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In vitro antibacterial efficacy of different natural linden honey against some gram-positive and gram-negative strains

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Linden honey is rich in organic acids, such as formic acid and malic acid, which have antibacterial and anti-inflammatory properties. This, in turn, helps prevent bacterial infections and inflammatory diseases that can lead to serious cardiovascular disorders. These bactericidal and antiviral properties are due to the high content of inhibin, lysozyme, and apidicin. These are bactericidal and antiviral enzymes that are responsible for the strong antibiotic properties of linden honey. The aim of the current study was *in vitro* antimicrobial profiling of different natural linden honey produced by Polish manufacturers, exhibiting inhibitory activity against Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™. The different natural linden honey produced by Polish manufacturers such as Beekeeping farm "Pszczółka" (Ustka, Poland), Beekeeping farm "Mazurskie Miody Bogdan Piasecki" (Tomaszkowo, Poland), Beekeeping farm "Sądecki Bartnik" (Stróże, Poland), Beekeeping farm "Zaczarowany Ogród", and Beekeeping farm "Karolczak Cezary" (Sławno, Poland) were used in the current study. The samples were stored in resalable vials at 5 °C in the dark but were allowed to adjust to room temperature before investigation. The testing of the antibacterial activity of different natural linden honey was carried out *in vitro* by the Kirby-Bauer disc diffusion technique. This study demonstrated that all samples of natural linden honey produced by Polish manufacturers demonstrated mild antibacterial activity against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains. More sensitive to all samples of natural linden honey studied was *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain following *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain. *Pseudomonas aeruginosa*

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(Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey. The presented results revealed the antibacterial activities of different samples of linden honey produced by Polish manufacturers, which require further study to be correctly understood and explained.

Keywords: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* strains, Kirby-Bauer disc diffusion technique

Introduction

Linden honey is a variety of honey produced by bees from the nectar of linden (*Tilia* L.) flowers. *T. cordata* Mill. and *T. platyphyllos* Scop. are native to much of Europe, with their ranges extending from southern Finland to southern Italy and the Caucasus. *T. cordata* is the more abundant of the two species and its core region is central and eastern Europe (Eaton et al., 2016). Linden flowers contain many beneficial compounds that can also be found in linden honey, including tannins, organic acids, flavonoids, and pectins (Naef et al., 2004; Qiao et al., 2020; Bodor et al., 2020). Linden trees were among the most sacred trees in the Slavic tradition, perhaps that is why now linden honey is one of the most popular species in Poland (Ţenche-Constantinescu et al., 2015).

The visual and taste properties of linden honey make it quite distinctive. Linden honey has an intense and very "honey" flavor, sweet but sour. Some people even claim that the taste of linden honey is a bit sharp and sometimes it can even be slightly bitter. In turn, the color of linden honey is light yellow and straw-colored, but it can also be slightly greenish depending on the linden variety and the harvesting period (Konopleva, 2011; da Silva et al., 2016). Linden honey contains 74% glucose and fructose, about 1.5–3.0% sucrose and water. Micronutrients present in linden honey include essential oils, flavonoids, the glycoside tiliacin, the triterpene taraxerol, tannins, bitter compounds, and the enzyme lysozyme. The main mineral is potassium. Honey contains vitamins C, H, PP, and B vitamins (Dżugan et al., 2018). The glycemic index of this product, due to the content of simple sugars, is relatively high (although not the highest among honey). It is approximately 55, depending on the origin of linden honey, which places it among kinds of honey with an average glycemic index. It can therefore be included in the diet of diabetics in a very small and controlled amount (Atayoğlu et al., 2016).

Linden honey is worth using for colds, flu, upper respiratory tract infections, bronchitis, and pneumonia. It has diaphoretic, antitussive, expectorant, and antispasmodic properties. It has unique healing properties, supports the nervous and immune systems, and contains lots of antioxidants. It soothes inflammation and has an antipyretic effect. Due to its diuretic effect, reducing swelling and gently lowering blood pressure, linden honey is recommended for circulatory system diseases. For people with cardiovascular disorders, its antispasmodic and calming effects, which result from the presence of essential oils, are also important. For this reason, it is recommended to use honey in cases of insomnia, chronic stress, nervous hyperactivity, and even neurosis (Khalil, 2023).

