METHOD AND RESULTS REPORT

Antibacterial Activity of Honey

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Date: 06.03. 2018

Products:
Manuka honey – Manuka Honey MGO™ 550+
Manuka honey - Organic Manuka Honey TA25+
Local honey - Fir Honey ("Jedľová medovica")

Methods:
Agar well diffusion method for detection of antibacterial activity.

Aim of investigation:
The aim of this study is to compare antibacterial activity of several species of honey including two manuka honeys and one local honey against coagulase-positive *Staphylococcus aureus*.

Documentation
All test relating activities have to be documented on worksheets of protocols.
In reports for tests are documented:

1. Test sample: Identification,
2. Incubation period, incubation temperature
3. Attachement - pictures
4. Date, name and signature of person performing the test

TABLE OF CONTENS

1. Introduction

Honey is produced from many sources, and its antimicrobial activity varies greatly with origin and processing.
The antimicrobial activity in most honeys is associated with the enzymatic production of hydrogen peroxide, but also with its high osmolarity or acidity (low pH). However, some kind of honey, including manuka honey, showed significant antimicrobial activity even when the hydrogen peroxide activity is blocked. This activity is associated with the presence of phytochemical components like methylglyoxal (MGO).
Manuka honey is produced in New Zealand by bees that pollinate the native manuka bush and showed high antibacterial activity associated with activity of methylglyoxal **(1)**.
MGO is a compound found in most types of honey, but usually only in small quantities. In manuka honey, MGO comes from the conversion of another compound –dihydroxyacetone, that is found in the nectar of manuka flowers. Quantity of MGO in manuka honey varies in different type of manuka honey and those honeys are also labeled according their MGO activity or MGO content.

Labeling of manuka honey as MGO™ 550+ signifies, that 1 kg of this honey contains 550 mg of methylglyoxal.
Manuka honey labeled as TA 25+ mean that in those honey total antimicrobial activity is combination of peroxide activity and non-peroxide activity. Number is calculated by combining both the peroxide activity and non-peroxide activity. Total activity is in most cases referenced against a biological hydrogen peroxide assay test and does not express the activity of methylglyoxal.

In this study we have tested antibacterial activity associated with another than peroxide or osmolarity activity of three species of honey, including Manuka MGO™ 550+, Organic Manuka Honey TA25+ and one local honey form Slovakia – fir honey, against *Staphylococcus aureus*.

Peroxide activity in honey can be destroyed easily by heat or the presence of catalase **(2)**. Blood shows catalase activity, and this feature we have used to eliminate activity of hydrogen peroxide in tested honey. Dilution of the honey we have used to eliminate high osmolarity.

2. Principle

The purpose of this study was to determine the antibacterial activity against *Staphylococcus aureus* CCM 4516/ATCC 6538 associated with other than peroxide and high osmolarity activity of three samples of honey. Two samples of manuka honey: Manuka MGO™ 550+ and Organic Manuka Honey TA25+ and one sample of local honey Fir Honey ("Jedľová medovica") produced in Slovakia, Bardejov region.
For evaluating of antibacterial effect of the honeys we have used agar well diffusion bioassay. We have used Columbia Blood Agar with 7% Sheep Blood to eliminate hydrogen peroxide activity of honey. A 25 % (w/v) and 50% (w/v) solution of honey was prepared to eliminate antibacterial activity of high osmolarity.

3. Quality statement

All activities outlined in this protocol are subject to the valid standard operating procedures used at the microbiological laboratory of Eurofins BEL/NOVAMANN s.r.o., Piešťany, Slovakia.

4. Materials

4.1. Honey used

Manuka honey – Manuka Honey MGO^{TM} 550+
Manuka honey - Organic Manuka Honey TA25+
Local honey - Fir Honey ("Jedľová medovica")

All types of honey were diluted with sterile water to obtain 25 % and 50% solution.

4.2. Reference strains

The test organism suspension used for inoculation was prepared as overnight culture from microorganisms on gelatin discs (Czech Collection of Microorganisms, CCM) after appropriate dilution.

