (MRSA) strains, have become a major problem, not only in hospitals, but also in the wider community. In this context, honey is a potential candidate as an antibacterial drug. Antibacterial properties of honey with different botanical origin: rape, lime, multifloral, honeydew and buckwheat, were analysed against *S. aureus* MSSA (methicillin susceptible) and MRSA strains. All tested honey samples were active against MSSA strain, while rape and lime honey did not inhibit growth of MRSA strain. Buckwheat honey possessed the highest antibacterial properties. It was demonstrated that the antibacterial properties were correlated with hydrogen peroxide content, antioxidant properties and phenolics content. It can be stated that the tested honey, especially buckwheat, may have antibacterial potential against *S. aureus*.

Rezumat

Dezvoltarea rezistenței bacteriene la antibiotice face necesară căutarea de noi substanțe cu proprietăți antimicrobiene. În zilele noastre, infecțiile cu tulpini de *S. aureus*, în special cele determinate de tulpini rezistente la meticilină (MRSA), au devenit o problemă majoră, nu numai în spitale, ci și în comunitate, în general. În acest context, mierea este un candidat potențial ca și produs antibacterian. Proprietățile antibacteriene ale mierii de diferite origini botanice: rapiță, tei, multiflorală, mană și hrișcă au fost testate pe tulpini de *S. aureus* sensibile (SAMS) și rezistente (MRS) la meticilină. S-a demonstrat că proprietățile antibacteriene sunt corelate cu nivelul peroxidului de hidrogen, proprietățile antioxidante și conținutul în fenoli al mierii. Rezultatele obținute au relevat că diferitele tipuri de miere, în special mierea de hrișcă, prezintă proprietăți antimicrobiene, putând fi utilizată în infecțiile cu *S. aureus*.

Keywords: honey, antibacterial and antioxidant activity, *Staphylococcus aureus*

Introduction

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics the occurrence of resistance is increasing worldwide. Nowadays, infections with *S. aureus* strains, especially those caused by methicillin resistant (MRSA) strains, have become a major problem, not only in hospitals, but also in the wider community. Previously, MRSA infections were almost exclusively associated with hospital or health-care contact, but new strains of MRSA have emerged, that cause community infection in patients without previous healthcare contact [1, 12, 28]. The so-called community or community associated (CA-MRSA) spread rapidly among healthy individuals. Outbreaks of community MRSA infections are now epidemic in the United States. Additionally, CA-MRSA strains have been reported in Canada, Asia, South America, Australia and throughout Europe, including Norway, the Netherlands, Denmark and Finland, countries with historically low prevalence of MRSA [9]. A fundamental biological property of *S. aureus* is the ability to asymptomatically colonize healthy people. Approximately 30% of human population are asymptomatic nasal carriers of *S. aureus* and they are presumed to be an important source of spread of *S. aureus* strains among individuals [9].

There is now an increasing awareness of these organisms as potentially dangerous pathogens, difficult to eradicate and control because of their resistance

to commonly used antibiotics. The World Health Organization alerted that infectious diseases may become non-curable owing to high levels of multiple drug resistant pathogens [33]. Therefore, alternative antimicrobial approaches are urgently needed, so it leads to a re-evaluation of the therapeutic use of ancient remedies, such as plants [16, 25, 29] and honey [15, 22, 31].

Based on the honey properties, an alternative medicine branch called apitherapy, has been developed in the recent years, offering treatments based on honey and other bee products against many diseases including bacterial infections. Nowadays, different types of honey are sold with standardized levels of antibacterial activity. Manuka honey, has been reported to have an inhibitory effect of around 60 species of bacteria, including aerobes and anaerobes, gram-positive and gram-negative ones [14, 25].

Honey is a remarkable viscous liquid produced by bees from the nectars of various plants. It is considered one of the last untreated natural food substances [14]. It has occupied a proeminent place in traditional medicines throughout world history, since 2100 - 2000 BC [11, 14]. The composition of honey and their therapeutic properties, is influenced by a number of factors such as geographical origin, botanical sources of nectar, environmental and climatic conditions as well as processing techniques [30].