Linden honey has strong antibiotic, diaphoretic, antitussive, expectorant, and antispasmodic properties. Linden honey is recommended for urinary and genital system infections, digestive system disorders due to its antibiotic activity, and rheumatic diseases due to its anti-inflammatory effect. Honey eliminates pathogenic microorganisms such as staphylococci, streptococci, yeast fungi, *Klebsiella pneumonia*, *Escherichia coli*, and *Candida albicans* (Lusby et al., 2005; Irish et al., 2006; Kuncic et al., 2012; Almasaudi, 2021). The effectiveness of honey against microorganisms depends on the type of honey produced, which depends on its botanical origin, the health of the bees, its origin and the processing method (Almasaudi, 2021).

In the current study, *in vitro* antimicrobial profiling of different natural linden honey produced by Polish manufacturers was performed, exhibiting inhibitory activity against Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™.

Materials and methodology

Natural linden honey

The different natural linden honey produced by Polish manufacturers such as Beekeeping farm "Pszczółka" (Ustka, Poland; 54° 34′ 43″ N, 16° 52′ 09″ E), Beekeeping farm "Mazurskie Miody Bogdan Piasecki" (Tomaszkowo, Poland; 53° 43′ 04″ N 20° 24′ 32″ E), Beekeeping farm "Sądecki Bartnik" (Stróże, Poland; 49° 39′ 24″ N, 20° 58′ 48″ E), Beekeeping farm "Zaczarowany Ogród", and Beekeeping farm "Karolczak Cezary" (Sławno, Poland; 54° 21′ 44″ N, 16° 40′ 49″ E) were used in the current study. The samples were stored in resalable vials at 5 °C in the dark but were allowed to adjust to room temperature before investigation.

Determination of the antibacterial activity of honey samples by the disk diffusion method

The testing of the antibacterial activity of linden honey was carried out *in vitro* by the Kirby-Bauer disc diffusion technique (Bauer et al., 1966). In the current study, Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ were used. All strains used in the current study originated from ATCC (American Type Culture Collection), a nonprofit, global biological resource center and standards organization and the leading developer and supplier of authenticated cell lines and microorganisms. Strains were purchased in Pol-Aura Sp. z o.o. (Zabrze, Poland).

The strains were inoculated onto Mueller-Hinton (MH) agar dishes. Sterile filter paper discs impregnated with linden honey sample were applied over each of the culture dishes. Isolates of bacteria with linden honey samples were then incubated at 37 °C for 24 h. The Petri dishes were then observed for the zone of inhibition produced by the antibacterial activity of linden honey. A control disc impregnated with 96% ethanol was used in each experiment. At the end of the 24-h period, the inhibition zones formed were measured in millimetres using the vernier. For each strain, eight replicates were assayed (n = 8). The Petri dishes were observed and photographs were taken. The susceptibility of the test organisms to the linden honey was indicated by a clear zone of inhibition around the discs containing the linden honey and the diameter of the clear zone was taken as an indicator of susceptibility. The following zone diameter criteria were used to assign susceptibility or resistance of bacteria to the phytochemicals tested: Susceptible (S) ≥15 mm, Intermediate (I) = 10–15 mm, and Resistant (R) ≤10 mm (Okoth et al., 2013; Tkachenko et al., 2022).

Statistical analysis

Zone diameters were determined and averaged. Statistical analysis of the data obtained was performed by employing the mean ± standard error of the mean (S.E.M.). All variables were randomized according to the phytochemical activity of the linden honey tested. All statistical calculation was performed on separate data from each strain. The data were analyzed using a one-way analysis of variance (ANOVA) using Statistica v. 13.3 software (TIBCO Software Inc., USA) (Zar, 1999).

Results and discussion

Figures 1 and 2 summarize the results obtained by the mean diameters of the inhibition zone around the growth of *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains induced by different natural linden honey produced by Polish manufacturers.

After applying different natural linden honey to *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain, we noted a statistically non-significant increase in the zone of growth inhibition by 17.72% ($p > 0.05$) for linden honey ("Zaczarowany Ogród"), by 13.95% ($p > 0.05$) for linden honey ("Sądecki Bartnik") and by 4.83% ($p > 0.05$) for linden honey ("Mazurskie Miody Bogdan Piasecki") compared to the control samples (9.54 ±0.85 mm). Linden honey from Beekeeping farm "Pszczółka" and Beekeeping farm "Karolczak Cezary" caused the decreased inhibition zone diameters after application on culture dishes with *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain (by 14.89% and 14.15%, $p > 0.05$, respectively) compared to the control samples (Figure 1).

