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ORIGINAL ARTICLE

Antibacterial potential of Croatian honey against antibiotic resistant pathogenic bacteria

Ivana Gobin[1], Goranka Crnković[1], Maja Magdalenić[1], Gabrijela Begić[1], Ana Babić[1], Dražen Lušić[2], Darinka Vučković[1]

[1]Department of Microbiology and Parasitology, [2]Department of Environmental Health; Faculty of Medicine, University of Rijeka, Braće Branchetta 20, 51000 Rijeka, Croatia

ABSTRACT

Aim To determine antimicrobial activity of honey against clinical bacterial strains and their respective reference strains.

Methods Twelve samples of Croatian honey from various botanical origin were evaluated for their antimicrobial activity against four clinical antibiotic resistant pathogens and their respective reference strains: *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli*. Antibacterial susceptibility was checked out by using broth microdilution method and interpreted according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) recommendations.

Results Significant differences in the antibacterial activity of tested honey samples were noticed. Fir honeydew honey and Mint honey showed the best antibacterial potential, while the Locust tree honey, Rapeseed honey and Spring pasture honey expressed the weakest antimicrobial activity.

Conclusion Croatian honey, prominently honeydew honey, has the potential to become an important additive to therapeutic techniques available to a medical practitioner against resistant pathogens, but the exact mechanisms of its activity should be investigated further.

Key words: antimicrobial activity, honeydew, pathogens, therapy

Corresponding author:
Darinka Vučković
Faculty of Medicine, University of Rijeka
B. Branchetta 20, HR-51000 Rijeka, Croatia
Phone: +385 51651 172;
Fax: +385 51651 177;
E-mail: darinka.vuckovic@medri.uniri.hr
Ivana Gobin ORCID ID: https://orcid.org/0000-0002-8956-4675

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INTRODUCTION

For a thousand of years honey has been used for the treatment of wounds. However, broader application in medicine is limited due to the lack of scientific support. Nowadays, the bacterial resistance to antimicrobial agents and numerous kinds of antibiotics, including the major resort drugs, is increasing worldwide (1-3). Therefore, alternative antimicrobial strategies are urgently needed and the potent activity of honey against antibiotic resistant bacteria resulted in renewed interest for its application (4-6). An additional advantage of topical use of honey as an antimicrobial agent is the fact that bacterial resistance to honey has not yet been described. The reason lies in the fact that honey inhibits bacteria using several mechanisms at the same time, which prevents them from developing resistance (7,8). Antibacterial activity of different honey is mainly due to hydrogen peroxide production, as well as the presence of various phytochemicals. In addition, honey is hygroscopic, which means that it can dehydrate bacteria, and its high sugar content and low pH level can also prevent the microbes from growth (7-9). In addition, unknown floral or bee components contribute to antibacterial activity (8-10). There are many types of honey that originate from different geographic areas and are made from different floral sources. Studies have shown that honey with these marked differences can have different antimicrobial activities and mechanisms of action (7,11). In addition, the antibacterial potency of diverse, locally produced honey is not possible to predict. Some studies showed that even honey collected from a single location can have significant batch-to-batch variation in antibacterial activity (10). The unpredictable antibacterial activity of such unstandardised honey preparations hampers its introduction as an antimicrobial agent (6).

Croatia has a large floral biodiversity and honey produced from these plants has been sold commercially in Croatia and in EU countries. However, data on antibacterial activity of different types of honey produced in Croatia are very scarce. Therefore, the aim of this study was to investigate the antibacterial activity of domestic honey from different floral origin, as well as honeydew honey, a bee product obtained from excretion of plant sucking insects, on selected pathogen bacteria.

MATERIAL AND METHODS

Honey samples, bacteria and study design

Twelve honey samples (HS) from different plant origin were obtained during the year 2014 (Table 1) and its antimicrobial activity was tested using bacterial strains originated from the collection of the Department of Microbiology and Parasitology, Faculty of Medicine, University of Rijeka, Croatia. Honey samples originated from different geographic areas in the Western part of Croatia: Sage honey, Maple honeydew, Fir honeydew, Chestnut honey,

Locust tree honey, Lime tree honey, Indigo bush honey, Rapeseed honey, Maple honey, Mint honey, Spring pasture honey and Autumn pasture honey.

