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EXPERIMENTAL STUDY

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

Zemanova M[1], Slobodnikova L[2], Cambal M[1], Labas P[1]

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), septicaemia, 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,*

[1]1st Department of Surgery, Faculty of Medicine, Comenius University in Bratislava and University Hospital Bratislava, Bratislava, Slovakia, and [2]Institute of Microbiology, Faculty of Medicine, Comenius University in Bratislava and University Hospital Bratislava, Bratislava, Slovakia

Address for correspondence: M. Cambal, MD, PhD, MHA, MPH, 1st Department of Surgery, Faculty of Medicine, Comenius University in Bratislava and University Hospital Bratislava, Mickiewiczova 13, SK-813 69 Bratislava, Slovakia.

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 oedema, 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 significantly shortens healing time, reduces financial expenses and improves life quality of people suffering from chronic nonhealing wounds (24, 25, 26, 27, 28, 29).

Wound types successfully being treated by honey nowadays include infected wounds after trauma or surgery (e.g. amputation wounds, or abdominal postoperative wounds) (26), burns (30, 31), pressure sores (26), skin ulcers (32), diabetic foot ulcers (33), chronic venous ulcers (34), sickle cells ulcers (35), tropical ulcers (25), herpetic skin lesions (36), skin lesions from meningococcal septicaemia (37) or ophthalmic surface infections (18, 25).

In order to be used safely, honey has to be germ-free. Even if active and metabolising microorganisms cannot grow in honey, moulds, yeasts and spore-forming bacteria can persist in honey in the form of spores. In adequate conditions (e.g. in the wound cavity, digestive tract), spores can germinate and may start severe human infections (38, 39). Therefore, all medicine-grade honeys should be free of vital spores able to germinate, what should be obtained by a suitable sterilising process, not diminishing the antimicrobial and healing-stimulatory activity of honey (18, 40, 41).

Up till now, several approved medical grade honey therapeutic preparations are available, such as manuka honey, kanuka honey, or Revamil honey (standardized, medical-grade honey, produced under controlled conditions in greenhouses) (42, 43).

However, in order to search for new sources of valuable biologically active honey with high antimicrobial activity, several studies were done on honey of various geographical origin (44, 45, 46, 47).

In accordance with these efforts, the aim of our study was to evaluate the antibacterial properties of four kinds of Slovak honey from ecological beekeeping and to compare them with an approved medicine-grade Manuka honey and with a commercial honeydew-honey antibacterial activity. Furthermore, the potential impact of honey sterilisation methods (filtration or gamma-irradiation) and the long-lasting storage of honey on bactericidal activity was examined.

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 beekeeping of *Apis mellifera* in pollution-free area in north-west Slovakia, and harvested by pressing to minimalize 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.

Tab. 1. The tested types of honey and the non-honey sugar solution control (the letters correspond with the honey labels in the Figures 1 and 2).

Figure label	The tested honey	Specification
A	Ecological Honeydew Honey	Polyfloral honeydew honey, ecological, Slovakia
B	Ecological Summer Forrest Honey	Polyfloral nectar summer forest honey, ecological, Slovakia
C	Ecological Summer Honey Sediment	Polyfloral nectar summer honey sediment, ecological, Slovakia
D	Ecological Spring Meadow Honey	Polyfloral 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

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

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 1.10^{6} 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 ($p \leq 0.1$). Concerning the bactericidal potential (Fig. 2), the best

