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ANTIBACTERIAL ACTIVITY OF CHESTNUT HONEY (*Castanea sativa* Mill.) AGAINST *Helicobacter pylori* AND CORRELATION TO ITS ANTIOXIDANT CAPACITY

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Summary

One of the proven therapeutic properties of honey is its antimicrobial activity. The aim of this study was to examine the antimicrobial activity of chestnut honey against *Helicobacter pylori* and to evaluate a relationship between the content of phenols, antioxidant capacity and antimicrobial activity. The antimicrobial activity of honey was determined by the agar well diffusion method, and the inhibitory effect of different honey concentrations (20%, 50% and 75%) was evaluated. The phenolic content was determined by the Folin-Ciocalteu method while the total antioxidant capacity was determined by the FRAP assay. Water activity and hydrogen peroxide content were also determined. The results showed that the zones of inhibition of *H. pylori* ranged from eight to 21 mm depending on the sample and the concentration of honey, where the concentration of honey of 20% did not have inhibitory effect. The phenolic content ranged from 204.94 to 233.82 mg of GA/kg while FRAP values were between 392.71 and 441.53 μM Fe (II). The honey sample that showed the highest antimicrobial activity against *H. pylori* also had the highest total antioxidant capacity. However, the same correlation was not observed in the other analysed samples. Further research is needed to determine the contribution of individual components of honey to its antimicrobial activity.

Keywords: chestnut honey, *Helicobacter pylori*, antibacterial activity, antioxidant capacity

Introduction

One of the proven therapeutic properties of honey is its antimicrobial effect. Low pH value of honey, high osmotic pressure, hydrogen peroxide, phytochemicals (phenolic components, methylglyoxal), antimicrobial peptides (bee defensin 1 and 2) and lysozyme are considered the main factors responsible for the antimicrobial activity of honey (Manyi-Loh et al., 2010; Samie et al., 2014; Ronsisvalle et al., 2019). Chemical composition and consequently therapeutic properties depend primarily on honeys botanical origin. Scientific studies have shown that darker honeys, like chestnut honey and honeydew honey, have stronger inhibitory effect on microorganisms compared to lighter honey types (Gradvol et al., 2015; Küçük et al., 2007; Güneş et al., 2016). Darker honeys have higher phenolic content and antioxidant capacity as well as higher enzyme activity, especially important is glucose oxidase activity that catalyses production of hydrogen peroxide, a major antibacterial substance in honey (Flanjak et al., 2016a; Flanjak et al., 2016b; Strelec et al., 2018). Chestnut honey (*Castanea sativa* Mill.) is characteristic for the continental area of Croatia and one of the most important unifloral types of honey produced in the Republic of Croatia.

Helicobacter pylori infection is one of the most common human chronic microbial infection worldwide. It is estimated that 50% human population harbors *H. pylori* bacterial strains and to some percent, it causes gastritis and peptic ulcers (Nzeako and Al-Namaani, 2006; Samie et al., 2014). Different treatment regimens for successful eradication of *H. pylori* have been proposed. Generally, a combination of two antibiotics (clarithromycin or amoxicillin and metronidazole) and a proton pump inhibitor or an anti-ulcer agent is most widely used therapy. However, the problem of resistance to antibiotics is growing and the alternative therapies are investigating intensively. Natural products, like plant extracts, honey and probiotics alone or in a combination with antibiotics are evaluated as possible anti-*H. pylori* agents. Studies have shown that honey as *in-vitro* anti-*H. pylori* activity that is mostly related to inactivation of *H. pylori* urease but further studies are needed to prove honeys' antimicrobial activity against *H. pylori in-vivo* (Ayala et al., 2014; Samie et al., 2014; Debraekeleer and Remaut, 2018).

The aim of this study was to examine the antimicrobial activity of chestnut honey (*C. sativa* Mill.) against *H. pylori* and to evaluate a relationship between the content of phenols, antioxidant capacity and antimicrobial activity.

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Materials and methods

Honey samples

Botanical origin of five chestnut honey (*Castanea sativa* Mill.) samples collected in 2019 was confirmed based on the results of pollen analysis (Deutsches Institut für Normung, 2002), electrical conductivity (Bogdanov, 2009) and sensory analysis (International Organization for Standardization, 1987).