- *Staphylococcus aureus* CCM 4516/ATCC 6538

4.3. Nutrient media and solutions

The culture media was prepared according to actual SOP and media are tested for growth ability and for sterility before they are used for testing.
Columbia Agar was purchased in ready form at plates.

Tryptone Soya Agar (TSA) Biolab
Columbia Blood Agar with 7% Sheep Blood (CBA) – HPL SERVIS
Tryptone sodium chloride solution (TS) – Centralchem, Biolab
Sterile distilled water
McFarland Standard, BioMérieux

TSA was used as control media for comparing of antibacterial activity of the samples of honey.
CBA was used to evaluate of antibacterial efficacy of non-hydrogen peroxide activity of samples of honey.
TS was used as diluent for preparation of test suspension of microbiology strain.
McFarland Standard was used as comparing standard to adjust turbidity of test suspension of inoculum of *Staphylococcus aureus*.

4.4. Equipment and materials

- Incubator 36-37°C- Automatic pipettes, Brand, Merck
- Vortex, VWR
- Automatic pipettes

- Sterile tips for automatic pipettes, Brand, Merck
- Sterile tubes 15ml, Biolab
- Sterile swab applicator, Biolab
- Digital caliper, Jiangsu S.LTD

5. Test procedure

The all procedures are performed under aseptic conditions to avoid contamination or cross-contamination.
For evaluating of antibacterial effect of the honeys we have used agar well diffusion bioassay. This assay followed Manual of Antimicrobial Susceptibility Testing **(3)** and Methods for in vitro evaluating antimicrobial activity: A review **(4)**.

5.1. Preparation of Test Suspension

- The test solution of *Staphylococcus aureus* was prepared from working culture.
  Working culture is subculture from the stock culture prepared by streaking on 90 mm TSA plates and incubated 24h/36-37°C.
- After incubation colonies was transferred into TS and suspended.
- The turbidity of the test suspension was standardized to turbidity equivalent to a 0,5 McFarland using a McFarland standard to obtain approximately 1-2 x 10^8 CFU/ml.
- Test suspension was used within 2 hours
- The number of CFU in test suspension was confirmed by diluting and inoculating of TSA using spread plate technique. Amount of 0,2 ml of suspension from suitable dilution was taken to TSA plate.

5.2. Preparation of samples of honey

- Three types of honey was alalysed: Manuka Honey MGO™ 550+, Organic Manuka Honey TA 25+, Fir Honey
- From each type of honey was prepared a suspension at concentration 25 % (w/v) and 50 % (w/v). Sterile distilled water was use as diluent.

5.3. Preparation of Agar Plates to testing

- Tryptone Soya Agar (TSA) and Columbia Blood Agar (CBA) was used warmed to room temperature.
- Vortex test suspension of *Staphylococcus aureus* to make sure it is well-mixed.
- Sterile swab was dipped into the suspension and the excess liquid from the swab was removed by pressing it against side of tube.
- Surface of culture media was inoculated by spreading over the entire agar surface.
- A hole with diameter approximately 8 mm was punched aseptically with a sterile metal pipe. At each agar medium was punched four holes.
- Each hole was marked from opposite site of petri dishes to recognize each sample and concentration
- The same procedure of preparing of holes was done but without inoculating with *Staphylococcus aureus* for preparation of blind attempt-nagative product controls

5.4. Testing procedure

- Using the method described above, we have prepared 2 x 5 parallel of CBA inoculated with *Staphylococcus aureus* and 2 x 5 parallel of CBA without *Staphylococcus aureus* (blind attempt-negative product controls)
- Using same procedure we have prepared 2 x 5 parallel of TSA inoculated with *Staphylococcus aureus* and 2 x 5 parallel of TSA without *Staphylococcus aureus* (blind attempt - negative product controls)
- Each hole has been marked from the back side of the Petri dish.
- Prepared agar plates were divided into 2 groups, and 50 % and 25% solutions of samples of honey were added into each hole:
  no.1 = Manuka Honey MGO™ 550+
  no.2 = Organic Manuka Honey TA25+
  no.3 = Fir Honey ("Jedľová medovica")
  V = Sterile distilled water
  Volume 100 µl of suspension of honey and sterile distilled water were added to the each hole.
- Petri dishes with samples were incubated at 36-37°C for 18-24 hours at aerobic conditions

5.5. Measuring zones

- zones have been measured form the back of the Petri dish and the outer circumference was measured
- for measured of diameter zone was used caliper
- in the case of double zone, innermost zone was measured
- the zone size is interpreted in mm as Zone Diameter of Inhibition (ZDI)

5.6. Acceptance criteria

Blind attempt-negative product controls shall be without any contamination.
All culture media and diluent shall be without any contamination.