We observed similar trends after *in vitro* application of different natural linden honey against *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain, where we also observed a statistically significant increase in the zone of growth inhibition by 40.14% ($p < 0.05$) for linden honey from Beekeeping farm "Pszczółka", by 41.98% ($p < 0.05$) for linden honey from Beekeeping farm "Mazurskie Miody Bogdan Piasecki". A statistically non-significant increase in the zone of growth inhibition of growth of *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain after *in vitro* application of linden honey was observed, i.e. by 13.38% ($p > 0.05$) for linden honey from Beekeeping farm "Sądecki Bartnik", by 10.29% ($p > 0.05$) for linden honey from Beekeeping farm "Zaczarowany Ogród", and by 15.07% ($p > 0.05$) for linden honey from Beekeeping farm "Karolczak Cezary" against the control samples (7.10 ±0.56 mm) (Figure 1).

Pseudomonas aeruginosa (Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey. After applying linden honey samples to *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain, a statistically non-significant

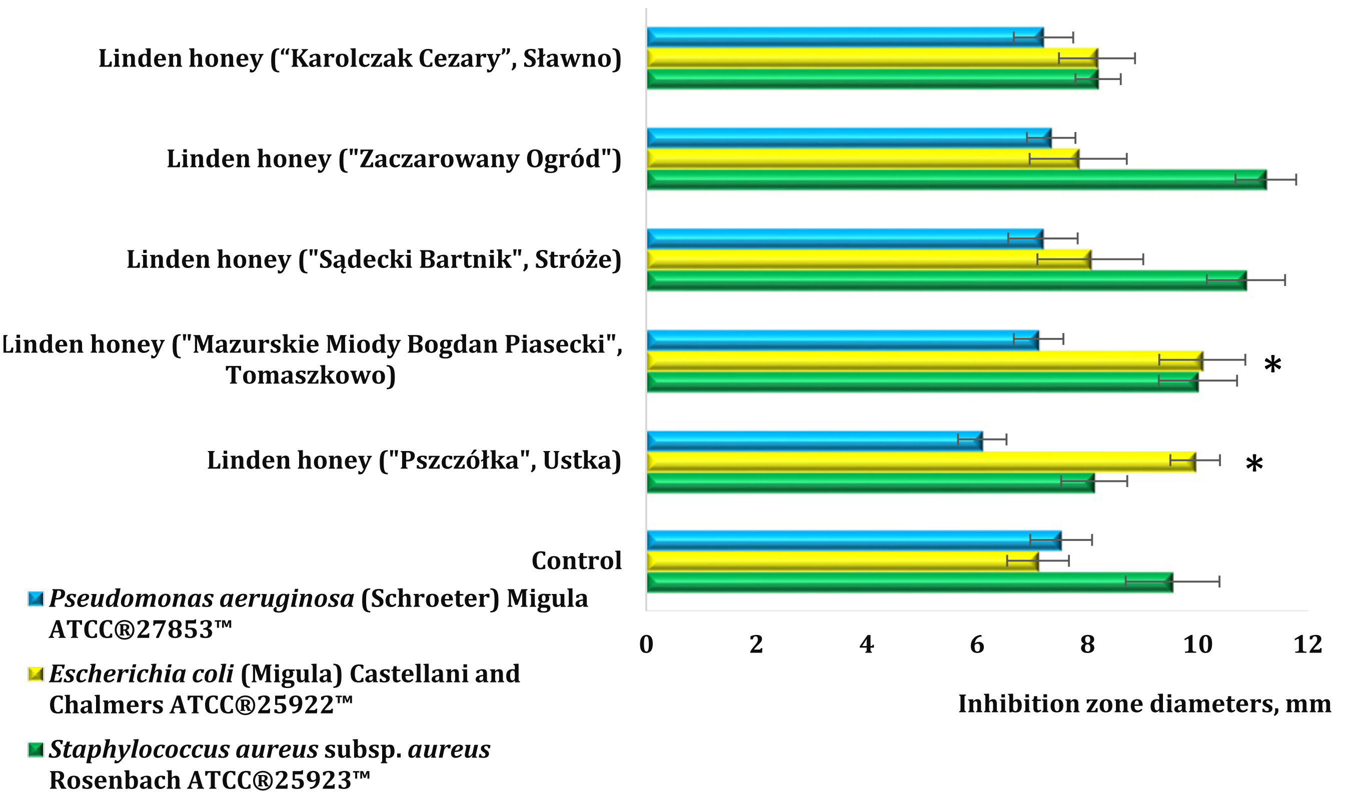

Figure 1 The mean inhibition zone diameters induced by different natural linden honey produced by Polish manufacturers against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ (M ±m, n = 8)
*– changes were statistically significant compared to the 96% ethanol

decrease in the zone of growth inhibition was noted, i.e. by 19.02% ($p > 0.05$) for linden honey from Beekeeping farm "Pszczółka", by 5.46% ($p > 0.05$) for linden honey from Beekeeping farm "Mazurskie Miody Bogdan Piasecki", by 4.39% ($p > 0.05$) for linden honey from Beekeeping farm "Sądecki Bartnik", by 2.40% ($p > 0.05$) for linden honey from Beekeeping farm "Zaczarowany Ogród", and by 4.26% ($p > 0.05$) for linden honey from Beekeeping farm "Karolczak Cezary" compared to the control samples (7.52 ±0.56 mm) (Figure 1).

The results of the current study revealed that all samples of natural linden honey produced by Polish manufacturers demonstrated mild antibacterial activity against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains. More sensitive to all samples of natural linden honey studied was *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain following to *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain. *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey.

