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ANTIBACTERIAL ACTIVITY OF ROYAL JELLY AND RAPE HONEY AGAINST METHICILLIN-RESISTANT *Staphylococcus aureus* STRAINS

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Abstract:

Antimicrobial resistance of pathogenic bacteria is a growing public health problem. Methicillin-resistant *Staphylococcus aureus* (MRSA) represents a significant burden on healthcare services because it is involved in severe difficult to treat infections in humans. Several non-antibiotic approaches regarding the treatment of infections caused by MRSA as well as of other resistant bacteria have been studied. Since ancient times royal jelly and honey have been considered both as foods and medicines, and recently have been identified as potential alternative medicines to treat bacterial infections, especially of the skin and soft tissue. The aim of the present study was to investigate the antibacterial effect of honey, royal jelly and their mixtures against MRSA *in vitro*. At least 3 decimal reductions of MRSA count were observed in Tryptone Soy broth with concentrations of 40% rape honey (RP), 20% and 30% royal jelly (RJ), 30% RJ:RH (1:100). In general, honey and royal jelly, individually or in combination, appeared to have a potential as alternative therapeutic agents against MRSA infections, but clinical studies are needed for confirmation.

Keywords: Honey, Royal jelly, Antibacterial activity, MRSA

Introduction

Although the therapeutic effect of honey in treating of infected wounds is known since the ancient times, more recently has been reported its inhibitory effect on a great number of bacteria including both aerobes and anaerobes, as well as Gram-positive and Gram-negative (Cooper et al., 2002) and also fungi and yeasts (Brady et al., 1996).

The high antibacterial effect of RJ has also been reported (Blum et al., 1959; Melliou & Chinou, 2005).

The antibacterial activity of royal jelly, rape honey, individually and in combination has been reported against resistant strain of *E. coli* (Dinkov et al., 2014) and *A. hydrophila* (ATCC 7965) (Stratev et al., 2015).

Antibiotic-resistant bacteria represent a critical problem in modern medicine world-wide (WHO, 2014) and consequently, scientific efforts have been developed to control bacterial infections with alternative medicines beyond conventional antibiotic therapy. Honey (Molan, 1997), propolis (Kujumgiev et al., 1999) and royal jelly (Fontana et al., 2004) are among these alternative therapeutic agents.

The aim of this study was to determine the antimicrobial effect of rape honey and royal jelly, individually or in combination against MRSA.

Materials and Methods

Test substances

Test substances were Bulgarian rape honey, royal jelly and mix of rape honey and royal jelly. The rape bee honey (RH) and royal jelly (RJ) were obtained from beekeepers, immediately after the flowering of rape from the region of Stara Zagora, Bulgaria. During the honey collection period bees were not treated with carbohydrate syrups or antimicrobial drugs. Until the analysis, RH and RJ samples were stored in sterilized jars and sterile plasic tubes at 0-4 °C, respectively. Water content, pH, free acidity, electrical conductivity, diastase and invertase activity, specific optical activity and hydroxymethylfurfurol (HMF) content were assayed as per the harmonized methods of the European honey commission (Bogdanov et al., 1997). The botanical origin of the samples was established by their melissopalynological, organoleptic, physical and chemical characteristics (Oddo et al., 2004; von der Ohe et al., 2004). All data referring to physical and chemical parameters of rape honey were statistically processed by the Student's t-test and presented as mean and standard deviation (SD) (Table 1).

Royal jelly was pipetted directly from queen's cells. The following parameters of samples were determined: sugars (fructose, glucose, sucrose) by HPLC according to Sesta (2006); proteins by Folin-Ciocalteu reagent; water content by refractometry; dry matter of the sample by subtracting the water content from 100; pH values -potentiometrically by pH meter Mi 150 (1% water solution of royal jelly); total acidity by titration with 0.1 N NaOH according to ON 2576693-84 about fresh and lyophilized royal jelly; electrical conductivity of 1 % water solution of royal jelly by conductometer (11, 15) (Table 2). All royal jelly samples were kept refrigerated at -20 °C in dark bottles. Solutions containing 10, 20, 30 and 40% (v/v) of each test substances were prepared in sterile Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany). To prevent photodegradation of glucose oxidase which is associated with antimicrobial activity in honey (Balkanska et al., 2012), all test substances were stored in the dark and dilutions were prepared immediately prior to testing (Sherlock et al., 2010).