Table 1. The origin of tested honey samples

Honey samples	Number of samples	Type
Sage honey *(Salvia officinalis L.)*	1, 2	monofloral
Maple honeydew *(Acer spp.)*	1	honeydew
Fir honeydew *(Abies alba Mill)*	1, 2	honeydew
Chestnut honey *(Castanea sativa Mill)*	1	monofloral
Locust tree honey *(Robinia pseudoacacia L.)*	1	monofloral
Lime treehoney *(Tilia spp.)*	1	monofloral
Indigo bush honey *(Amorfa fruticosa L.)*	1	monofloral
Rapeseed honey *(Brassica napus L.)*	1	monofloral
Maple honey *(Acer spp.)*	1	monofloral
Mint honey *(Mentha spp.)*	1	monofloral
Spring pasture honey	1	polifloral
Autumn pasture honey	1	polifloral

Methods

No mechanical treatment or heat was used in sample preparations. The HS was stored at +4 °C in hermetically closed glass bottles until used. The sediments of the HS were analysed according to the method recommended by the International Commission for Bee Botany of International Union of Biological Sciences (12). The antibacterial activity of tested HS was investigated using the reference bacterial strains: *Staphylococcus aureus* ATCC 25923, *Acinetobacter baumannii* ATCC BAA–1605 (multidrug resistant), *A. baumannii* ATCC 19606 (drug sensitive), *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 as well as several clinical bacterial isolates such as methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa* (multidrug resistant) and extended spectrum beta-lactamase (ESBL) - positive *E. coli*. The bacteria were stored at -80 °C in glycerol broth (10% glycerol) (Biolife, Italy). For the experiments, bacteria were thawed at room

temperature, resuspended in Mueller-Hinton broth (MHB) (Difco, MD, USA) for 24 h at 37 °C and 120 rpm (Unimax 1010, Heidolph, Germany).

Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined using a broth dilution method, and interpreted according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) recommendations (13). Initial inoculum for all experiments was approximately 1.0×10^6 CFU/mL as determined by the turbidity of the bacterial suspension and confirmed retrospectively by plating the inoculum on Mueller-Hinton agar (MHA) (Difco, MD, USA) for 24 h at 37 °C. Further, twofold serial dilutions in MHB were prepared from a stock solution of each honey sample to get final concentrations ranging from 0.025 to 0.8 g/mL. The MIC was defined as the lowest concentration of a honey that produced no visible bacterial growth in the test tube when compared with the control. Minimal bactericidal concentration (MBC) was determined by transferring the broths used for MIC determination on the MHA. The broth with the lowest concentration of the tested honey that did not produce growth of the tested organism was considered MBC. Meropenem (Sigma, St Louis, MO, USA) was used as a positive control for growth inhibition when gram negative bacteria were used in the experiments, and vancomycin (Sigma, St Louis, MO, USA) when gram positive bacteria were used. The final antibiotic concentrations used in the assays as a control ranged between 0.00004 and 0.128 mg/mL for both antibiotics. For the interpretation of these results EUCAST recommendations were followed (13).

Statistical analysis

The differences between the tested honey samples with a respect to MICs and MBCs were statistically tested using Kruskal-Wallis test. The $p<0.05$ level of significance was applied.

RESULTS

The results of the antibacterial activity assay, performed with selected HS showed significant differences in MIC and MBC values when reference bacterial and antibiotic resistant strains were tested (Table 2 and 3). Analyzing MICs, the strongest antibacterial potential showed Fir honeydew honey 2 (MIC=0.11 g/mL), Fir honeydew honey 1 (MIC=0.12 g/mL) and Mint honey (MIC=0.13 g/mL) in a case of both reference and antibiotic resistant bacterial strains. On the contrary, the weakest overall antibacterial activity was showed by Locust tree honey (MIC=0.28 g/mL), Rapeseed honey (MIC=0.32 g/mL) and Spring pasture honey (MIC=0.40 g/

Table 2. Antibacterial activity of tested honey samples on referent bacterial strains

	Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of bacterial strain (g/mL)*									
	S. aureus **ATCC 25923**		***P. aeruginosa*** **ATCC 27853**		***E. coli*** **ATCC 25922**		***A. baumannii*** **ATCC BAA-1605**		***A. baumannii*** **ATCC 19606**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Honey samples										
Sage honey 1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1
Sage honey 2	0.1	0.1	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.2
Maple honeydew	0.05	0.05	0.2	0.4	0.4	>0.4	0.1	0.2	0.1	0.1
Fir honeydew 1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Fir honeydew 2	0.05	0.05	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Chestnut honey	0.1	0.1	0.2	0.4	0.2	>0.4	0.2	0.4	0.1	0.1
Locust tree honey	0.2	>0.4	0.4	>0.4	0.4	>0.4	0.2	0.4	0.2	0.4
Lime tree honey	0.2	0.2	0.4	0.4	0.4	>0.4	0.1	0.2	0.2	0.2
Indigo bush honey	0.05	0.2	0.2	0.2	0.4	0.4	0.1	0.1	0.1	0.1
Rapeseed honey	0.4	>0.4	0.4	0.4	0.4	>0.4	0.2	0.4	0.2	0.4
Maple honey	0.2	>0.4	0.2	0.4	0.2	>0.4	0.1	0.1	0.1	0.1
Mint honey	0.1	0.1	0.2	0.2	0.2	0.4	0.05	0.1	0.1	0.1
Spring pasture honey	0.4	>0.4	0.4	>0.4	0.4	>0.4	0.4	0.4	0.4	0.4
Autumn pasture honey	0.05	0.05	0.4	0.4	0.2	0.2	0.05	0.1	0.1	0.1
Drugs for positive control for growth inhibition										
Vancomycin	0.000001	0.000001	NT	NT	NT	NT	NT	NT	NT	NT
Meropenem	NT	NT	0.000000064	0.000000064	0.000000006	0.000000006	>0.000032	>0.000032	0.000000025	0.00000025

*MIC, concentration required for 99% bacteriostatic effect; MBC, concentration required for 99% of bacterial killing effect; NT, not tested

Table 3. Antibacterial activity of tested honey samples on antibiotic resistant bacterial strains

	Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of bacterial strain (g/mL)*									
	MRSA		*P. aeruginosa*		*E. coli*		*A. baumanii 771*		*A. baumanii 53154*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Honey samples										
Sage honey 1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1
Sage honey 2	0.1	0.1	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.2
Maple honeydew	0.05	0.05	0.2	0.4	0.4	>0.4	0.1	0.2	0.1	0.1
Fir honeydew 1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Fir honeydew 2	0.05	0.05	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1
Chestnut honey	0.1	0.1	0.2	0.4	0.2	>0.4	0.2	0.4	0.1	0.1
Locust tree honey	0.2	>0.4	0.4	>0.4	0.4	>0.4	0.2	0.4	0.2	0.4
Lime tree honey	0.2	0.2	0.4	0.4	0.4	>0.4	0.1	0.2	0.2	0.2
Indigo bush honey	0.05	0.2	0.2	0.2	0.4	0.4	0.1	0.1	0.1	0.1
Rapeseed honey	0.4	>0.4	0.4	0.4	0.4	>0.4	0.2	0.4	0.2	0.4
Maple honey	0.2	>0.4	0.2	0.4	0.2	>0.4	0.1	0.1	0.1	0.1
Mint honey	0.1	0.1	0.2	0.2	0.2	0.4	0.05	0.1	0.1	0.1
Spring pasture honey	0.4	>0.4	0.4	>0.4	0.4	>0.4	0.4	0.4	0.4	0.4
Autumn pasture honey	0.05	0.05	0.4	0.4	0.2	0.2	0.05	0.1	0.1	0.1
Drugs for positive control for growth inhibition										
Vancomycin	0.000001	0.000001	NT	NT	NT	NT	NT	NT	NT	NT
Meropenem	NT	NT	>0.000032	>0.000032	0.000000006	0.000000006	>0.000032	>0.000032	0.00000025	0.00000025

*MIC, concentration required for 99% bacteriostatic effect; MBC, concentration required for 99% of bacterial killing effect; NT, not tested

mL). For *S. aureus* MIC values ranged from 0.05 g/mL (Fir honeydew honey, Maple honeydew honey and Autumn pasture honey) to 0.4 g/mL (Rapeseed honey and Spring pasture honey), and this bacterium was the most susceptible to all tested honey samples. From all gram negative bacteria, antibiotic sensitive strain of *A. baumannii* was the most sensitive strain with MIC values ranged from 0.1 g/mL to 0.4 g/mL. Suprisingly, multidrug resistant *A. baumannii* strain was also sensitive to all tested honey samples with MIC values ranged from 0.05 g/mL (Autumn pasture honey and Mint honey) to 0.4 g/mL (Spring pasture honey). When comparing sensitivity to honey of antibiotic sensitive and resistant strains of tested bacterial species no statistically significant differences have been proven.