Among the features of linden honey, it is worth highlighting its antibacterial, expectorant, diaphoretic, and antipyretic properties. It may have a calming and slightly soporific effect. In practice, it has an antitussive, warming, soothing effect and regulates blood pressure. Also, linden honey has a mild diuretic effect on the human body. Due to the above-mentioned features of linden honey, it is certainly worth emphasizing its help in the fight against colds, flu symptoms, and diseases of the respiratory, circulatory, and nervous systems (Khalil, 2023). Apart from colds and periods of reduced immunity, linden honey is worth using due to its beneficial effect on the nervous system in the face of insomnia or exhaustion of the body. In states of nervousness or increased stress, linden honey can also be great. Moreover, it can be a great ingredient in the diet of athletes due to the content of easily digestible carbohydrates. Convalescents can also benefit from its properties, because not only is it a source of easily accessible energy (which they need in excess), but also due to its ingredients it supports the immune system. Linden honey can be successfully used, for example, in peptic ulcer disease; it alleviates the symptoms associated with it (Khalil, 2023).

The factors influencing the antibiotic effect of honey are quite complex. They can be divided into three groups: physical, chemical, and biological (Kędzia and Hołderna-Kędzia, 2017). Physical factors include high osmotic pressure resulting from the high sugar content in honey, as well as low pH caused by the presence of organic acids. Chemical factors include primarily hydrogen peroxide produced as a result of an enzymatic reaction (glucose oxidase), as well as methylglyoxal found in manuka honey and, in some varieties of honey, high content of phenolic compounds, including phenolic acids and flavonoid compounds. However, biological factors include peptides – lysozyme and defensin-1, probably the same substance, only named differently (Patton et al., 2006; Al-Waili et al., 2011; Kwakman and Zaat, 2012; Almasaudi, 2021). Other phytochemical factors such as tetracycline, peroxides, amylase, fatty acids, phenols, ascorbic acid, terpenes, benzyl alcohols, and benzoic acid make honey active against pathogenic bacteria and have either bacteriostatic or bactericidal effects. All of these factors vary depending on the nectar source and storage conditions (Almasaudi, 2021).

Gram-positive target strains were most susceptible to honey samples. In contrast, according to previous observations, Gram-negative microbes were less sensitive to all honey samples (Kwakman et al., 2011; Mandal and Mandal, 2011). The difference in susceptibility to honey and other antibacterial agents between Gram-positive and Gram-negative microbes may be due to cell wall composition. Gram-positive bacteria do not have an outer membrane protecting the peptidoglycan layer, unlike Gram-negative bacteria, which makes it easier for antimicrobial agents to penetrate and cause damage (Matzen et al., 2018).