6. Personnel

RNDr. L. Jendeková, PhD. – responsible for testing plan, performance and testing
Mgr. S. Mateičková – responsible for testing, aseptic area preparation, media preparation and media control

7. Literature

(1) Molan PC: The antibacterial activity of honey. 2. variation in the potency of the antibacterial activity. *Bee World* 1992; 73:59-76
(2) Mandal MD, Mandal S Honey: Its medicinal property and antibacterial activity, *Asian Pac J Trop Biomed 2011*; 1(2):154-160

(3) MB Coyle, Manual of Antimicrobial Susceptibility Testing , American Society for Microbiology, © 2005
(4) M Balouiri, M. Sadiki, S K Ibnsouda, Methods for in vitro evaluating antimicrobial activity: A review, J Pharm Analysis 6 2016; 71-79

8. Results

In the Results Worksheet find the results of antibacterial activity of three samples of honey:
no.1 = Manuka Honey MGO™ 550+
no.2 = Organic Manuka Honey TA25+
no.3 = Fir Honey ("Jedľová medovica")
against *Staphylococcus aureus*.

The results listed in **Table III. and picture no. 1** shows that all three tested honeys were free of any microbial growth. Results were expressed as blind attempt control/negative product control.

Table IV. shows antibacterial effect of tested honeys against *Staphylococcus aureus* CCM 4516/ATCC 6538, which was expressed by measuring of zone diameter of inhibition.

For all tested honey types at a 50 % concentration, there was detected inhibition of growth of *Staphylococcus aureus* at TSA medium (**picture no. 5**). The largest zone of inhibition was detected around sample no. 3 (Fir Honey "Jedľová medovica") – average 21,72mm. Sample no.1 (Manuka Honey MGO™ 550+) showed average of inhibition zone 17,40mm and sample no.2 (Organic Manuka Honey TA25+) showed zone of inhibition in diameter 16,53mm.

At the same cultivation medium at a concentration of 25 % showed (**picture no. 2 and 3**) inhibition of *Staphylococcus aureus* only sample no.3 (Fir Honey "Jedľová medovica") – average 16,08mm and sample no.1 (Manuka Honey MGO™ 550+) – average 12,00mm. Sample no.2 (Organic Manuka Honey TA25+) at a concentration of 25% has no inhibition effect on *Staphylococcus aureus* at TSA medium.

At Columbia Blood Agar (CBA) there was detected inhibition effect against *Staphylococcus aureus* only at sample no.1 (Manuka Honey MGO™ 550+) at a concentration of 50% (**picture no. 6**). Average of zone diameter of inhibition was 12,59mm. This type of inhibition is due to non-peroxide activity, because peroxide was inhibited by blood.

At a concentration of 25 % we did not detected any antibacterial effect against *Staphylococcus aureus* of any type of honey (**picture no. 4**).

The results described above indicate that, all three types of honey showed antibacterial activity against *Staphylococcus aureus* only at Tryptone Soya Agar (TSA) but only Manuka Honey MGO™ 550+ showed antibacterial activity at Columbia Blood Agar (CBA).

According those results we can conclude, that only Manuka Honey MGO™ 550+ has antibacterial activity not only associated with peroxide activity but also with non-peroxide antibacterial activity presented with methylglyoxal.