Analyzing bactericidal concentration of honey samples to all tested bacteria strains, the best results showed Fir honeydew honey 2 (MBC=0.13 g/mL), Fir honeydew honey 1 (MBC=0.14 g/mL) and Sage honey 1 (MBC=0.16 g/mL). There were no significant differences in sensitivity to tested honey samples among antibiotic resistant and sensitive bacterial strains. Again, the highest MBCs were determined for Rapeseed honey (MBC=0.56 g/mL), Locust tree honey (MBC=0.64 g/mL) and Spring pasture honey (MBC=0.64 g/mL) regarding the bacterial sensitivity to antibiotic. *S. aureus* and *A. baumannii* regardless of sensitivity to antibiotics, were the most sensitive to all HSs, especially to Fir honeydew and Mint honey with MICs ranging from 0.05 g/mL – 0.1 g/mL, and MBCs ranging from 0.05 g/mL – 0.1 g/mL, respectively.

DISCUSSION

There are several studies in which dark honey from the conifer forests of the mountainous regions of central Europe has been found to have particularly high antimicrobial activity (8,9,14,15). This honey is not from the nectar source, but from honeydew, produced by aphids sucking the sop from the leaves of the trees (14,15). This coincides with our results which showed that two samples of honeydew (Fir honeydew 1 and Fir honeydew 2) possess the best antibacterial activity according to the MIC as well as MBC values shown. Antibiotic resistant pathogens like MRSA, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterococcus faecalis* are major causes of severe infections in hospitalized patients leading to longer hospital stays and higher mortality rates worldwide (2).

A good example of antibiotic resistant bacterium is *A. baumannii, which* is an opportunistic pathogen that usually infects immunocompromised individuals through open wounds, catheters and

breathing tubes (16). It is well documented that *A. baumannii* rapidly develops resistance to antibiotics and it is a real challenge to treat infections caused by this bacterium. Our results showed that *A. baumannii* as well as *S. aureus* were the most sensitive to honey, especially to Fir honeydew honey and Mint honey. These results suggest that they might be used in treating bacterial infections, especially those caused with *S. aureus* and *A. baumannii*. This is in line with the report of other authors who found that honey was effective against antibiotic-resistant bacteria that colonize burn wounds, such as MRSA, vancomycin-resistant *Enterococcus* spp. (VRE) and multiple-resistant Gram-negative rods, including *P. aeruginosa*, *Acinetobacter* spp. and members of the Enterobacteriaceae family (17-20). Furthermore, recent reviews on the successful usage of honey as a dressing on infected wounds show that many authors support the use of honey when treating infected wounds and some suggest even the prophylactic use of honey on the wounds of patients susceptible to MRSA and other antibiotic-resistant bacteria (21-23).

Large variations in antimicrobial activity exist among honey collected from different environments, which is possibly related to spatial and temporal variations in sources of nectar (10,24,25). Other reasons behind the recognition of honey as an effective antimicrobial agent are the use of non-standardized laboratory methods for testing. While antibiotic susceptibility testing uses standardized methods like EUCAST Recommendations, for antibacterial testing of natural products there are no such recommendations (13). It is very difficult to compare the results of different scientific studies and the results are very often confusing. Most commonly used in the research are the diffusion method (agar well or disk diffusion methods) and the antimicrobial activity of honey is measured by a size of the inhibition zone. However, it was found that a disc impregnated with various concentrations of honey added to an agar plate became dry because of vaporization of fluid from the disc when the media were incubated at 37 °C for 24 hours. Also, honey is a mixture of different molecules that will not equally well diffuse through the agar and the results will not be precise. Therefore, we consider that assessment of the inhibitory and bactericidal concentration is a much better choice of testing because honey will easily come into contact with bacteria in the liquid media and such a complex mixture of compounds will better demonstrate its effect. By using this method, it was easy to find the MIC and MBC of tested honey that inhibited the growth of pathogens.

In our study, the weakest antibacterial potential was obtained by Locust tree honey, Rapeseed honey and Spring pasture honey. For these honey samples, we can assume that they do not have the necessary ingredients or synergy among active components that would affect the bacteria. Namely, antibacterial activity of honey is quite a complicated issue due to the involvement of multiple ingredients, and large differences in their concentrations between the different types of honey. Closer determination of antibacterial compounds and their activities, and their involvement in the antibacterial activity of honey could enable the standardization and help removing the main obstacles for the application of honey in the therapy of infections.

In conclusion, some honey of Croatian origin, like Fir honeydew and Mint honey, could be potentially applied as an antibacterial agent in medicine. We also demonstrated that locally produced honey possess excellent antibacterial activity comparable to the commercial honey. Our work shows that discovery and research of the antimicrobial potential of different honey samples are of great importance and may contribute to the fight against bacterial resistance.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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