Analyses

Water content and hydroxymethylfurfural (HMF) content were determined according to the methods prescribed by International Honey Commission (Bogdanov, 2009). Water activity was determined using HygroLab 3 water activity measuring system which is calibrated in range 0.000 to 1.000 aw range. Semi-quantitive method (Strelec et al., 2018) using MQuant™ peroxide test strips (Merck, Germany) was used for estimation of hydrogen peroxide content. Phenolic content and antioxidant capacity (FRAP assay) were determined according to the methodology described by Flanjak et al. (2016a).

Antibacterial activity

The clinical specimen of *Helicobacter pylori* strain 3639 was isolated from a biopsy of the gastric mucosa of a patient treated under the diagnosis of chronic gastritis. Sample was homogenised and cultured on Columbia brood agar base (OXOID; Basingstoke, UK) with 7% defibrillated sheep blood plus *Helicobacter pylori* Selective Supplement (Dent) (OXOID, Basinstoke, UK). *H. pylori* cultures were incubated under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) with addition of Campy Gen sachet (OXOID, Basinstoke, UK) at 37 °C for 5 days. Identification of grown colonies were Gram strained and observed under microscope. In addition, urease, oxidase and catalase activity tests were performed.
Agar well diffusion method (Dastaouri et al., 2008; Manyi-Loh et al., 2010) was used to test the antibacterial activity. Clinical specimen of *H. pylori* was suspended in sterile saline and adjusted to 4.0 McFarland standard (corresponding to 1.2 x 10^9 CFU/mL). The suspension was smeared with a sterile cotton swab on selective nutrient media. Three wells were made in each Petri dish with sterile tip and in every well 100 µL of honey solution diluted with sterile saline (20%, 50% and 75%; respectively) was added. Amoxicillin (2µg) was used as positive control. Petri dishes were incubated at 37 °C for seven days under the microaerophilic conditions. Antibacterial activity was evaluated by measuring the zone of inhibition against the test microorganism.

Results and discussion

Five honey samples labelled by the beekeepers as unifloral chestnut honey (*Castanea sativa* Mill.) were subjected to botanical origin determination and according to the results presented in Table 1 uniflorality of samples was confirmed. All samples had *C. sativa* pollen share higher than 80% (90 – 98 %) that is a prescribed limit in national regulation (Ministry of Agriculture, Fisheries and Rural Development, 2009) for unifloral chestnut honey. Also, all samples had electrical conductivity higher than 0.8 mS/cm (0.81 – 1.73 mS/cm) that is a minimum for chestnut honey prescribed in national and international regulations (Codex Alimentarius Commission, 2001; Council of the European Union, 2002; Ministry of Agriculture, 2015). Sensory attributes of analysed samples (aroma, taste and colour) was characteristic for chestnut honey (Persano Oddo and Piro, 2004). Besides, the water content (18.0 ± 1.2 %) and HMF content (4.24 ± 2.88 mg/kg) of samples indicate that the collected samples were fresh and properly processed. After botanical origin confirmation and quality assessment, the antibacterial activity of different honey solutions against *H. pylori* was tested. The inhibitory effect of three concentrations of honey solution (20%, 50% and 7 %) on *H. pylori* growth was tested and the results, expressed as zone of inhibition (mm), are presented in Table 2 and Fig. 1. The concentration of 20% of honey solution had no inhibitory effect on *H. pylori* while at concentration of 50% two samples and at 75% three samples had inhibitory potential against *H. pylori*. The inhibitory potential at 50% (zone of inhibition 8 and 18 mm) is in accordance or slightly higher to literature data for the same chestnut honey concentration (Küçük et al., 2007; Kolayli et al., 2008; 2017). Based on the diameter of the inhibition zone (Table 2), the inhibitory potential of chestnut honey samples can be classified as very low (5.5 – 10 mm) or low (11 – 15 mm) and for sample 2 even high inhibitory potential (16 mm or higher) against *H. pylori* (Kolayli et al., 2008; Küçük et al., 2007). At the same time, two samples showed no inhibitory effect to growth of *H. plyori* at any of tested concentrations. Antimicrobial activity of honey is a result synergistic effect of different physical (acidity, osmolarity) and chemical (H_2O_2, phenolic components, lysozyme, bee defensins, methylglyoxal) factors and the contribution of each factor to overall antimicrobial activity is not clear yet (Maddocks and Jenkins, 2013; Gradvol et al., 2015; Samie et al., 2014; Debraekeleer and Remaut,