Table 1. Physicochemical parameters of rape honey

Parameters	Mean	SD	Maximum	Minimum
Water content (%)	16.8	0.2108	17	16.6
Free acidity (meq.kg^{-1})	36.3	1.1595	38	35
pH	3.232	0.01032	3.25	3.22
Conductivity (mS.cm^{-1})	0.128	0.00105	0.13	0.127
Diastase activity (Ghote), (DN)	12.9	0.1051	13.1	12.8
Hydroxymethilfurfurol (HMF), (mg.kg $^{-1}$)	14.89	0.3528	15.36	14.4
Invertase activity (IN)	10.643	0.0241	10.69	10.62
Specific optical rotation, $[\alpha]_D^{20}$	(–) 12	0.8164	(–) 13	(–) 11

Table 2. Physicochemical characteristics of royal jelly

Parameters	Mean	SD	Maximum	Minimum
Water content (%)	62.7	1.43452	63.7	60.2
pH	3.97	0.07776	4.06	3.78
Total acidity (mL 0.1n NaOH/g)	4.08	0.38084	4.51	3.31
Electrical conductivity (μS/cm)	197	14.0791	224	180
Proteins (%)	16.94	1.37065	19.36	14.81
Fructose (%)	4.83	0.75832	6.19	3.59
Glucose (%)	3.85	0.99522	5.65	2.7
Sucrose (%)	1.70	0.86652	4.25	0.64

Bacterial strains and preparation of inoculum

Three MRSA isolates belonging to Prof. D. Sergelidis collection were used in our study. These isolates belonged to spa types t127 (isolated from goat carcass), t4038 (isolated from unpasteurized goat's milk) and t548 (isolated from marinated anchovies). They were stored in cryo-tubes containing Tryptone Soy broth (Merck, Darmstadt, Germany) supplemented with 15% glycerol at -80 °C. Prior to experiments the MRSA strains were incubated for 35 °C in TSB (Merck, Darmstadt, Germany) for 24 h and then a loopfull was streaked onto Blood agar and incubated for 24 h at 35 °C. Three to four colonies were taken from the Blood agar and suspended in 5 mL sterile physiological solution for preparation of bacterial suspension adjusted to the 0.5 McFarland standard ($1.5x10^8$ CFU/mL). Decimal dilutions to 10^{-4} in 9 mL sterile TSB were prepared from the initial suspension.

Experimental design

Prior to the experiment, 50% (w/v) dilutions of RH, RJ and RH and RJ mix in TSB (Merck, Darmstadt, Germany) were prepared. Thereafter, serial dilutions of the 50% stock solutions were prepared in order to obtain 40%, 30%, 20% and 10% (v/v) RH, 30%, 20% and 10% (v/v) RJ, and 40%, 30%, 20% and 10% RH and RJ mix, respectively. TSB (Merck, Darmstadt, Germany) was used as a control.

The tubes were inoculated with the bacterial cultures from each MRSA isolate according to the method described by Patton et al. (2006). The inoculated tubes were incubated at 35 °C for 48 h. In order to the determination of survived staphylococci after 24 and 48 h, serial 10-fold dilutions in 0.1% peptone water supplemented with 2.5% NaCl were prepared. Thereafter, 0.1 mL from each tube was spread plated onto Baird Parker agar (Merck, Darmstadt, Germany) containing 0.0025% w/v potassium tellurite and rabbit plasma fibrinogen. Typical *S. aureus* colonies were counted after incubation at 35 °C for 24 h. For the detection of survivors at populations lower than 10 CFU/g, the first dilution was incubated for enrichment at 35 °C for 24 h and then 0.1 mL was spread plated onto Baird Parker agar.

The experiment was performed twice and the results are presented as mean values.

Results and Discussion

There were not survived cells of MRSA t127 (3.34 $\log_{10}$ reduction) after 24 h incubation in TSB with 40% RH, with 20 and 30% RJ and with 30 and 40% mix of RJ:RH (1:100). A reduction of 1.95 $\log_{10}$ and 1.23 $\log_{10}$ was observed in TSB with 30% RH and 20% RJ:RH after 48 h incubation (1:100) (Table 3). The counts in the other concentrations of all substances were more than 8 $\log_{10}$ after 48 h.

A reduction of 3.17 $\log_{10}$ of MRSA t548 was observed after 24 h incubation in TSA with 10, 20 and 30% RJ and with 40% RJ:RH (1:100) (Table 4). Although a reduction almost 3.17 $\log_{10}$ was observed after 24 h in TSA with 40% RJ:RH (1:100), staphylococcal cell count reached 3.54 $\log_{10}$ at 48 h. The counts in the other concentrations of all substances were more than 7 $\log_{10}$ after 48 h.

