EXPERIMENTAL STUDY

Excellent antibacterial activity of Slovak honeys on bacteria mostly infecting chronic wounds

Zemanova M1, Slobodnikova L2, Cambal M1, Labas P1

1st Department of Surgery, Faculty of Medicine, Comenius University in Bratislava and University Hospital Bratislava, Bratislava, Slovakia. zemanova.maria@gmail.com

ABSTRACT

INTRODUCTION AND AIM OF STUDY: Chronic wounds are commonly colonized by various bacterial species and colonization frequently turns into wound infection, severely impairing healing process. With increasing antimicrobial resistance, the antimicrobial treatment of chronic wounds may be extremely challenging. Rediscovery of old and forgotten antimicrobial therapeutic options, such as apitherapy, may contribute to solving the problem of incurable chronic wound infections. Aim of this study was to evaluate the antimicrobial properties of four kinds of Slovak honey from ecological beekeeping against the most common bacterial species contaminating and infecting chronic wounds, and to compare these antimicrobial activities with those of the approved medical-grade Manuka honey. The impact of honey sterilisation methods and long-lasting storage on the bactericidal activity was also examined.

MATERIAL AND METHODS: Antimicrobial activity of honey was detected against 7 bacterial collection strains by broth microdilution antimicrobial susceptibility test according to EUCAST. The results were statistically analysed by Fisher’s exact test.

RESULTS AND CONCLUSIONS: Slovak ecologically produced honey samples demonstrated an excellent in vitro antibacterial activity, superior to the monofloral medical-grade Manuka honey activity. Neither the gamma-irradiation, nor the three-year-long storage had impact on the bactericidal activity of the tested honey (Tab. 4, Fig. 2, Ref. 53). Text in PDF www.elis.sk

KEY WORDS: chronic wounds, apitherapy, bacterial resistance, antibacterial activity, honey, medical-grade honey.

Introduction

Chronic wounds currently represent enormous medical, economic and social problem all over the world. They are defined as wounds which are not healed to full anatomical and functional integrity within 3 months (1).

According to The Wound Healing Society, there are four main groups of chronic wounds based on aetiology – venous ulcers, arterial insufficiency ulcers, diabetic foot ulcers and pressure ulcers (2).

These wounds are commonly colonized by various bacterial species, most commonly Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii (3), Escherichia coli, Enterococcus faecalis and Proteus mirabilis (4). Unfortunately, colonization frequently turns into wound infection, which severely impairs healing processes (5, 6) and if not treated successfully, may lead to several complications such as cellulitis, haemorrhage, or gangrene, leading to radical surgical procedures (such as extremity amputation), sepsicaemia, renal failure, or even death of the patient (1, 7, 8).

Nowadays, in the era of increasing antimicrobial resistance among microorganisms, extensive research on new and more effective antibiotics is facilitated (9). On the other hand, alternative non-antibiotic treatments are applied, and the old and forgotten antimicrobial therapeutic options are being rediscovered.

The current available alternative topical treatment methods of chronic wounds include topical antiseptic agents, such as chlorine dioxide, sodium chloride, acetic acid, cadexomer iodine, cetrimide, chlorhexidine gluconate, povidone iodine, sodium hypochlorite, hydrogen peroxide, or silver dressings (10). The old, for years neglected non-antibiotic antimicrobial treatment methods are represented by phage therapy, (7, 11) maggot therapy (12, 13), phytotherapy (14) or apitherapy. Apitherapy includes therapeutic usage of bee products, such as honey, propolis, royal jelly or bee venom therapy (15, 16, 17, 18, 19, 20, 21).

Honey is a unique natural compound. The definition of honey according to Codex Alimentarius is “natural sweet substance produced by honey bees from nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate,
store and leave in honey comb to ripen and mature*. There are two known honey types according to Codex Alimentarius: blossom or nectar honey (honey coming from plant nectars) and honeydew honey (from excretions of insects that suck secretions of living parts of plants) (22).

Honey is the only compound digestible for all animal species, it is non-toxic and has long lifespan, and, what is from the therapeutic points of view the most important - for microorganisms it is not possible to grow in honey. Thanks to the complex composition of honey, microbes cannot develop resistance to it (23). This is what makes honey so unique in nature and what made honey to be one of the oldest natural remedies widely used for treatment of various diseases for thousands of years. In medical use, honey was preferred for its ability to stimulate immunity, to treat infection and after local application to heal wounds. In fact, honey is the oldest wound dressing material known to humans (18).

As the antibiotic era started, honey was forgotten and replaced by various antibiotics. But nowadays, in the era of worldwide spread of resistant and polyresistant microbial strains, honey gives the hope to solve the problem of incurable wound infections.

In fact, the contemporary „western“ medicine of Europe, New Zealand and Australia has already accepted honey as a medicine for treatment of various wounds, skin and mucosal lesions (18).

The positive effect of honey on wound healing is due to its ability to rapidly eliminate microorganisms colonizing and infecting wounds, its ability of wound deodorization, and painless autolytic debridement of wound bed. Through reducing edema, honey improves circulation in capillaries and tissue oxygenation. It promotes tissue regeneration, stimulates angiogenesis, epithelisation and scar contraction. Its pain reducing effect has been proven as well. Due to its healing properties, honey signifies and scar contraction. Its pain reducing effect has been...

### Materials and methods

**The tested honey**

Six different types of honey were used in the tests (Tab. 1). The ecological Slovak honey (A–D), produced by Warré hives of *Apis mellifera* in pollution-free area in north-west Slovakia, and harvested by pressing to minimize the honey oxidation, was provided directly by the beekeeper. The commercial honeydew honey and the medicinal-grade Manuka honey were purchased in a retail-shop.

The honey samples were tested in their natural non-treated state, after filtration through bacteriological filter (0.45 mm; Merck Millipore Ltd., Ireland), and after gamma-irradiation. Gamma irradiation was performed in authorized company for radiation sterilization Bioster, Czech Republic, holder of ISO 9001, EN 46001 and ISO 11137/1,2,3 certificates. To evaluate the impact of the storage on antibacterial activity, the testing with the gamma-irradiated honey was repeated three years after the initial testing. Throughout the whole study, the honey was kept in cold, dry and dark conditions.

| Figure label | The tested honey | Specification |
|--------------|------------------|--------------|
| A            | Ecological Honeydew Honey | Polypollar honeydew honey, ecological, Slovakia |
| B            | Ecological Summer Forrest Honey | Polypollar nectar summer forest honey, ecological, Slovakia |
| C            | Ecological Summer Honey Sediment | Polypollar nectar summer honey sediment, ecological, Slovakia |
| D            | Ecological Spring Meadow Honey | Polypollar nectar spring meadow honey, ecological, Slovakia |
| E            | Medical Manuka Honey | Medical-grade monofloral Manuka honey, Activon Tube, New Zealand |
| F            | Commercial Honeydew Honey | Commercial polyfloral honeydew honey, Slovakia |
| G            | Glucose Solution | “Non-honey” sugar solution |
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The tested bacterial strains

Collection strains of seven bacterial species, selected with respect to the spectrum of bacteria most commonly contaminating and infecting the human chronic wounds, were included into the study (Tab. 2). The strains were purchased from the Czech Collection of Microorganisms, Brno, Czech Republic.

Antimicrobial activity testing

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of honey was evaluated using broth microdilution assay according to EUCAST, corresponding to ISO 20776-1:2019 (EUCAST, 2020) (48). Sterile U-shaped 96-wells microtiter plates (Roll s.a.s., Italy) and Mueller-Hinton Broth (OXOID UK) were used in the assay.

Prior to each of the test runs, aliquots of honey were freshly diluted in the antimicrobial susceptibility testing medium. Serial 1:1 geometric dilutions of honey, ranging from 50 % (w/V; equal to 500 mg.mL⁻¹) to 3.125 % (w/V, equal to 31.25 mg.mL⁻¹), were prepared.