2018; Quraisiah et al., 2020). One for the reasons is the fact that although in unifloral honey one botanical species prevails (nectar and pollen) and gives a specific melissopalynological, physicochemical and sensory characteristics, there is no 100% unifloral honey. Those botanical species present in lower amounts can contribute to variations in honey properties. Besides, processing and manipulation after extraction of honey by the beekeeper can effect on chemical composition and properties of honey. The difference in antimicrobial potential within the same honey type was also reported by Kolayli et al. (2008) and Gradvol et al. (2015). Antimicrobial activity of honey is mostly attributed to the presence of hydrogen peroxide (H_2O_2) that is a product of the conversion of glucose into gluconic acid catalysed by enzyme glucose oxidase (Strelec et al., 2018). The H_2O_2 content in all analysed honey samples was 147.05 μmol/L h (Table 1) determined by semi-quantitative method. The obtained results in this study are in compliance to previous results for chestnut honey (Strelec et al., 2018). Along with H_2O_2, the high osmolarity and acidity of honey contribute to its antimicrobial activity. Water activity (a_w) of analysed chestnut honey samples was between 0.55 and 0.60 (Table 1). Those a_w values are low enough to create inhospitable environment for most microorganisms (Maddocks and Jenkins, 2013). As mentioned above the non-peroxide substances, like phenolic components, contribute to antimicrobial activity. Many studies available prove the correlation between honey's antioxidant capacity, phenolic content and composition and antibacterial activity (Güneş et al., 2016; Kolayli et al., 2008; 2017; Küçük et al., 2007; Ronsisvalle et al., 2019). Generally, darker honeys (e.g. chestnut honey and honeydew honey) have higher total phenolic content, higher antioxidant capacity and possess higher antimicrobial activity than lighter honey types (e.g. black locust honey, lime honey). Total phenolic content of analysed chestnut honey samples was between 204.94 mg GA/kg and 233.82 mg GA/kg and antioxidant capacity determined by FRAP assay between 392.71 μM (Fe(II)) and 441.53 μM (Fe(II)) (Table 1). The obtained results are in compliance to the literature data (Bertocelj et al., 2007; Beretta et al., 2005; Flanjak et al., 2016a). The honey sample that showed the highest antimicrobial activity against *H. pylori* also had the highest total antioxidant capacity. However, the same correlation was not observed in the other analysed samples. This indicates that phenolic components contribute to the antimicrobial activity of honey but overall antimicrobial activity is a result of synergistic action of different components of honey, only some of which were determined in this study.

Table 1. Specific pollen share, physicochemical characteristics, phenolic content and antioxidant capacity (FRAP assay) of analysed honey samples

Sample	Specific pollen (%)	Water content (%)	Electrical conductivity (mS/cm)	HMF content (mg/kg)	Water activity	Hydrogen peroxide content (μmol/L h)	Phenolic content (mg GA/kg)	FRAP (μM (Fe(II))
1	90	17.2	1.06	8.98	0.58	147.05	219.66	413.50
2	97	17.2	1.09	2.47	0.56	147.05	211.17	441.53
3	88	17.1	1.73	1.50	0.55	147.05	233.82	436.00
4	96	19.2	0.81	3.89	0.59	147.05	204.94	404.82
5	98	19.5	0.81	4.27	0.60	147.05	211.17	392.71
Min-Max	90 - 98	17.1 - 19.5	0.81 - 1.73	1.50 - 8.98	0.55 - 0.60	147.05 - 147.05	204.94 - 233.82	392.71 - 441.53
Mean±SD	94 ± 4	18.0 ± 1.2	1.10 ± 0.38	4.24 ± 2.88	0.58 ± 0.02	147.05 ± 0.00	216.15 ± 11.18	417.71 ± 20.68

Table 2. Antibacterial activity of chestnut honey solutions against *H. pylori*

Honey concentration (%)	Zone of inhibition (mm)				
	sample 1	sample 2	sample 3	sample 4	sample 5
20	0	0	0	0	0
50	8	18	0	0	0
75	15	21	0	12	0

Fig. 1. Zone of inhibition of selected chestnut honey solutions (sample 2) against *H. pylori* on chocolate agar

Conclusion

The obtained data indicate the potential use of chestnut honey in the treatment of *H. pylori* infections but further research is needed to determine the contribution of individual components of chestnut honey to its antimicrobial activity. Also, the efficiency of honey as an alternative ot complementary anti-H. pylori agent sholud be confirmed in in-vivo studies in future research.

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