Results Worksheet

Table I.: Reference strains			
Organism	CCM/ATCC	Manufacturer	Expiry date
Staphylococcus aureus	4516/ 6538	Czech Collection of Microorganisms (CCM), Brno	05/2019

Table II.: Cultivation Media – control of sterility			
Media/Batch no.	Incubation temperature	Results	Test date
Tryptic Soya Agar/01.02.18	36-37°C	sterile	27.02.2018
Columbia Blood Agar with 7% Sheep Blood /KACO00090118	36-37°C	sterile	27.02.2018
Tryptone sodium chloride solution/01.02.18	36-37°C	sterile	27.02.2018
Sterile distilled water /26.02.18	36-37°C	sterile	27.02.2018

Table III. Blind attempt (mm)		**Honey concentration (%)**					
		50			**25**		
		sample no. 1	sample no. 2	sample no. 3	sample no. 1	sample no. 2	sample no. 3
Cultivation media	CBA	0	0	0	0	0	0
	TSA	0	0	0	0	0	0

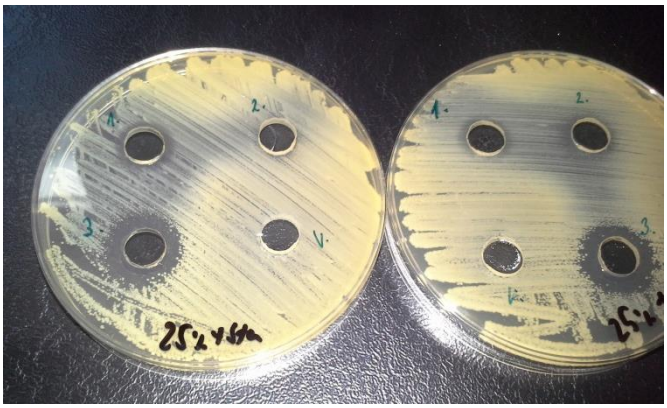
Results Worksheet

Table IV. Zone Diameter of Inhibition (mm)		**Honey concentration 50 %**			**Honey concentration 25 %**		
		sample no. 1	sample no. 2	sample no. 3	sample no. 1	sample no. 2	sample no. 3
		Zone Diameter of Inhibition (mm)					
Cultivation media	CBA+St. aureus	12,32	0	0	0	0	0
	CBA+St. aureus	13,58	0	0	0	0	0
	CBA+St. aureus	12,30	0	0	0	0	0
	CBA+St. aureus	12,97	0	0	0	0	0
	CBA+St. aureus	11,79	0	0	0	0	0
	Average Zone diameter of inhibition (mm)	12,59	0	0	0	0	0
	TSA+St. aureus	18,64	17,07	22,54	13,02	0	16,06
	TSA+St. aureus	18,56	18,36	23,46	10,29	0	14,29
	TSA+St. aureus	17,24	16,82	21,17	12,69	0	16,53
	TSA+St. aureus	16,69	15,10	19,48	11,82	0	17,40
	TSA+St. aureus	15,87	15,30	21,94	12,20	0	16,13
	Average Zone diameter of inhibition (mm)	17,40	16,53	21,72	12,00	0	16,08

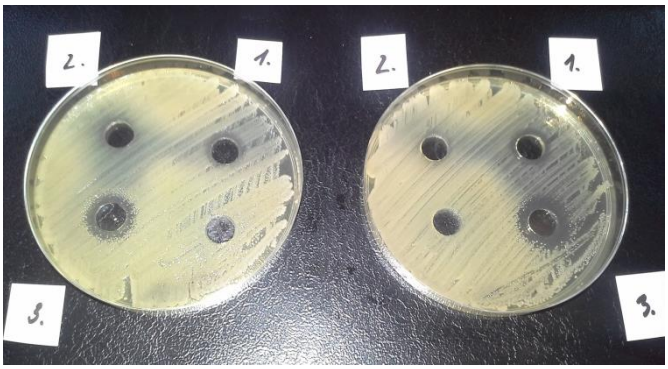
sample no. 1 = Manuka Honey MGO™ 550+
sample no. 2 = Organic Manuka Honey TA 25+
sample no. 3 = Fir Honey ("Jedľová medovica")

Attachement

no. 1
Blind attempt control/negative product control
at a concentration of 25 % and 50 %
(Alpha hemolysis around holes is caused by hydrogen peroxide produced by honeys)