As a “non-honey” control, sugar solution containing glucose in amount corresponding to sugar content in honey (80 % w/V) was used. The particular serial dilutions of honey and the glucose solution were applied into the corresponding wells of the microtiter plate in 100 mL volumes.

Bacterial inoculae were prepared in sterile physiologic solution from overnight bacterial cultures grown on blood agar. Bacterial suspensions were standardized using DEN-1 McFarland Densitometer (BioSan, Latvia) to reach 1.10⁶ CFU.mL⁻¹. The standardized suspensions were added to the corresponding microtiter wells in 10 μL aliquots (except to the sterility control wells, which contained bacteria-free medium or medium with the diluted honey samples only). Honey-free wells, inoculated by the tested bacteria, were used as growth control.

The MIC values were evaluated visually after an overnight incubation at 35 °C. MICs were determined as the lowest concentration of honey that completely inhibited the growth of the tested bacterial strain. MBCs were determined by sub-culturing the samples from wells without visible signs of bacterial growth; solid culture medium free of honey was used. After an overnight incubation at 35 °C, the MBCs were determined as the lowest concentration of the tested honey at which 99.9 % of bacterial inoculum was inactivated. The non-treated honey and the honey sterilised by filtration or gamma-irradiation were parallelly tested in the same runs. Three independent runs were performed for each bacterial species.

Analysis of results and their design

The results were submitted to statistical analysis by Fisher's exact test; the graphs and tables design was performed by the computer program Microsoft Excel (MS-office 2019; Microsoft Corporation).

Results and discussion

Antibacterial activity of the tested honey samples

Antibacterial activities of four kinds of ecologically produced honey from pollution-free area of north-west Slovakia were compared with those of medical grade Manuka honey and commercial honeydew honey. Bacteria most commonly isolated from chronic wounds were used in the tests; they included two Staphylococcus aureus strains (one was methicillin-susceptible, the second was methicillin-resistant), and one strain of Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis, Klebsiella pneumoniae and Proteus mirabilis (Tab. 2).

All the tested honey samples showed antibacterial inhibitory activity; however, some of them only at the highest (50 %) tested concentration (Fig. 1). Ecologically produced honeydew honey (honey A) and summer honey sediment (honey C) had significantly lower MICs to the majority of the tested bacterial strains in comparison with medical grade Manuka honey (p ≤ 0.1). Concerning the bactericidal potential (Fig. 2), the best

Tab. 2. Bacterial collection strains used in the study.

| Bacterial species                | Collection number of strain | Note       |
|---------------------------------|----------------------------|------------|
| Staphylococcus aureus (1)       | CCM 4750                   | MRSA       |
| Staphylococcus aureus (2)       | CCM 4223                   | MSSA       |
| Enterococcus faecalis           | CCM 4224                   |            |
| Pseudomonas aeruginosa          | CCM 3955                   |            |
| Escherichia coli                | CCM 3954                   |            |
| Klebsiella pneumoniae           | CCM 4415                   |            |
| Proteus mirabilis               | CCM 7188                   |            |

CCM – Czech Collection of Microorganisms; MRSA – methicillin-resistant S. aureus; MSSA – methicillin-susceptible S. aureus

Fig. 1. Bacteriostatic activity of honey samples (expressed in % w/V). A – Ecological Honeydew Honey, B – Ecological Summer Forrest Honey, C – Ecological Summer Sediment Honey, D – Ecological Spring Meadow Honey, E – Medical Manuka Honey, F – Commercial Honeydew Honey, G – Glucose Solution, MRSA – methicillin-resistant S. aureus MSSA – methicillin-susceptible S. aureus.
results were detected again with ecologically produced honeydew honey (honey A) and summer honey sediment (honey C), which inactivated the tested S. aureus strains at the 6.3% concentration, P. aeruginosa and P. mirabilis at 12.5%, E. coli at 25% and 12.5% concentration, respectively, and E. faecalis and K. pneumoniae at 25% concentration. The results were significantly superior (p ≤ 0.01) in comparison with those received with medical grade Manuka honey. These two kinds of honey were followed by ecologically produced honey from mixed flower sources (summer forest – honey B, and spring meadow – honey D) with MBCs from 6.3% to 50% concentrations. These results were also significantly better (p ≤ 0.1) than results obtained with Manuka honey, which inactivated the tested bacterial strains at concentrations from 25% to >50%. The less potent was commercial honeydew honey, with MBCs >50% for all of the tested bacterial strains except to P. aeruginosa (MBC = 50%). The glucose solution, when applied at the highest tested concentration, inhibited only the growth of glucose solution, when applied at the highest tested concentration.

Due to this high osmolarity and hygroscopicity, honey draws the moisture out of the surrounding environment causing dehydration of bacteria, which are not able to grow in these conditions (49). Even if the impact of osmotic and hygroscopic activity of honey should be considered in the final antibacterial activity of honey, this effect alone was not proved to be sufficient enough in inactivation of bacteria, as it is shown in our results with glucose solution in concentrations corresponding to sugar content of honey (sample G). Further, honey has a strong hydrogen peroxide activity. This activity is due to the content of glucose oxidase, which degrades glucose into gluconic acid, with hydrogen peroxide continuously formed as a secondary product, even if honey is being diluted. The antimicrobial activity of honey is also attributed to its low acidity. The gluconic acid, formed in honey during degradation reactions of glucose, yield the final pH between 3.2 and 4.5, which is inappropriate for growth of the majority of bacteria. Phytochemical plant products (e.g. monophasic and polymethenic compounds, or flavonoids), methyleglyoxal (the conversion compound of dihydroxycetone, found in the nectar of Manuka flowers), and antimicrobial peptides of bee origin (such as bee-derived defensin-1) belong to the last proposed antimicrobial tools of honey. All these compounds act as non-peroxide antimicrobial factors (18, 23, 49). The honey composition and its antibacterial activity is dependent on the floral origin of honey, geographical origin, climate conditions, way of beekeeping, way of honey processing.

The final antibacterial activity of honey on various bacterial species of Gram-negative and Gram-positive bacteria is probably due to the complex nature of honey. First of all, honey contains approximately 80% of sugars, mainly glucose and fructose. Sugars bind water and give hyperosmolar activity to honey. Tab. 3. Impact of sterilisation methods (filtration and gamma-irradiation) on antibacterial activity of honey samples (MBCs, expressed in μg.mL⁻¹).

| Bacterial species | Honey A | Honey B | Honey C | Honey D | Treatment |
|------------------|---------|---------|---------|---------|-----------|
| S. aureus (1)    | 62.5    | 62.5    | 62.5    | 125     | n.a.      |
| S. aureus (2)    | 62.5    | 62.5    | 62.5    | 125     | n.a.      |
| E. faecalis      | 250     | 500     | 250     | 500     | n.a.      |
| P. aeruginosa    | 125     | 125     | 125     | 250     | BF        |
| E. coli          | 250     | 500     | 250     | 500     | BF        |
| K. pneumoniae    | 125     | 125     | 125     | 250     | GI        |
| P. mirabilis     | 125     | 125     | 125     | 250     | BF        |
Conclusions

Slovak ecologically produced honey of polyfloral origin demonstrated an excellent in vitro antibacterial activity against bacteria commonly colonising and infecting chronic wounds. This activity was significantly superior to the monofloral medical-grade Manuka honey and to the commercial honeydew honey. Gamma-irradiation did not influence the bactericidal activity of honey, neither did it the three-years-long storage in cold, dry and dark conditions.

The highest therapeutic potential was presented by the samples of Slovak ecologically produced honeydew honey and summer honey sediment. They seem to be the most suitable candidates for a medical-grade honey preparation for chronic wounds topical therapy.
treatment as a bio-alternative, especially for patients in whom other approved treatment methods were not effective. Supportive clinical studies are necessary prior to their introduction to the therapeutic armamentarium.

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