It is known that consumption of antioxidants has been related to the several preventive effects against different diseases such as cancer, coronary diseases, inflammatory disorders, neurological degeneration and aging [18]. Honey contains phenolic acids, flavonoids, certain enzymes (glucose oxidase and catalase), ascorbic acid, carotenoid-like substances, organic acids, amino acids and proteins displaying antioxidant activities [18]. Moreover, honey has antibacterial properties that is significant in the context of the growing resistance of many bacterial strains to the most commonly used antibiotics [6, 22].

The aim of this study was to evaluate the anti-bacterial properties of selected honey types collected in the Southern-Eastern part of Poland against *S. aureus* MSSA and MRSA strains and its correlation with hydrogen peroxide, antioxidants and phenolic content. According to our knowledge there is little data describing the antibacterial properties of honey collected in this part of Poland, especially against *S. aureus* MRSA strain.

Materials and Methods

Sample collection

25 honey samples were collected from apiaries localized in Southern-Eastern part of Poland during 2011. Among them were: rape honey (5 samples), lime (5 samples), buckwheat honey (5 samples), multifloral honey (5 samples) and honeydew honey (5 samples). 25% (m/V) of freshly prepared honey water solutions were used for determination of antioxidant activity, phenolic compounds, ascorbic acid and antibacterial properties. Hydrogen peroxide was determined in 30% (m/v) water solutions of honey after 24 h of incubation at room temperature.

Antibacterial activities

The bactericidal activity was assessed against the laboratory strains *Staphylococcus aureus* ATCC 25923 (methicillin susceptible, MSSA) and ATCC 43300 (methicillin resistant, MRSA) by growth rates obtained from the Bioscreen C analyser. Overnight cultures in Mueller-Hinton (MH) broth medium were used. Optical densities of bacterial cultures were measured using a UV-VIS Helios Lambda spectro-photometer. For inoculation, bacterial biomass was added in appropriate volume, that the starting cell density distributed to Bioscreen C microplates was OD_{600nm} = 0.1 in total volume of 200 µL. 100 µL double-concentrated MH medium was mixed with 100 µL of 25% (m/v) honey extracts. Bacterial growth was monitored turbidimetrically at 600 nm for 24 hours (with measurements performed every 1 h) at 37^{o}C. The results are presented as relative values of growth (%) in stationary phase compared with the control – bacterial growth on MH medium, without the addition of honey.

Hydrogen peroxide determination

Hydrogen peroxide was determined according to Kwakman *et al*. [23]. Briefly, honey samples (40 µL) were mixed with 135 µL of reagent, consisting of 50 µg/mL O-dianisidine and 20 µg/mL horseradish peroxidase, type IV in 10 mM phosphate buffer (pH 6.5). After 5 min incubation at room temperature, reactions were stopped by addition of 120 µL of 6 M H_2SO_4 and absorption was measured at 540 nm. Hydrogen peroxide was used for calibration curve. Results were expressed as mM of hydrogen peroxide *per* 1 kg of honey.

Antioxidant activities:

ABTS - radical cation decolourization assay. The TAC (total antioxidant capacity) was estimated by 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) radical cation decolourization assay ($ABTS^{\cdot+}$) for fast and slow antioxidant as described by Wnuk *et al*. [32]. A fresh solution of 2,2'-azinobis (3-ethylbenzthiazo-line-6-sulphonic acid) radical cation ($ABTS^{\cdot+}$) was prepared by dissolving 19.5 mg of $ABTS^{\cdot+}$ and 3.3 mg of dipotassium peroxodisulfate in 7 mL of 0.1 mol/L phosphate buffer, pH 7.4. The solution was stored for 16 hours in the dark in order for the reaction to be complete. The $ABTS^{\cdot+}$ solution was then diluted in the 0.1 mol/L phosphate buffer to obtain an absorbance of about 1.0 at 414 nm. Aliquots (20 µL) of measured samples were added to 980 µL of $ABTS^{\cdot+}$ solution and mixed thoroughly. The decrease in the absorbance of the mixture was measured spectrophotometrically at 414 nm exactly

after 10 seconds (for fast antioxidants) and after 3 minutes (for slow antioxidants), after mixing the sample with the $ABTS^{\cdot+}$ solution. The results were expressed as mmoles of Trolox *per* 1 kg of honey.