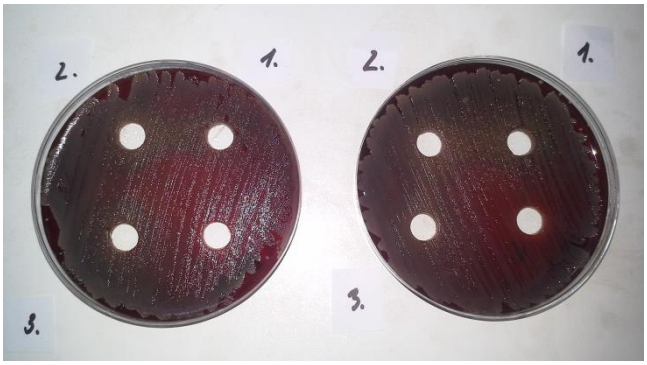


no. 2
Zones of inhibition at a concentration of 25 % at TSA
(1 = Manuka Honey MGO™ 550+, 2= Organic Manuka Honey TA25+, 3 = Fir Honey "Jedľová medovica")



no. 3
Zones of inhibition at a concentration of 25 % at TSA
(1 = Manuka Honey MGO™ 550+, 2= Organic Manuka Honey TA25+, 3 = Fir Honey "Jedľová medovica")

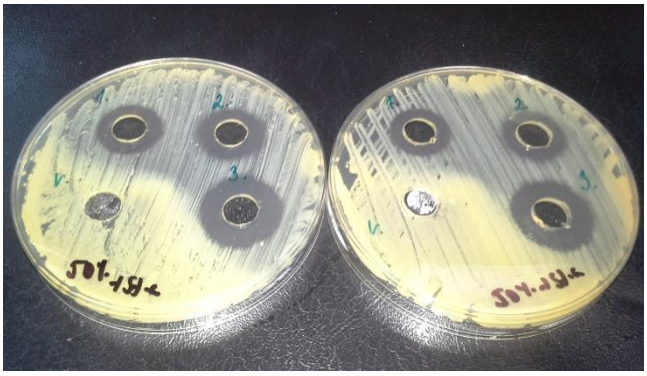
Attachement



no. 4

No zones of inhibition at a concentration of 25 % at CBA

(1 = Manuka Honey MGO™ 550+, 2= Organic Manuka Honey TA25+, 3 = Fir Honey "Jedľová medovica"

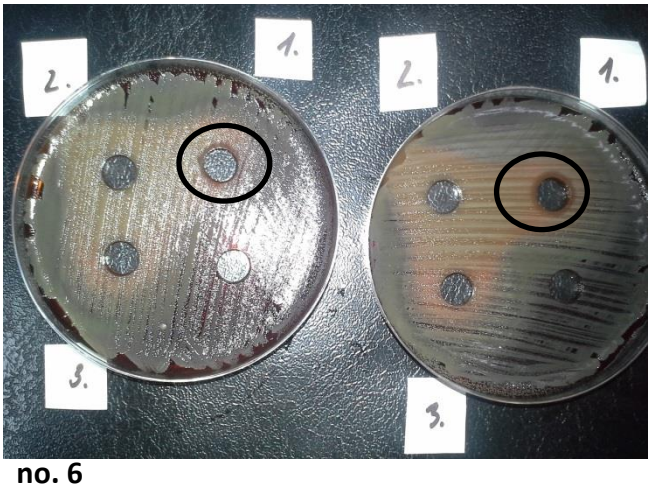


no. 5

Zones of inhibition at a concentration of 50 % at TSA

(1 = Manuka Honey MGO™ 550+, 2= Organic Manuka Honey TA25+, 3 = Fir Honey "Jedľová medovica")

Attachement



no. 6

Zones of inhibition at a concentration of 50 % at CBA

(1 = Manuka Honey MGO™ 550+, 2= Organic Manuka Honey TA25+, 3 = Fir Honey “Jedľová medovica”

Alpha hemolysis around holes is caused by hydrogen peroxide produced by honeys)

Date: 06.03. 2018

Name: RNDr. Lýdia Jendeková, PhD.

Signature: