DOI 10.2478/JAS-2018-0012 J. APIC. SCI. VOL. 62 NO. 1 2018

DE GRUYTER OPEN

Original Article

ROYAL JELLY ALIPHATIC ACIDS CONTRIBUTE TO ANTIMICROBIAL ACTIVITY OF HONEY

Walerij Isidorov[1*]
Stanisław Witkowski[2]
Piotr Iwaniuk[3]
Monika Zambrzycka[3]
Izabela Swiecicka[3]

[1] Forest Department, Bialystok University of Technology, Poland
[2] Department of Natural Product Chemistry, Institute of Chemistry, Białystok University, Poland
[3] Department of Microbiology, Institute of Biology, Białystok University, Poland

*corresponding author: isidorov@uwb.edu.pl
Received: 13 November 2017; accepted: 10 May 2018

Abstract

Honey is valued for its therapeutic qualities which are attributed among others to its antibacterial multifactorial properties. However, all the factors that influence these properties have not been identified. The present study is focused on the antibacterial action of fatty acids originating from royal jelly, the larval food of honeybees. Aliphatic C8-C12 acids characteristic of this bee product had previously been identified in more than fifty different samples of honey originating from seven countries and in eleven samples of Polish herbhoney. Experiments were performed to ascertain the influence of acidity on the antimicrobial activity of the acids. In acidic nutrient media all tested aliphatic hydroxyacids and unsaturated dicarboxylic acids demonstrated antibacterial action against different microbes with minimal inhibitory concentrations between 0.048 and 3.125 mM. Our results confirm that part of the antibacterial activity of honey contributes to these compounds of bee origin.

Keywords: antimicrobial activity, honey, royal jelly acids

INTRODUCTION

Owing to antimicrobial and wound healing properties, honey is also widely used in apitherapy (Mizrahi & Lensky, 1996; Moore et al., 2001; Kwakman et al., 2010a; Al-Waili et al., 2011; Giles & Laheij, 2017). There has been an upsurge of interest in the use of honey to control the growth of foodborne pathogens (Taormina, Niemira, & Beuchat, 2001; Fidaleo, Zuorro, & Lavecchia, 2010; Lee & Lee, 2016). The antibacterial action of honey is attributed to high osmolarity, as well as to the content of methylglyoxal, MGO (Weston, Brocklebank, & Lu, 2000; Mavric et al., 2008; Sultanbawa et al., 2015), the bee protein defensin-1 (Valachová, Bučeková, & Majtán, 2016), phenolic acids (Aljadi & Yusoff, 2003) and hydrogen peroxide (Brudzinski, 2006; Brudzinski et al., 2011; Brudzinski & Sjaarda, 2015; Brudzinski et al., 2017; Grecka et al., 2018). Besides these well characterized antibacterial factors, honey is thought to contain other unknown compounds with bacteriostatic or bactericidal activity (Kwakman et al., 2010b; Al-Waili et al., 2011; Bučeková & Majtán, 2016; Fyle et al., 2017). This belief is based on the preservation of antibacterial activity by honey after the successive neutralization of H_2O_2, MGO and defensin-1 (Kwakman et al., 2010a). Moreover, Bogdanov (1997) concluded that the high "residual" activity of honey was associated with its acidic fraction and was of bee origin.

The acidic components of bee origin are represented in honey solely by the set of C_8, C_{10} and C_{12} hydroxy fatty acids and unsaturated dicarboxylic acids typical of royal jelly(RJ) (Isidorov et al., 2009; Isidorov et al., 2011). A long time ago, Blum, Novak, & Taber (1959) determined that

the main RJ acid, 10-hydroxy-2(E)-decenoic acid (10-HDA), possessed antibacterial activities. According to these authors, 10-HDA was less than one fourth as active as penicillin against *Micrococcus pyrogenes* and less than one fifth as active as chlortetracycline against *Escherichia coli.* The main goal of the present study was to evaluate the antibacterial activity of some of the RJ acids against a wide range of microorganisms, which had not been previously investigated. The inhibitory spectra of these acids were compared with the antimicrobial activity of acidic fraction extracted from lyophilised royal jelly.

MATERIAL AND METHODS

Materials

10-Hydroxy-2(E)-decenoic and 2-dodecene-1,12-dioic (2-DDecDA) acids were purchased from Cayman Europe (Tallinn, Estonia). 2-Hydroxyoctanoic (2-HO-C_8), 8-hydroxyoctanoic (8-HO-C_8), 9-hydroxydecanoic (9-HDAA), 10-hydroxydecanoic (10-HDAA) and 12-hydroxydodecanoic (12-HDAA) acids, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) with added 1% trimethylchlorosilane were purchased from Sigma-Aldrich (Poznań, Poland). A commercial preparation of lyophilized royal jelly (lot 01032014) was obtained from Bartpol® (Poland). The genuine multifloral honey was supplied by Bartnik Sądecki® (Stróże, Poland) and pine herbhoney by Doctor Miodek™ (Białystok, Poland).

Synthesis of dec-2(E)-ene-1,10-dioic acid

Dec-2(E)-ene-1,10-dioic acid (2-DecDA) was obtained by the oxidation of 10-HDA with pyridinium dichromate (PDC) according to the method by Corey & Schmidt (1979). Commercial 10-HDA (74.4 mg, 0.4 mM) and 526 mg of PDC was dissolved in 3 mL of dry dimethyl formamide and stirred at ambient temperature under argon overnight. Water (5 mL) was next added and extracted with diethyl ether (3 × 5 mL). The combined extracts were washed with saturated NH_4Cl solution and water. After anhydrous Na_2SO_4 -drying and evaporation to dryness, the crude solid product was purified through column (silica, ethyl acetate) chromatography. Fifty-five milligrams of crystalline 2-DecDA was obtained (69% yield with a melting point of 166–169°C).

Extraction and analysis of lyophilized royal jelly

The sample preparation and GC-MS investigation of RJ acidic fraction were performed as previously described (Isidorov et al., 2009). In short, the friable contents of five capsules of Bartpol® lyophilized royal jelly with a total mass of 505±10 mg was transferred to a retort and extracted (3 × 25 mL) with diethyl ether. The joint extracts were filtered, and the solvent was removed at 50 °C. The residue left on the walls (140± 5 mg) was washed with 10 mL of ether (extractions were performed in triplicates); 0.5 mL of this solution was evaporated and derivatized by BSTFA in dry pyridine. The trimethylsilyl (TMS) derivatives were analysed by capillary GC on a HP 7890A gas chromatograph with a 5975C VL MSD mass selective detector (for more details and chemical composition of the extract, see the Supplementary Online Material).

To identify the separated components, both mass spectral data and calculated linear temperature-programmed retention indices (I^T) were used. Mass spectrometric identification was carried out with an automatic GC-MS data processing system supplied by NIST as well as with the authors' previously published data (Isidorov et al., 2009; Isidorov et al., 2011; Isidorov, Bakier, & Grzech, 2012; Isidorov et al., 2015; Isidorov, 2015).

Extraction and analysis of honey

Acidic compounds were extracted from both genuine honey and herbhoney as described previously (Isidorov et al., 2011; Isidorov et al., 2015). Briefly, 10g of honey were diluted in 50 ml of water and filtered through C18 extraction discs (Mallinckrodt Baker Inc.). The adsorbed compounds were eluted with 50 mL of diethyl ether. The solvent was evaporated and 220 µL of pyridine and 80 µL of BSTFA/TMCS was added to the dry residue. The reaction mixture was sealed and heated for 0.5 h at 60°C to obtain trimethylsilyl derivatives. The TMS derivatives and identification were separated as

described above.

Microorganisms and culture media

The antimicrobial activity of aliphatic hydroxy and dicarboxylic acids was tested against *Staphylococcus aureus* PCM 2267 (PCM, Polish Collection of Microorganisms), *Bacillus subtilis* ATCC 6634 (American Type Culture Collection), *B. cereus* ATCC 14579, *B. cereus* F4810/72, emetic reference strain, *B. thuringiensis* IS5056, *Paenibacillus larvae* LMG 9820 (Belgian Coordinated Collection of Microorganisms), *E. coli* PCM 2268, *Pseudomonas aeruginosa* PCM 2270, and fungi *Candida albicans* ATCC 10231. All the microorganisms kept at -80°C in the storage medium (Luria-Bertani, LB broth and glycerol at a ratio 1:1) were inoculated onto nutrient agar (bacteria) or Sabouraud agar (fungi) and incubated overnight at 37°C.

Screening for antimicrobial activity

The antimicrobial activity of the acids was assessed by the minimal inhibitory concentration (MIC) in accordance with the Clinical and Laboratory Standard Institute protocols. Royal jelly aliphatic acids used in this study were dissolved in DMSO at a concentration of 100 mM and filtered with the 0.22-μm pore size Rotilabo-Spitzenfilter filter. To test the antimicrobial activity of all bacteria (except *P. larvae*), the solutions were serially diluted 2-fold in 2x concentrated Mueller-Hinton broth of pH 7.0 or 5.1 (established before autoclaving with hydrochloric acid) in a U-shaped 96-well microtiter plate with a final volume of 100 μL and ranged from 0.049 to 50 mM. For *P. larvae,* the solutions were serially twofold diluted in 2x concentrated Mueller-Hinton broth, pH 7.2, as this bacterium does not grow in acidic medium.

The microorganisms were cultured overnight in LB broth (*S. aureus, B. cereus, B. thuringiensis, E. coli, P. aeruginosa, C. albicans*) or Mueller-Hinton broth (*P. larvae*) at 37°C with shaking (200 rpm). The cultures were suspended to a final optical density of 0.2–0.3 at a wavelength of 600 nm measured with a V-670 spectrophotometer (Jasco, Japan).

For the assay, 100 μL of the bacterial suspensions was added to each well in the microtiter plate containing diluted acids and incubated for 48 hours (*P. larvae*) or overnight (the rest of the microorganisms tested) at 37°C. To obtain comparable data, all the bacteria were treated under the same conditions. The MIC values were determined as the lowest concentration of the acids in the wells with no bacterial growth observed visually. All the tests were carried out in quadruplicate. Microorganisms cultured in LB or Mueller-Hinton broth, as appropriate, without the acids were applied as a positive control, while the LB or Mueller-Hinton broth supplemented with 10% DMSO was used as a solvent control.

RESULTS

Acidic fraction extracted from lyophilised royal jelly by diethyl ether included 28 TMS derivatives of C_8, C_{10}, C_{12} and C_{14} hydroxyacids whose share in the total ion current was 91.8%. In accordance with Melliou and Chinou (2005) and our previous data (Isidorov et al, 2009; 2012), the most abundant the identified compounds were 10-HDA, 10-HDAA, 2-DecDA, and 3,10-dihydroxydecanoic acid (the total list of identified compounds is presented in Tab. 1S in the Supplementary Online Material).

The presence of aliphatic acids characteristic of RJ, the larval food of the honeybee (*Apis mellifera* L.), was demonstrated recently in different honeys from different countries (Isidorov et al., 2011; 2015). In this study we present the typical chromatograms (previously non published) of acidic fractions extracted from the representatives of genuine honey and herbhoney. Fig. 1 shows a fragment of typical chromatograms of multifloral and pine herbhoney TMS derivatives. Besides the peaks of phenol carboxylic acids, the chromatograms also showed clear signals of hydroxy acids and unsaturated dicarboxylic acids that were detected in the royal jelly extract. The share of C_8–C_{10} royal jelly acids in the total ion current was 26.8% and 36.6% in the acidic fraction of multifloral and pine herbhoney, respectively.

The antimicrobial activities of the aliphatic

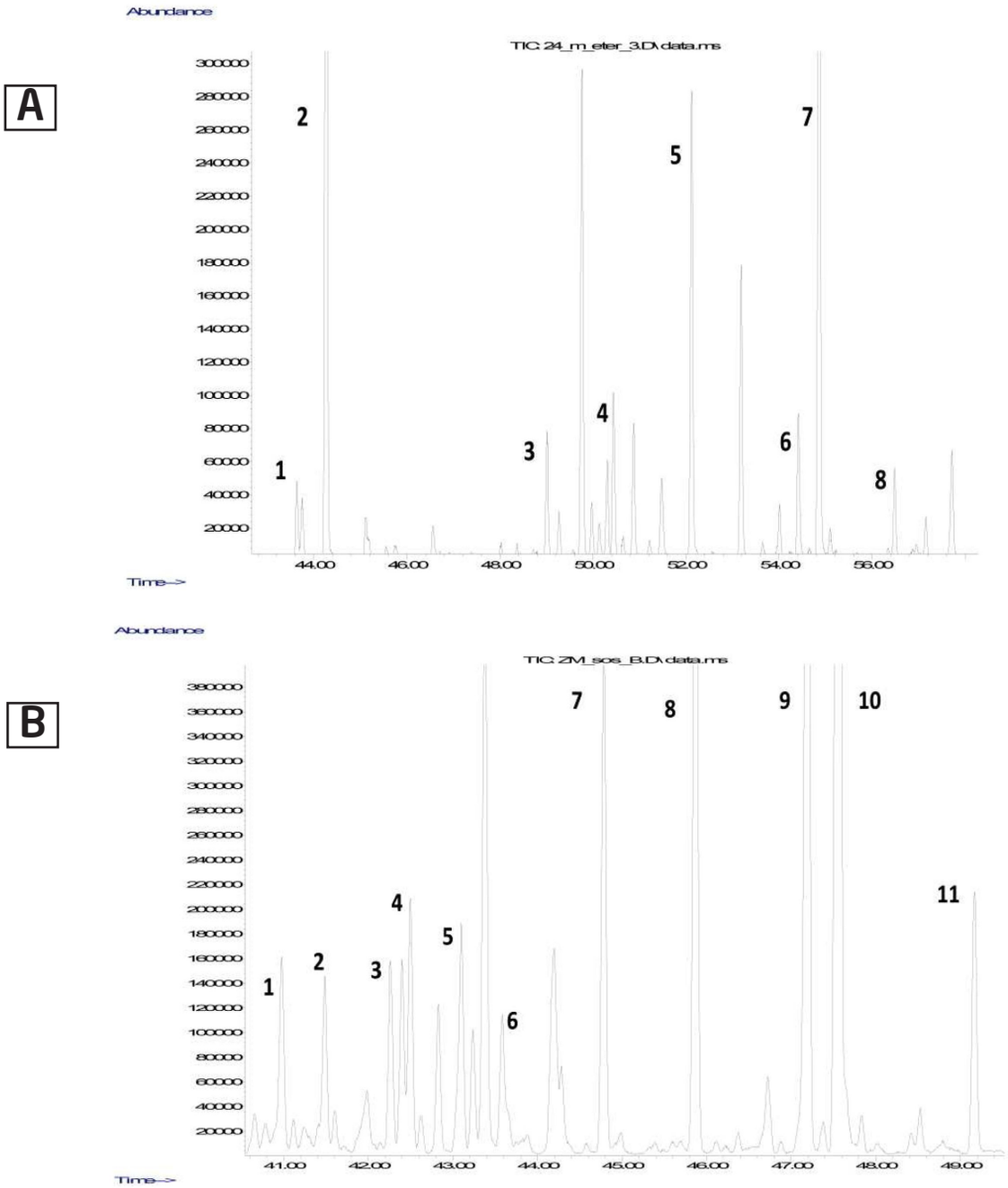

Fig. 1. Part of chromatograms of TMS derivatives of acids extracted from multifloral honey (A) and pine herbhoney (B).

A: (1) 8-hydroxyoctanoic acid, (2) 4-hydroxybenzeneacetic acid, (3) 9-hydroxydecanoic acid, (4) 10-hydroxydecanoic acid, (5) 10-hydroxy-2-decenoic (10-HDA) acid, (6) *p*-coumaric acid, (7) 2-decene-1,10-dioic acid, (8) 3,10-dihydroxydecanoic acid (the extract also contains trace amounts of 7-hydroxyoctanoic, 2-octene-1,8-dioic, 3-hydroxydecanoic, and 9-hydroxy-2-decenoic acids).
B: (1) 2-octene-1,8-dioic acid, (2) 8-hydroxyoctanoic acid, (3) 9-hydroxy-2-decenoic acid, (4) - homovanillic acid, (5) 9-hydroxydecanoic acid, (6) 10-hydroxydecanoic acid, (7) 10-hydroxy-2-decenoic (10-HDA) acid, (8) sebacic acid, (9) *p*-coumaric acid, (10) 2-decene-1,10-dioic acid, (11) 3,10-dihydroxydecanoic acid (the extract also contains trace amounts of 7-hydroxyoctanoic, and 3-hydroxydecanoic acids).

Table 1.
Antimicrobial activity of some of the royal jelly acids and the acidic fraction extracted from lyophilised RJ at pH 7.0

Microorganism	Minimal inhibitory concentration, mM (μg/mL)						RJ extract (μg/mL)
	2-Hydroxy-octanoic (2-HOC_8)	12-Hydroxy-dodecanoic (12-HDAA)	10-HDA	2-Octene 1,8-dioic (2-OenDA)	2-Decene-1,10-dioic (2-DecDA)	2-Dodecene-1, 12-dioic (2-DDecDA)	
Gram-positive bacteria							
Staphylococcus aureus	30.0 (4806.3)	12.50 (2704.0)	12.50 (2328.1)	10.00 (1720.2)	5.0 (1001.2)	10.0 (2282.3)	250.0
Bacillus subtilis	12.5 (2002.6)	7.81 (1689.5)	7.81 (1454.6)	n. d.*	n. d.	12.50 (2853.5)	250.0
Bacillus cereus ATCC 14597	7.50 (1201.6)	31.25 (6760.0)	6.25 (1164.0)	5.00 (860.1)	2.50 (500.6)	2.50 (570.7)	125.0
Paenibacillus larvae	6.25 (1001.0)	6.25 (1375.0)	1.56 (290.5)	n. d.	2.50 (500.6)	6.25 (1426.8)	125.0
Gram-negative bacteria							
Pseudomonas aeruginosa	7.50 (1201.6)	62.50 (13250.0)	12.50 (2328.1)	5.00 (860.1)	6.25 (1251.4)	6.25 (1426.8)	125.0
Escherichia coli	15.0 (2403.2)	125.0 (27040.0)	12.50 (2328.1)	5.00 (860.1)	12.50 (2502.8)	6.25 (1426.8)	250.0
Fungi							
Candida albicans	3.75 (600.8)	15.63 (3381.1)	1.56 (290.5)	0.31 (53.4)	6.25 (1251.4)	6.25 (1426.8)	125.

* n. d. - not determined

acids typical of RJ were tested against the most known human pathogens associated with food and involved in such food toxicity or intestinal infections as gram-positive staphylococci and bacilli. These are represented in our study by *S. aureus*, *Bacillus cereus*, an emetic *B. cereus* reference strain F4810/72 and gram-negative bacilli, e.g. *E. coli* and *P. aeruginosa*. In addition, we expanded the number of tested bacteria by adding *B. subtilis*, a model gram-positive organism, and *B. thuringiensis,* representing bacteria often used as bio-insecticides. Considering the increased numbers of American foulbrood among honey bees (Skubida et al., 2014), we also tested the antimicrobial effect of RJ acids against *P. larvae*, an etiological agent of this disease. In this study, we used strain *P. larvae* LMG 9820. To have a global view of the antimicrobial spectra of the chemicals used in the study, we also tested their activity against pathogenic fungi using a *C. albicans* strain.

To test the antimicrobial activity of the selected RJ acids, we used the tube dilution assays. The sensitivity of microbes were tested under neutral and slightly acidic conditions. As can be seen from data in Tables 1 and 2, the tested acids demonstrated higher activity in experiments with acidic nutrient media.

DISCUSSION

The inhibitory activity of RJ against both gram-positive and gram-negative bacteria had been demonstrated many years ago by McCleskey & Melampy (1938), but their nature and the chemical composition of this honey bee product were not established for a long time. At the end of the 1950s, the aliphatic 10-hydroxy-2-decenoic acid (10-HDA) was identified as the main component of diethyl ether extracts from RJ (Butenandt & Rembold, 1957; Barker et al., 1959). At the same time, the biological properties of RJ and its constituents were investigated, particularly the antimicrobial action of 10-HDA. Barker et al. (1959) stated that this hydroxy acid showed little or weak activity against a representative range of bacteria and fungi (the antibacterial assays were performed

Table 2.

Antimicrobial activity of some of the royal jelly acids and the acidic fraction extracted from lyophilised RJ at pH 5.1

Microorganism	Minimal inhibitory concentration, mM (μg/mL)							RJ extract, μg/mL
	2-Hydroxy-octanoic (2-HOC_8)	8-Hydroxy-octanoic (8-HOC_8)	9-Hydroxy-decanoic (9-HDAA)	10-Hydroxy-decanoic (10-HDAA)	12-Hydroxy-dodecanoic (12-HDAA)	10-HDA	2-Decene-1,10-dioic (2-DecDA)	2-Dodecene-1,12-dioic (2-DDecDA)	
Gram-positive bacteria									
Staphylococcus aureus	0.78 (125.0)	1.49 (239.0)	0.048 (9.0)	3.12 (587.3)	0.048 (10.4)	0.098 (18.2)	0.78 (156.2)	0.39 (89.0)	62.5
Bacillus subtilis	0.78 (125.0)	3.12 (500.1)	0.048 (9.0)	0.78 (146.8)	0.098 (21.2)	0.098 (18.2)	0.39 (78.1)	0.048 (11.0)	31.2
Bacillus cereus ATCC 14597	3.12 (500.1)	6.25 (1001.3)	0.048 (9.0)	0.39 (73.4)	0.39 (84.4)	0.78 (145.3)	0.195 (39.0)	0.78 (178.1)	62.5
Bacillus cereus F4810/72	0.78 (125.0)	3.12 (500.1)	0.048 (9.0)	0.39 (73.4)	0.195 (42.2)	0.39 (72.6)	0.048 (9.6)	0.78 (178.1)	62.5
Bacillus thuringiensis	1.56 (250.0)	6.25 (1001.3)	0.048 (9.0)	0.78 (146.8)	0.39 (84.4)	0.39 (72.6)	0.39 (78.1)	0.39 (89.0)	125.0
Gram-negative bacteria									
Pseudomonas aeruginosa	6.25 (1001.3)	12.50 (2002.6)	12.50 (2353.1)	12.50 (2353.1)	6.25 (1352.0)	6.25 (1164.0)	1.25 (250.3)	2.50 (570.7)	250.0
Escherichia coli	6.25 (1001.3)	12.50 (2002.6)	12.50 (2353.1)	12.50 (2353.1)	6.25 (1352.0)	3.125 (582.0)	2.50 (500.6)	5.00 (1141.4)	250.0
Fungi									
Candida albicans	12.50 (2002.6)	12.50 (2002.6)	6.25 (1176.6)	3.12 (587.3)	0.195 (42.2)	6.25 (1164.0)	0.098 (19.6)	0.62 (142.7)	62.5

by Glaxo Ltd.). In contrast, at the same time, another working group had demonstrated the high activity of 10-HDA against many bacteria and fungi (Blum et al., 1959).

This discrepancy is supposed to be related to the differences in antimicrobial assay conditions, namely, the acidity of nutrient medium. According to McCleskey and Melampy (1938), the sterilisation of test cultures required only a few minutes at a natural acidity of RJ (pH 4.6), but required two days at pH 7.0. Blum et al. (1959) had observed high bactericidal 10-HDA activity under slightly acidic conditions, but after neutralization, the activity was considerably lower. To definitively ascertain the influence of acidity (i.e. ionisation state of tested acids) on the antimicrobial activity of RJ acids, we performed two series of experiments with the neutral and acidic nutrient media.

As can be seen from Tab. 1, which presents the results of antibacterial assays under neutral conditions, the majority of the tested acids demonstrated only weak antibacterial activity (MIC >1000 µg/mL). Moderate action of 10-HDA against *P. larvae* and *C. albicans* as well as unsaturated dicarboxylic acids 2-DecDA and 2-OenDA against tested gram-positive bacteria was observed. Another unsaturated dicarboxylic acid, 2-DodecDA (known as traumatic acid), also moderately inhibited the growth of *B. cereus* with MIC ~570 µg/mL.

In agreement with observations by Blum et al. (1959), 10-HDA and RJ acids demonstrated much higher activity against bacteria and fungi when acidification of the nutrient medium was adjusted to pH 5.1, i.e. typical acidity of genuine honeys (Fyfe et al., 2017). Tab. 2 presents the MIC values, which as a rule are an order of magnitude lower than that detected under neutral conditions in Tab. 1 and higher inhibition was observed in the assays with 9-HDAA against gram-positive bacteria. Interestingly, an emetic *B. cereus* reference strain F4810/72 demonstrated the highest susceptibility in relation to nearly all tested acids. Also remarkable, the growth inhibition of the diethyl ether extract of lyophilised royal jelly (its acidic fraction) was more pronounced under both neutral and acidic conditions, than separate acids found in this honey bee product. This testifies to a significant synergistic effect of different RJ extract components.

Overall, 47 hydroxy and dicarboxylic acids were registered in crude royal jelly (Isidorov et al., 2012). Ten acids characteristic of this bee product were identified in different quantities and in different combinations in the analysed honey and herbhoney samples: saturated 7- and 8-hydroxyoctanoic, 3-, 9- and 10-hydroxydecanoic, and 3,10-dihydroxydecanoic acids, as well as unsaturated 9-hydroxy-2-decenoic, 10-hydroxy-2-decenoic, 2-octene-1,8-dioic, 2-decene-1,10-dioic acids (Isidorov et al., 2011; 2015). One of them, 2-DecDA was also detected in New Zealand manuka, kanuka and white clover unifloral honeys in concentrations from 3.5 to 181.2 µg/g (Tan et al., 1988). Recently, 2-decenedioic acid and its glycoside were investigated in some Scottish honeys, but the exact chemical structures of the components were not determined (Fyfe et al., 2017).

The quantity of the aliphatic royal jelly acids in different samples of genuine honey and herbhoney varied from 6 to 209 µg/g (n = 65; Isidorov et al., 2012, and unpublished data). It was proposed that RJ components produced by the mandible and hypopharyngeal glands of worker bees enter the honey at the time of nectar processing (Isidorov et al., 2011). Hence, the content of these bee-derived compounds varies depending on the bee family's physiological conditions i.e. number of young workers capable of participating in nectar processing and accessibility of protein-containing forage, which is a precursor of RJ in workers.

The combination of data presented above concerning the antibacterial activity of RJ acids and their content in honey (Isidorov et al., 2011; Isidorov et al., 2015) allows us to conclude that they are just "unknown compounds of bee origin" with bacteriostatic or bactericidal activity (Bogdanov, 1997; Kwakman et al., 2010a). RJ acids may influence microorganisms individually or participate in additive/synergistic effects with other antimicrobial agents such as hydrogen peroxide, methylglyoxal, polyphenols and the bee protein defensin-1.

ACKNOWLEDGEMENTS

This work was performed in the framework of task S/ZWL/1/2014 of Białystok University of Technology (V.I.) as well as the Department of Natural Product Chemistry of Białystok University (S.W.), and supported from the funds of the Polish Ministry of Science and Higher Education under project Specific Scientific Equipment No. 8636/E-342/SPUB/2016/1 (I.S.).

REFERENCES

Aljadi, A.M., & Yusoff, K. M. (2003). Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties. *Turkish Journal of Medicine, 33,* 229-236.

Al-Waili, N. S., Salom, K., Butler, G., & Al Ghamdi, A. A. (2011). Honey and microbial infections: A review supporting the use of honey for microbial control. *Journal of Medicinal Food, 14,* 1079-1096. DOI:10.1089/jmf.2010.0161

Barker, S. A., Forster, A. B., Lamb, D. C., & Hodgson, N. (1959). Identification of 10-hydroxy-Δ^2- decenoic acid in royal jelly. *Nature, 183,* 996-997. DOI: 10.1038/18399a0

Blum, M. S., Novak, A. F., & Taber, S. (1959). 10-Hydroxy-Δ^2-decenoic acid, an antibiotic found in royal jelly. *Science, 130,* 452-453.

Bogdanov, S. (1997). Nature and origin of the antibacterial substances in honey. *Lebensmittel-Wissenschaft und Technologie, 30,* 748-753. DOI: 10.1006/fstl.1997.0259

Brudzinski, K. (2006). Effect of hydrogen peroxide on antibacterial activity of Canadian honeys. *Canadian Journal of Microbiology, 52,* 1228-1237.

Brudzinski, K., Abubaker, K., St-Martin, L., & Castle., A. (2011). Re-examining the role of hydrogen peroxide in bacteriostatic and bactericidal activities of honey. *Frontiers in Microbiology, 2,* 213-218. DOI: 10.3389/fmicb.2011.00213

Brudzinski, K., & Sjaarda, C. (2015). Honey glycoproteins containing antimicrobial peptides, Jelleins of the major royal jelly protein 1, are responsible for the cell wall lytic and bactericidal activities of honey. *Plos One 10*(4):e0120238. DOI: 10.1371/journal.pone.0120238

Brudzynski, K., Miotto, D., Kim, L., Sjaarda, C., Maldonado-Alvarez, L., Fukś, H. (2017). Active macromolecules of honey form colloidal particles essential for honey antibacterial activity and hydrogen peroxide production. *Scientific Reports, 7,* 7637. DOI:10.1038/s41598-017-08072-0

Bučeková, M., & Majtán, J. (2016). The MRJP1 honey glycoprotein does not contribute to the overall antibacterial activity of natural honey. *European Food Research & Technology, 242,* 625-629. DOI: 10.1007/s00217-016-02665-5

Butenant, A., & Rembold, H. (1957). Über den Weiselzellenfuttersaft der Honig Biene. *Zeitschrift für Physikalische Chemie, 308,* 285-299.

Core, E. J., & Schmidt, G. (1979). Useful procedures for the oxidation of alcohols involving pyridinium dichromate in aprotic media. *Tetrahedron Letters, 20,* 399-402. DOI: 10.1016/S0040-4049(01)93515-4

Fidaleo, M., Zuorro, A., & Lavecchia, R. (2010). Honey: a natural antimicrobial agent against foodborne pathogens? *Journal of Biotechnology, 150S,* S298. DOI: 10.1016/j.jbiotec.2010.09.253

Fyfe, L., Okoro, P., Paterson, U., Coyle, Sh., McDougall, G. J. (2017). Compositional analysis of Scottish honeys with antibacterial activity against antibiotic-resistant bacteria reveals novel antimicrobial components. *LWT – Food Science &Technology, 79,* 52-59. doi.org/10.1016/j.lwt.2017.01.023

Giles, S. L., & Laheij, R. J. F. (2017). Successful treatment of persistent *Clostridium difficile* infection with Manuka honey. *International Journal of Antimicrobial Agents, 49,* 522-523. doi.org/10.1016/j.ijantimicag.2017.02.005

Grecka, K., Kuś, P. M., Worobo, R. W., & Szweda, P.

(2018). Study of the anti-staphylococcal potential of honeys produced in Northern Poland. *Molecules, 23*, 260. DOI:10.3390/molecules23020260

Isidorov, V.A. (2015). Identification of Biologically and Environmentally Significant Organic Compounds. Mass Spectra and Retention Indices Library of Trimethylsilyl Derivatives. PWN, Warszawa. 429 pp.

Isidorov, V. A., Czyżewska, U., Isidorova, A. G., & Bakier, S. (2009). Gas chromatographic and mass spectrometric characterization of the organic acids extracted from some preparations containing lyophilized royal jelly. *Journal of Chromatography B, 877,* 3776-3780. DOI: 10.1016/j.jchromb.2009.09.016

Isidorov, V. A., Czyżewska, U., Jankowska, E., & Bakier, S. (2011). Determination of royal jelly acids in honey. *Food Chemistry, 124*, 387-391. DOI: 10.1016/j.foodchem.2010.06.044

Isidorov, V. A., Bakier, S., & Grzech, J. (2012). Gas chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. *Journal of Chromatography B, 885-886,* 109-116. DOI: 10.1016/j.jchromb.2011.12.025

Isidorov, V. A., Bagan, R., Bakier, S., & Swiecicka, I. (2015). Chemical composition and antimicrobial activity of Polish herbhoneys. *Food Chemistry, 171*, 84-88. DOI: 10.1016/j.foodchem.2014.08.112

Kwakman, P. H. S., te Velde, A. A., de Boer, L., Vandenbrouke-Grauls, Ch. M. J. E., Zaat, S. A. J. (2010a). Two major medical honeys have different mechanisms of bactericidal activity. *PLoS One, 6,* e 17709. DOI: 10.1371/journal.pone.0017709

Kwakman, P. H. S., te Velde, A. A., de Boer, L., Speeijer, D., Vandenbrouke-Grauls, Ch. M. J. E., Zaat, S. A. J. (2010b). How honey kills bacteria. *The FASEB Journal, 24,* 2567-2582, DOI: 10.1096/fj.09-150789

Lee, S. K., & Lee, H. (2016). Antibacterial activity of solvent fractions and bacterial isolated of Korean domestic honey from different floral sources. *Food Science and Biotechnology, 25,* 1507-1512. DOI:10.1007/s10068-016-0234-0

Mavric, E. , Wittmann, S., Barth, G., & Henle, T. (2008). Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermium scoparium*) honeys from New Zealand. *Molecular Nutrition & Food Research, 52,* 483-489. DOI: 10.1002/mnfr.200700282

McCleskey, C. S., & Melampy, R. M. (1938). Bactericidal activity of "Royal Jelly" of honey bees. *Journal of Microbiology, 38*, 324.

Melliou, E., & Chinou, I. (2005). Chemistry and bioactivity of royal jelly from Greece. *Agricultural and Food Chemistry, 53,* 8987-8992. DOI: 10.1021/jf051550p

Mizrahi, H., & Lensky, Y. (Eds.). (1996). *Bee products: Properties, applications and apitherapy.* NY: Plenum Press.

Moore, O. A., Smith, L. A., Campbell, F., Seers, K., McQuay, H. J., Moore, R. A. (2001). Systematic review of the use of honey as a wound dressing. *BMC Complementary & Alternative Medicine, 1, 2-7*. http://www.biomedcentral.com/1472-6882/1/2

Russell, K. M., Molan, P. C., Wilkins, A. L., & Holand, P. T. (1988). Identification of some antibacterial constituents of New Zealand Manuka honey. *Journal of Agricultural & Food Chemistry, 38,* 10-13. DOI: 10.1021/jf00091a002

Skubida, M., Pohorecka, K., Bober, A., & Zdańska, D. (2014). Five-year investigation of *Paenibacillus larvae* expansion in Polish apiaries: Analyses of results. In: Mat. 51th Sci. Beekeep. Conf., Szczyrk, pp. 55-56.

Sultanbawa, Y., Cozzolino, D., Fuller, S., Cusack, A., Currie, M., Smyth, H. (2015). Infrared spectroscopy as a rapid tool to detect methylglyoxal and antibacterial activity in Australian honeys. *Food Chemistry, 172,* 207-212. DOI: 10.1016/j.foodchem.2014.09.067

Tan, S.-T., Holland, P. T., Wilkins, A. L., & Molan, P. C. (1988). Extractivities from New Zealand honeys. White clover, manuka and kanuka unifloral honeys. *Journal of Agricultural & Food Chemistry, 36*, 453-460. DOI: 10.1021/jf00081a012

Taormina, P. J., Niemira, B. A., & Beuchat, L. R. (2001). Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power. *International Journal of Food Microbiology, 69,* 217-225.

Valachová, I., Bučeková, M., & Majtán, J. (2016). Quantification of bee-derived peptide Defensin-1 in honey by competitive enzyme-linked immunosorbent assay, a new approach in honey quality control. *Czech Journal of Food Sciences, 34,* 233-243. DOI: 10.17221/422/2015-cjfs

Weston, R. J., Brocklebank, L. K., & Lu, Y. R. (2000). Identification and quantitative levels of antibacterial components of some New Zealand honeys. *Food Chemistry, 70,* 427-435. DOI: 10.1016/S0308-8146(00)00127-8

Supplementary data
associated with the article titled: " Royal jelly aliphatic acids contribute to the antibacterial activity of honey" by Valery A. Isidorov, Stanisław Witkowski, Piotr Iwaniuk, Monika Zambrzycka and Izabela Swiecicka

A. GC-MS analysis of extract from lyophilized royal jelly

Acidic fraction of RJ extracted by diethyl ether was derivatised by BSTFA. The obtained solution of TMS derivatives was analysed by a GC–MS method on a HP 7890 gas chromatograph with the 5975 VL MSD Triple-Axis Detector (Agilent Technologies, USA). The apparatus was fitted with an HP-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness), with electronic pressure control and split/splitless injector. The latter worked at 250°C in the split (1:50) mode. The helium flow rate through the column was 1 mL/min. The initial column temperature was 50°C, rising to 320°C at 3°C/min, and the higher temperature was maintained for 15 min. The MSD detector acquisition parameters were as follows: the transfer line temperature was 280°C, the MS source temperature 230°C and the MS quad temperature 150°C. The electron impact mass spectra were obtained at 70 eV of ionization energy. After integration, the fraction of separated components in the total ion current (TIC) was calculated.

To identify the separated components, both mass spectral data and calculated linear temperature-programmed retention indices (I^T) were used. Mass spectrometric identification was carried out with an automatic system of GC-MS data processing supplied by NIST and home-made mass spectra libraries. The latter contains more than 1750 spectra of TMS derivatives prepared from commercial preparations of flavonoids, other phenolics, terpenoids, aliphatic acids, alcohols and carbohydrates.

Retention indices were calculated from the results of the separation C_8–C_{40} *n*-alkanes solutions in hexane and the TMS derivatives. The obtained I^T values were compared with NIST collection (NIST 2013) as well as with the authors' previously published data (Isidorov et al., 2009; Isidorov et al., 2014a; 2014b; Isidorov, 2015). Identification was considered reliable if the results of a computer search in the mass spectra library were confirmed by the experimental I^T values, *i.e.*, if their deviation from the averaged literature values did not exceed ±10 u. i. (inter-laboratorial deviation for low polar stationary phases). Apart from the semi-quantitative composition (total ion current fraction) of extract, Tab. 1 contains some analytical parameters that were used to confirm the results of identification (calculated and literature retention indices, *m/z* values of the most intensive ions in the mass spectra, and the mass number of molecular ions M^+, if detected in the mass spectra). The list of identified substances includes 28 TMS derivatives of C_8, C_{10}, C_{12} and C_{14} hydroxyacids whose share in the total ion current is 91.8%.

Table 1S.

Relative chemical composition (% of TIC) of ether extract from lyophilized royal jelly

Compound, TMS	RI^{Exp}	RI^{Lit}	Target ions, m/z	M+	%
Lactic acid	1074	1074	73,117,147,191,190	-	0.07
Benzoic acid	1247	1250	179,105,135,77,194	194	0.01
Octanoic acid	1270	1266	201,75,73,117,129	-	0.03
Glycerol	1294	1293	147,205,73,218,117	-	0.05
Nicotinic acid	1295	1296	180,106,136,78,51	-	0.02
Pyrocatechol	1322	1330	73,254,199,75,147	254	trace
Succinic acid	1325	1321	147,73,247,75,129	-	0.01
2-Hydroxyoctanoic acid	1472	1468	215,75,73,81,131	-	0.09
3-Hydroxyoctanoic acid	1483	1486	73,147,173,233,247	-	0.3
4-Hydroxy methylbenzoate	1494	1494	209,224,193,135,73	224	0.02
7-Hydroxyoctanoic acid	1558	1555	117,73,75,217,147	-	0.8
NN	1604	-	117,147,73,230,81	-	0.01
8-Hydroxyoctanoic acid	1628	1624	147,73,289,75,55	304	5.6
4-Hydroxybenzoic acid (paraben)	1632	1636	267,223,183,282,73	282	0.01
4-Methylheptanedioic acid	1654	1653	117,73,75,217,274	-	0.01
3-Hydroxydecenoic acid?	1661	-	147,73,75,233,199	-	trace*
3-Hydroxydecanoic acid (3-HDA)	1669	1667	73,147,233,201,275	332	1.5
8-Hydroxy-2-octenoic acid	1677	1675	147,73,287,95,81	302	0.07
Octanedioic (suberic) acid	1710	1706	73,75,187,303,147	-	0.03
NN	1726	-	303,73,75,147,213	-	0.01
NN	1737	-	73,131,303,117,147	-	0.01
9-Hydroxydecanoic acid	1750	1750	117,73,217,317,147	332	0.5
NN	1759	-	145,73,230,147,287	-	0.02
4-Hydroxy hydrocinnamic acid	1766	1764	179,193,310,73,75	-	0.01
Vanillic acid	1775	1776	297,312,73,267	312	trace
3,4-Dihydroxy-2-phenylethanol	1781	1781	267,370,193,179,73	370	0.01
8-Hydroxy-2-decenoic acid	1786	1784	131,73,211,315,81	330	0.02
Glycerol-1-phosphate	1798	1796	357,299,73,356,315	-	2.7
9-Hydroxy-2-decenoic acid	1802	1801	117,73,147,286,315	330	trace
10-Hydroxydecanoic acid (10HDAA)	1822	1820	317,73,75,147,227	332	15.2
NN	1836	-	289,73,147,171,199	-	0.1
α-Fructofuranose	1846	1846	217,73,437,147,525	-	0.02
10-Hydroxy-2-decenoic acid (10-HDA)	1876	1875	147,315,73,81,75	330	52.0
Decanedioic (sebacic) acid	1907	1904	73,331,75,215,129	-	4.1
10-Hydroxydodecanoic acid	1931	1931	331,131,73,75,217	360	0.4
3,9-Dihydroxydecanoic acid	1938	1936	73,117,147,305,233	-	0.4
11-Hydroxydodecanoic acid	1946	1944	117,73,147,204,217	358	0.7
2-Decene-1,10-dioic acid	1960	1958	73,75,136,164,119	344	4.5
10-Hydroxy-2-dodecenoic acid	1991	-	329,131,73,253,300		0.04
NN	1997	-	117,73,253,343,314	-	0.2
3,10-Dihydroxydecanoic acid (3,10-DDA)	2011	2011	73,147,233,405,75	420	8.3
12-Hydroxydodecanoic acid	2018	2016	345,73,329,147,255	360	0.3
NN	2025	-	317,73,318,147,129	-	0.2
β-Glucopyranose	2030	2029	204,191,73...525	-	0.02
Hexadecanoic acid	2054	2051	313,73,75,129,132	328	0.06

12-Hydroxy-2-dodecenoic acid	2069	2070	343,147,73,253,315	358	0.2
NN	2071	-	315,73,147,225,403	-	0.5
3-Hydroxydecanedioic acid	2090	2088	73,147,233,217,303	-	0.05
Dodecanedioic acid	2100	2099	359,73,75,217,234	-	0.1
3,10-Dihydroxydodecanoic acid	2110	2111	73,131,147,233,419	-	0.02
3,11-Dihydroxydodecanoic acid	2126	2125	117,73,147,305,233	448	0.4
NN (dihydroxydodecanoic acid?)	2133	-	331,73,117,147,433	448	0.03
NN	2138	-	117,73,75,373,357	-	0.05
10,11-Dihydroxydodecanoic acid	2152	2151	331,73,332,147,433	448	0.2
11,12-Dihydroxydodecanoic acid?	2172	2172	73,103,271,129,217	-	0.1
3,12-Dihydroxydodecanoic acid	2198	2198	147,73,233,433,189	-	0.1
14-Hydroxytetraceadecanoic acid?	2214	-	345,73,147,75,346	-	trace
Oleic acid	2222	2218	339,73,75,117,55	354	trace
Octadecanoic acid	2250	2250	73,147,73,129,132	356	0.03
3,13-Dihydroxytetraceadecanoic acid	2316	2314	73,117,147,305,233	-	0.01
n-Heptacosane	2700	2700	57,71,85,43,41	-	0.01
n-Nonacosane	2900	2900	57,71,85,43,41	-	0.01

*trace - below 0.01% of TIC.

REFERENCES

Isidorov, V. A., Brzozowska, M., Czyżewska, U., & Glinka, Ł. (2009). Gas chromatographic investigation of phenylpropenoid glycerides from aspen (*Populus tremula* L.) buds. *Journal of Chromatography A, 1198–1199*. 196–201. DOI: 10.1016/j.chroma.2008.05.038

Isidorov, V., Szczepaniak, L., & Bakier, S. (2014a). Rapid GC/MS determination of botanical precursors of Eurasian propolis. *Food Chemistry ,142*, 101–110. DOI: 10.1016/j.foodchem.2013.07.032

Isidorov, V., Szczepaniak, L., Wróblewska, A., Pirożnikow, E., Vetchinnikova, L. (2014b). Gas chromatographic-mass spectrometric examination of chemical composition of two Eurasian birch (*Betula* L.) bud exudates and its taxonomical implication. *Biochemical Systematics and Ecology, 52*, 41–48. DOI: 10.1016/j.bse.2013.12.008

Isidorov, V.A. (2015). Identification of Biologically and Environmentally Significant Organic Compounds. Mass Spectra and Retention Indices of Trimethylsilyl Derivatives. PWN, Warsaw, 430 pp.

NIST Chemistry WebBook (2013). National Institute of Standards and Technology, Gaithersburg, MD 20899. http://webbook.nist.gov.chemistry.