Ferric reducing/antioxidant power (FRAP) assay. A manual assay was used based upon the methodology of Benzie and Strain [2]. FRAP reagent was freshly prepared with 1 mM 2,4,6-tripyridyl-2-triazine (TPTZ), 2 mM ferric chloride in 0.25 M sodium acetate buffer, pH 3.6. Aliquots (200 µL) of prepared, properly dissolved samples were added to 1.8 mL of FRAP reagent, mixed vigorously, incubated for 10 minutes at room temperature and measured spectrophotometrically at 593 nm. A standard Trolox solution was used for the calibration curve and the results were expressed as mmoles of Trolox equivalent *per* 1 kg of honey.

Total phenolic content

The total polyphenol content was measured using Folin-Ciocalteu reagent according to Jurca *et al*. [19]. For the standard curve, gallic acid was used. The results were expressed as mg of gallic acid equivalents (GAE) *per* 1 kg of honey.

Ascorbic acid content

Ascorbic acid content was determined reflectometrically according to the appropriate manuals of Merck Reflectoquant®. Results were expressed as mg of ascorbic acid *per* 1 kg of honey.

Statistical analysis

All analyses were carried out in triplicate and the data were expressed as mean ± standard deviations (SD). The Shapiro-Wilk test was used to check the normal distribution of data and Brown-Forsyth test to check the homogeneity of variances. To compare the mean values of examined parameters, one-way ANOVA with Tuckey post-hoc test was used. The statistical hypotheses were tested with $p \leq 0.05$. The Principal Component Analysis (PCA) was applied to reveal the relationships among all investigated properties and the similarities among honey types. The statistical analysis of the data was performed using STATISTICA version 12.0 (StatSoft, Inc., Poland).

Results and Discussion

Antibacterial activities

It has been established that many natural substances of bee products, such as honey, bee pollen displaying antioxidant activities, may also have strong anti-bacterial activities against different microorganisms [5, 6, 22].

Antibacterial activity of honey is mainly due to the acidity, high osmotic pressure, volatile compounds, flavonoids, polyphenolic acids. Moreover, enzymes like glucose oxidase and catalase are also responsible for the antibacterial potential of honey. These properties are strictly related to the botanical and geographical origin of honey [22].

That's why antibacterial properties of honey from different botanical and geographical origin have been investigated, providing data on the honey types that could be successfully applied as medical-grade honey in therapy [21, 22, 34]. Hydrogen peroxide plays very important role regarding antimicrobial properties of honey. It is produced by glucose oxidase in enzymatic oxidation of glucose [22]. It was previously reported that the highest level of hydrogen peroxide was formed in 30% water solutions after 24 h of incubation at room temperature [23].

The antibacterial properties and hydrogen peroxide concentration are shown in Figure 1.

It can be seen that the antibacterial properties depend on the honey type and bacterial strain. Rape, lime, multifloral and honeydew honey inhibit the growth of methicillin susceptible strain (MSSA) from around 40 to 65%, while buckwheat honey possesses the highest anti-bacterial properties against this bacterial strain and inhibits its growth in around 75%. On the other hand, rape and lime honey didn't inhibit the growth of MRSA strain; furthermore, the addition of lime honey improved the bacterial growth. Also Brudzynski *et al*. [6] observed that the addition of Canadian honey stimulated the growth of *Bacillus subtilis*. Rape, lime, multifloral and honeydew honey inhibited MSSA growth by about 35 - 55%. MRSA was inhibited less than MSSA strain. The highest antibacterial potential against both strains was observed for buckwheat honey (growth inhibition by about 80%) (Figure 1). Kuś *et al*. [22] determined higher antibacterial properties of Polish buckwheat and lime honey. For these honey types, they established the minimal inhibitory concentration, required to inhibit the growth of 90% of bacteria (MIC 90), at a level of 6.25% against *S. aureus* strain. In our study, the buckwheat honey, with the highest antibacterial properties, inhibited *S. aureus* growth by about 80% at a concentration of 12.5%. It means that MIC 90 of the tested honey samples was higher than 6.25%. These differences may be due to different botanical and geographical sources of honey or different bacterial strains used for analysis. Kędzia *et al*. [20] described that among different Polish honey types, the highest antibacterial properties against *S. aureus* MSSA possessed the buckwheat and honeydew honey. Also, Maeda *et al*. [24] reported antibacterial properties of Irish honey against CA-MRSA *S. aureus* strains.

We can observe that the honey with the strongest antibacterial properties has the highest hydrogen peroxide content (Figure 1). Strong negative correlations ($r = -0.45$ and $r = -0.58$ for MSSA and MRSA strains, respectively) are presented in Figure 2.

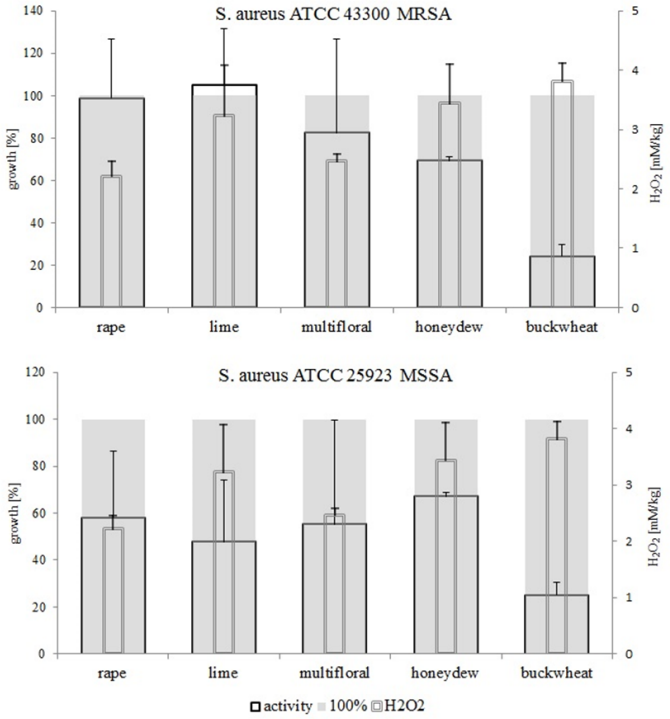
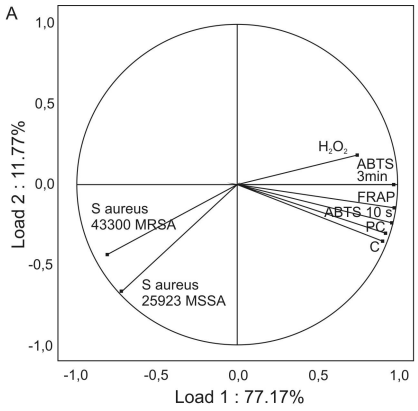


Figure 1.

Relative growth of *S. aureus* strains with addition of honey (25%; w/v) and hydrogen peroxide concentration of honey; data expressed as M ± S.D.



	Correlation matrix	
	S aureus 43300 MRSA	S aureus 25923 MSSA
H_2O_2	-0.445917	-0.582120
FRAP	-0.756931	-0.631796
PC	-0.591506	-0.469966
ABTS 10 s	-0.681762	-0.569552
ABTS 3 min	-0.775822	-0.693373
C	-0.577489	-0.451771

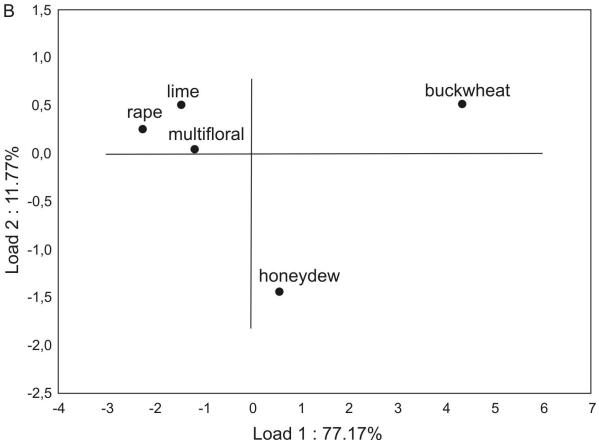


Figure 2.

Results of PCA analysis. A – the relationships among examined parameters with the correlation values; B – the similarity among the types of honey with respect of all examined parameters

Our results show that the higher hydrogen peroxide concentration is the lower bacterial growth develops. Some authors also noticed strong correlations between these two parameters for honey of different floral and geographical origin. Kwakman *et al*. [23] observed this aspect in respect to *E. coli*, *S. aureus* MRSA, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, and Brudzynski *et al*. [6, 7] for Canadian honey against *E. coli*.

The antioxidant activity depends largely on the content of flavonoids and polyphenolic acids. Moreover, strong correlation was observed between the colour of honey, and antioxidant properties as well as the content of polyphenolic compounds [26]. In this work we aimed to detect if there are any correlations between phenolic compounds, antioxidant properties of honey and their antibacterial potential.

Mean values of antioxidant properties, ascorbic acid and phenolic contents are shown in the Table I.

Table I

Phenolic content, FRAP and $ABTS^{\cdot+}$ values of the analysed honey

Honey	**Phenolic content** (mg gallic acid/kg)	**FRAP value** (mmoles Trolox/kg)	**$ABTS^{\cdot+}$ 10 s** (mmoles Trolox/kg)	**$ABTS^{\cdot+}$ 3 min** (mmoles Trolox/kg)	**Ascorbic acid** (mg/kg)
	Mean ± SD Range				
Rape (n = 5)	127.92 ± 25.49^{A} 99.49 - 165.48	673.27 ± 50.33^{A} 601.49 - 732.67	0.86 ± 0.06^{A} 0.79 - 0.94	1.66 ± 0.15^{A} 1.47 - 1.84	76.4 ± 6.54^{A} 70 - 86
Lime (n = 5)	167.11 ± 55.52^{A} 118.78 - 229.44	723.02 ± 118.85^{AB} 626.24 - 883.66	0.89 ± 0.26^{A} 0.66 - 1.32	1.78 ± 0.37^{A} 1.41 - 2.36	89.2 ± 10.83^{AB} 70 - 102
Buckwheat (n = 5)	822.94 ± 131.07^{C} 671.07 - 1032.49	2427.72 ± 236.44^{D} 2028.47 - 2626.24	3.56 ± 0.28^{C} 3.07 - 3.73	3.80 ± 0.06^{C} 3.70 - 3.73	324.8 ± 100.11^{C} 196 - 442
Multifloral (n = 5)	188.22 ± 12.07^{AB} 172.59 - 206.09	1036.63 ± 193.24^{B} 852.72 - 1222.77	1.16 ± 0.22^{A} 0.93 - 1.47	2.15 ± 0.29^{A} 1.82 - 2.55	119.6 ± 31.29^{AB} 80 - 154
Honeydew (n = 5)	297.66 ± 29.28^{B} 268.02 - 337.06	1674.50 ± 205.75^{C} 1440.59 - 1919.55	1.99 ± 0.29^{B} 1.67 - 2.24	3.20 ± 0.34^{B} 2.81 - 3.59	176.8 ± 39.41^{B} 108 - 206

SD- standard deviation; ^{A}, ^{B}, ^{C}, ^{D}, - the same Arabic letters indicate no significant differences among the means

Buckwheat honey exhibited the highest antioxidant activity measured by The two methods whereas rape honey exhibited the lowest values in this respect. The poly-phenol content ranged from 99.49 mg GAE/kg for rape honey to 1032.49 mg GAE/kg for buckwheat honey, with mean values 127.92 mg GAE/kg to 822.94 mg GAE/kg, respectively. These values are similar with data obtained by other authors describing Polish honey. Kuś *et al*. detected phenolic concentration at a level of around 200 mg GAE/kg and 1200 mg GAE/kg for lime and buckwheat honey, respectively [22]. Wilczyńska observed higher phenolic content for buckwheat honey from northern part of Poland (1100 - 1800 mg GAE/kg), and 870 mg GAE/ kg for buckwheat honey from southern part of Poland [31] together with a higher content of phenolic compounds in rape honey collected in northern part of Poland (176 - 358 mg GAE/kg).

The total phenolic content, antioxidant and antibacterial activities varied between honey types. The highest properties were determined for dark honey like: buck-wheat, honeydew, while the pale honey (rape, lime) showed a lower antioxidant and antibacterial properties. Wilczyńska also observed the same correlation in honey from different parts of Poland [31].

The mean values of antioxidant properties, phenolic and ascorbic acid content of honey of different floral origin are decreased in the order: buckwheat honey > honeydew > multifloral > lime > rape. Several authors have concluded that the antibacterial performance of honey is strongly associated with the phenolic content of the honey [17, 27]. We can observe that antibacterial properties of analysed honey increased in the same order as their phenolic content. General observation can be made that dark honey samples (buckwheat and honeydew) were characterized by considerably higher phenolic content than the other samples, like rape and lime honey. This trend was in accordance with other authors describing Polish [22, 31], Slovenian and Italian honey [3, 4].

Vitamin C (ascorbic acid), a strong antioxidant, is associated with health and vital force, and therefore food products are well accepted by the consumer when a high content of vitamin C is indicated. The mean vitamin C content in honey samples is showed in Table I. The results showed that vitamin C is present in all samples. In particular, buckwheat honey presented the highest vitamin C content (324.8 mg/kg honey; $p < 0.05$). The lower level of ascorbic acid was detected for rape honey (76.4 mg/kg). In multifloral honey, the content ranged between 80 mg/kg honey and 154 mg/kg, depending on the sample. However, a great dispersion in the values was stated. Similar vitamin C content was described for Nigerian honey – from 138.6 to 273.2 mg of ascorbic acid *per* 1 kg honey, with mean values of 211.5 mg/kg [8]. In Romanian honey, vitamin C content was twice as more, for example for multifloral honey ranged from 610 - 960 mg/kg honey. Maybe so high concentration are

due to different methods used and the geographical and botanical origin of honey [13]. In unifloral honey, the content ranged between 2.68 mg/kg honey (citrus honey) and 3.92 mg/kg honey (chestnut honey). These values were similar to those found in citrus and eucalyptus honey, while they were lower than those found in Italian honey by Ciulu *et al*. [10]. We can observe a significant correlation between the antibacterial properties of all honey types against two analysed strains and antioxidant properties and phenolic content (Figure 2). Strong correlation was also observed by Kuś *et al*. for Polish honey against *S. aureus* and *P. aeruginosa* [22].

Taking into considering all the tested properties of honey we can conclude that rape, lime and multifloral honey are quite similar but honeydew and buckwheat honey clearly differ from others (Figure 2).

Conclusions

These results demonstrate that several honey samples collected from Southern-Eastern part of Poland may have therapeutical potential against *S. aureus*. The most promising proved to be buckwheat honey. It was also confirmed that the botanical origin of honey is very important in its antibacterial potential. The correlations between the antibacterial properties and the antioxidant, phenolic and hydrogen peroxide content suggests that these substances can be important predictors of the honey's antibacterial activity.

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