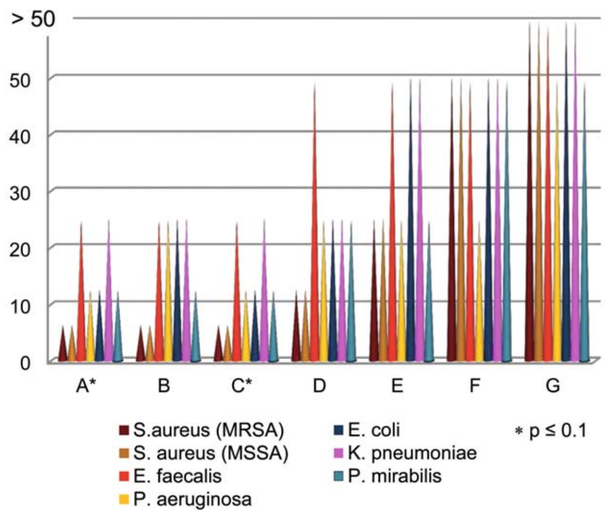

Fig. 1. Bacteriostatic activity of honey samples (expressed in % w/V). A – Ecological Honeydew Honey, B – Ecological Summer Forrest Honey, C – Ecological Summer Sediment Honey, D – Ecological Spring Meadow Honey, E – Medical Manuka Honey, F – Commercial Honeydew Honey, G – Glucose Solution, MRSA – methicillin-resistant S. aureus MSSA – methicillin-susceptible S. aureus.

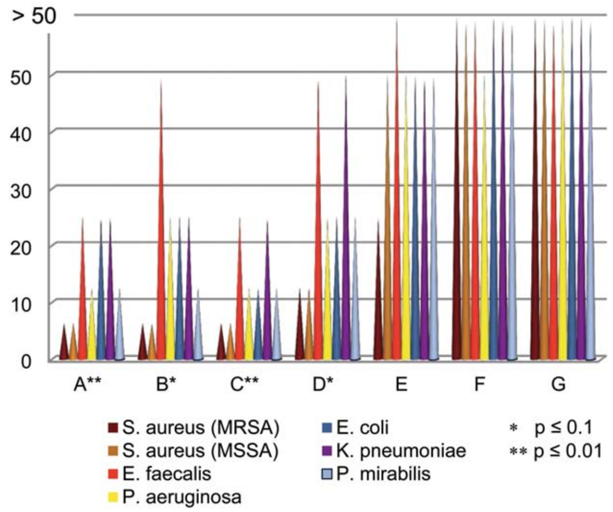

Fig. 2. Bactericidal activity of honey samples (expressed in % w/V). A – Ecological Honeydew Honey, B – Ecological Summer Forrest Honey, C – Ecological Summer Sediment Honey, D – Ecological Spring Meadow Honey, E – Medical Manuka Honey, F – Commercial Honeydew Honey, G – Glucose Solution, MRSA – methicillin–resistant 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 ($p \leq 0.01$) 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 ($p \leq 0.1$) 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 *P. aeruginosa* and *P. mirabilis*, but had no bactericidal effect. The most susceptible to the tested honey samples were the strains of *S. aureus*, without respect to their susceptibility or resistance to methicillin. Gram-negative bacteria were, in general, less susceptible; the most susceptible from this bacterial group were *P. aeruginosa* and *P. mirabilis*. The tested *E. faecalis* strain showed susceptibility to the tested honey samples with values between those of staphylococci and the Gram-negative bacteria.

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. 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 to 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. monophenolic and polyphenolic compounds, or flavonoids), methylglyoxal (the conversion compound of dihydroxyacetone, 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 process-

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.
	62.5	62.5	62.5	125	GI
	125	125	125	250	BF
S. aureus (2)	62.5	62.5	62.5	125	n.a.
	62.5	62.5	62.5	125	GI
	125	250	125	250	BF
E. faecalis	250	500	250	500	n.a.
	250	500	250	500	GI
	>500	>500	500	>500	BF
P. aeruginosa	125	250	125	250	n.a.
	125	250	125	250	GI
	250	500	250	500	BF
E. coli	250	250	125	250	n.a.
	250	250	125	250	GI
	500	500	250	500	BF
K. pneumoniae	250	250	250	500	n.a.
	250	250	250	500	GI
	500	500	500	500	BF
P. mirabilis	125	125	125	250	n.a.
	125	125	125	250	GI
	250	250	125	500	BF

A – Ecological Honeydew Honey; B – Ecological Summer Forrest Honey; C – Ecological Summer Sediment Honey; D – Ecological Spring Meadow Honey; n.a. – no treatment applied; BF – filtration through bacteriologic filter; GI – gamma irradiation; for the bacterial strains characteristics see the Table 2

Tab. 4. Impact of honey storage on antibacterial activity (MBCs, expressed in μg.mL^{-1}).

Bacterial species	Honey A	Honey B	Honey C	Honey D	Testing
S. aureus (1)	62.5	62.5	62.5	125	Time “0”
	62.5	125	62.5	125	+ 3 years
S. aureus (2)	62.5	62.5	62.5	125	Time “0”
	62.5	125	62.5	125	+ 3 years
E. faecalis	250	500	250	500	Time “0”
	250	500	250	500	+ 3 years
P. aeruginosa	125	250	125	250	Time “0”
	125	250	125	250	+ 3 years
E. coli	250	250	125	250	Time “0”
	250	250	125	250	+ 3 years
K. pneumoniae	250	250	250	500	Time “0”
	250	250	250	500	+ 3 years
P. mirabilis	125	125	125	250	Time “0”
	125	125	125	250	+ 3 years

For the legend see the Table 3.

ing and storage method (50, 51, 52). Therefore, it is very difficult (if not impossible) to produce particular types of honey, which would be unique in all aspects of the active molecules content and the final biologic activity. This fact is supported also by our results – the most potent honey samples (ecologically produced honeydew honey and summer honey sediment) had probably more rich content of antimicrobial molecules and antibacterial mechanisms with assumed synergistic microbicidal activity. Furthermore, the ecological honeydew honey (honey A) was more effective probably due to the additional content of potentially active components of insect origin (mostly of aphids and of some of the scale insects), and the antibiotic products of plant origin synthesized in reaction to plant tissue damage caused by insects. All these compounds may be contained in honeydew - the excretion of plant-sap sucking insects (53). The honeydew is then collected by bees and processed into honey. The second most effective honey sample was the Ecological Summer Sediment Honey. Its excellent bactericidal activity was probably the result of higher concentrations of active molecules, as this type of honey contained highly concentrated honey sediment. To confirm this hypothesis, qualitative and quantitative analysis of the major antibacterial compounds in the tested honey samples will be performed during the further study. The higher bactericidal effectivity of Slovak ecological honey samples used in our study may also point to the role of the beekeeping method (Warré hives are assumed to give more natural conditions for bee life than the other types of hives) (51), to the geographical impact on the honey quality (50) and to the better performance of polyfloral kinds of honey in comparison with monofloral honey (such as Manuka honey) (52).

Impact of honey sterilisation on antibacterial activity

During the apitherapy, the honey must be applied directly into the treated wound or skin defect; therefore, it is inevitable to guarantee its germ-free state. The risk for patient treated by apitherapy may impose the contamination of honey by bacterial of fungal spores. At the same time, the process of honey sterilisation must keep untouched the antimicrobial and heeling properties of honey. In our study, two ways of honey sterilisation were selected – filtration through bacteriologic filter and gamma-irradiation. Filtration requires that the honey be available in the form of solution. Medicinal application of honey in the form of solution may be useful in the patients treated by wound or infected body-cavities irrigation. The necessity to use freshly dissolved honey may be considered as a great disadvantage of such application, as it is a time- and work-consuming process, requiring sterile conditions. Moreover, if the honey was once diluted, it cannot be stored for any later applications. On the other hand, gamma-irradiation allows usage of concentrated honey, which can be long-lastingly stored and later used either in its concentrated form or in solution. The effect of these two sterilisation procedures on the honey antibacterial activity is shown in the Table 3.

Filtration, in general, increased the MBC values of the honey samples by one dilution. Due to the high complexity of honey, it is highly possible that some of the components with antibacterial activity were bound to the filter membrane during the honey filtration. On the other hand, the gamma irradiation process had no effect on antibacterial activity of the tested honey samples, and seems to be a superior sterilising procedure.

Impact of honey storage on antibacterial activity

Production of medicinal-grade honey requires a lot of time, labour and supplementary equipment. Moreover, the apitherapy is usually not provided as a single treatment. Therefore, it is important to be sure, that the honey will not lose its biological activities during storage. In our study, evaluation of the impact of long-lasting honey storage on the honey antibacterial properties was performed with honey samples sterilised by gamma-irradiation. The samples were re-tested for their bactericidal activity after three years storage at cold (4 to 6 °C) and dark place in closed containers to prevent the contact with air. All the tested honey samples preserved their antibacterial activity even after three-year storage and (with a few exceptions) no increase in MIC/MBC values was detected; the results are shown in the Table 4. One-fold dilution increase of the MBC values was detected only in the case of the Ecological Summer Forrest Honey (honey B) against both of the tested *S. aureus* strains.

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 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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