As demonstrated in the literature, honey has antibacterial activity (bacteriostatic and bactericidal effect), similar to antibiotics, against test organisms and provides alternative therapy against certain bacteria (Mohapatra et al., 2011). The antibacterial activity of honey likely depends on the pasture in which the bees were raised, climatic conditions, as well as the natural composition of the flower nectar (Abd-El Aal et al., 2007). Many studies revealed that honey was effective against methicillin-resistant *Staphylococcus aureus*, hemolytic streptococci, and vancomycin-resistant enterococci (Lusby et al., 2005).

The antioxidant and antibacterial activity of 21 ypes of honey derived from Mount Olympus (Mt. Olympus), a region with great plant biodiversity, were studied by Stagos et al. (2018). The antibacterial activity was examined against the growth of S. *aureus* and *P. aeruginosa* by the agar well diffusion assay and the determination of the minimum inhibitory concentration (MIC). The MIC of the tested honey types against S. *aureus* ranged from 3.125 to 12.5% (v/v), while the MIC of Manuka honey was determined to be 6.25% (v/v). The MIC values of the tested honey

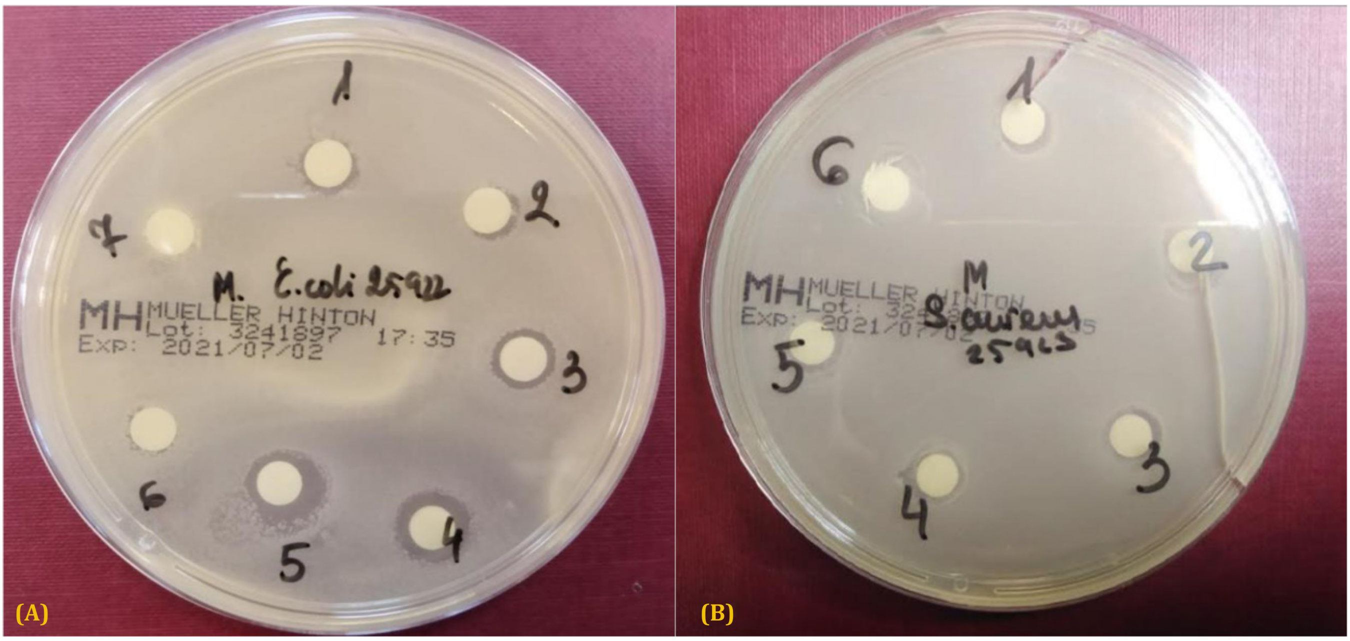

Figure 2 The mean diameters of the inhibition zone around the growth of *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ (A) and *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ (B) induced by different natural linden honey produced by Polish manufacturers

types against *P. aeruginosa* ranged from 6.25 to 12.5% (v/v) and the MIC of Manuka honey was determined at 12.5% (v/v). Moreover, the results suggested that the presence of hydrogen peroxide and proteinaceous compounds in the honey types accounted, at least in part, for the antibacterial activity. Better free radical scavenging efficiency has been demonstrated in these types of honey compared to Manuka honey. Moreover, antioxidant activity was observed in four types of honey tested that were converted into powder by freeze-drying. Data showed that the three powdered honey retained their antioxidant properties after lyophilization, making them suitable for further bioactivity assessment and application (Stagos et al., 2018).

The physicochemical characteristics and antioxidative, antibacterial and antiproliferative effects of nineteen samples of different honey types (acacia, linden, heather, sunflower, phacelia, basil, anise, sage, chestnut, hawthorn, lavender and meadow) collected from different locations in the Western Balkans (Republic of Serbia, Kosovo, Bosnia and Herzegovina, and Northern Macedonia) were evaluated by Sakač et al. (2022). Antibacterial activity was estimated *in vitro* using agar diffusion tests and measuring minimal inhibitory concentration (MIC). Among investigated bacterial strains following resistant potencies were determined: *Escherichia coli* > *Escherichia coli* ATCC 8739 > *Enterococcus faecalis* > *Proteus mirabilis* > *Staphylococcus aureus* > *Staphylococcus epidermidis*. The linden honey from Fruška Gora (MIC values of 3.12% and 6.25% against *Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively) and phacelia honey (MIC values of 6.25% and 3.12% against *Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively) showed the strongest antibacterial activity (Sakač et al., 2022). The antibacterial potential of linden honey could be related to methyl syringate, which was found to be the most abundant component besides lindenin in linden honey and known to act as an antibacterial agent (Almasaudi et al., 2017; Sakač et al., 2022). As mentioned by Qiao et al. (2020), methyl syringate and lindenin were the most abundant components in rape and linden honey; moreover, their average contents reached 10.44 and 21.25 $mg.kg^{-1}$, respectively. The presence of two identified terpene acids, i.e., 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-diene-1-carboxylic acid (1) and 4-(1-methylethenyl) cyclohexa-1,3-diene-1-carboxylic acid (2), was confirmed in (Swiss) linden honey after solid-phase extraction and HPLC purification were identified by Frérot et al. (2006).

Hulea et al. (2022) investigated the antioxidant profile and the antimicrobial activity of four different types of monofloral honey (manuka, brassica rapeseed, acacia, and linden honey) against some bacterial/fungal ATCC strains and some multidrug-resistant strains isolated from chronic otitis in dogs. The antioxidant characterization of the analyzed honey samples showed the highest antioxidant activity and concentrations of total polyphenols and total flavonoids in Manuka honey, followed by linden honey. Manuka honey was proven to be the most effective on most clinical isolates concerning antimicrobial activity in comparison with brassica rapeseed, acacia, and linden honey. Except for *B. cepacia* and *P. vulgaris*, all the clinical isolates were sensitive to the antibacterial activity of honey. Regarding the ATCC strains, Manuka honey 10% was the most effective in inhibiting all the strains tested except for *P. aeruginosa*. The efficacy classification was Manuka honey > brassica rapeseed honey > acacia honey > linden honey (Hulea et al., 2022).

Correlation establishing between factors affecting the antibacterial nature of honey (osmolarity, acidity, content of hydrogen peroxide and non-peroxide components) and the honey harvest area environmental parameters as well as harvesting, processing and storage conditions is necessary to clarify the reasons for minor differences of the antibacterial efficacy of the same concentrations linden honey different manufacturers.

Conclusions

In the current study, we assessed *in vitro* antimicrobial profiling of different natural linden honey produced by Polish manufacturers was performed, exhibiting inhibitory activity against Gram-positive strains such as *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, and Gram-negative strains such as *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™. This study demonstrated that all samples of natural linden honey produced by Polish manufacturers demonstrated mild antibacterial activity against *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™, *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™, and *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strains. More sensitive to all samples of natural linden honey studied was *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC®25923™ strain following to *Escherichia coli* (Migula) Castellani and Chalmers ATCC®25922™ strain. *Pseudomonas aeruginosa* (Schroeter) Migula ATCC®27853™ strain was resistant to different natural linden honey. The presented results revealed the antibacterial activities of different samples of linden honey produced by Polish manufacturers, which, to be correctly understood and explained, require further study.

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical statement

This article doesn't contain any studies that would require an ethical statement.

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