The population of MRSA t4038 was reduced by 3.2 $\log_{10}$ after 24 h in TSB with 40% RH, 10, 20 and 30% RJ, 20-40% RJ:RH (1:100), and after 48 h in TSB with 30% RH and again with 20, 30 and 40% RJ:RH (1:100) (Table 5). The population reached 7 $\log_{10}$ after 24 h incubation in TSB with 10 and 20% RH and then after 48 h incubation it declined to 3.47 and 3.26 $\log_{10}$, respectively. In all other cases the population was grown by at least 7 $\log_{10}$. In general, there are not many references in the international literature on the antimicrobial activity of honey and royal jelly, and particularly for the *S. aureus* and MRSA.

Table 3. Antibacterial activity of Rape Honey (RP), Royal Jelly and mix RJ:RH (1:100) at several concentration in Tryptone Soy broth (TSB) against MRSA t127

Substance	Concentration	Initial inoculum	Counts after 24h	Counts after 48h
RH	10%	3.34 CFU/mL	>8 log CFU/mL	>8 log CFU/mL
	20%		>8 log CFU/mL	>8 log CFU/mL
	30%		3.53 CFU/mL	1.39 CFU/mL
	40%		0	0
RJ	10%	3.34 CFU/mL	>8 log CFU/mL	>8 log CFU/mL
	20%		0	0
	30%		0	0
RJ:RH (1:100)	10%	3.34 CFU/mL	>8 log CFU/mL	>8 log CFU/mL
	20%		3.58 log CFU/mL	2.11 log CFU/mL
	30%		0	0
	40%		0	0

Table 4. Antibacterial activity of Rape Honey (RP), Royal Jelly and mix RJ:RH (1:100) at several concentration in Tryptone Soy broth (TSB) against MRSA t548

Substance	Concentration	Initial inoculum	Counts after 24h	Counts after 48h
RH	10%	3.17 CFU/mL	>7 log CFU/mL	>7 log CFU/mL
	20%		>7 log CFU/mL	>7 log CFU/mL
	30%		>7 log CFU/mL	>7 log CFU/mL
	40%		3.54 log CFU/mL	>7 log CFU/mL
RJ	10%	3.17 CFU/mL	0	0
	20%		0	0
	30%		0	0
RJ:RH (1:100)	10%	3.17 CFU/mL	>7 log CFU/mL	>7 log CFU/mL
	20%		>7 log CFU/mL	>7 log CFU/mL
	30%		>7 log CFU/mL	>7 log CFU/mL
	40%		0	3.47 log CFU/mL

Table 5. Antibacterial activity of Rape Honey (RP), Royal Jelly and mix RJ:RH (1:100) at several concentration in Tryptone Soy broth (TSB) against MRSA t4038

Substance	Concentration	Initial inoculum	Counts after 24h	Counts after 48h
RH	10%	3.2 CFU/mL	>7 log CFU/mL	3.47 log CFU/mL
	20%		>7 log CFU/mL	3.26 log CFU/mL
	30%		3.29 log CFU/mL	0
	40%		0	0
RJ	10%	3.2 CFU/mL	0	0
	20%		0	0
	30%		0	0
RJ:RH (1:100)	10%	3.2 CFU/mL	>7 log CFU/mL	>7 log CFU/mL
	20%		0	0
	30%		0	0
	40%		0	0

The MIC of four varieties of honey from Algeria for *S. aureus* ranged between 20% and 21% (v/v), while the MIC of RJ was 2% (v/v). When honey and RJ were used jointly, all honey varieties had a more than 50% decrease in MIC with 1% (v/v) RJ (Boukraa et al., 2008). In another study in Algeria, the MIC of RJ was 1.7% (vol /vol) against *S. aureus* and 2% against *Escherichia coli* (Boukraa et al., 2009). When starch was added in RJ, a MIC decrease of 61% and 30% against *S. aureus* and *E. coli*, respectively. Manuka honey showed a MIC of 6% and 7% against methicillin-resistant and methicillin-sensitive *S. aureus* (Alzahrani et al., 2012).

The broad spectrum of antibacterial activity of honey is mainly against Gram-positive bacteria (Marcucci et al., 2001) and is highly complex due to the involvement of multiple compounds and due to the large variation in the concentrations of these compounds among honeys. The antimicrobial action of the hydrogen peroxide in honey that is produced by glucose oxidase (Dustmann, 1979; Taormina et al., 2001), the high osmolarity (honey consists of 80% w/v of sugars) (Dustmann, 1979), the presence of lysozyme and its high antimicrobial potential (Bogdanov, 1997) are well characterized (Molan, 1992). Recently, methylglyoxal (MGO) in manuka honey and the antimicrobial peptide bee defensin-1 in revamil honey have been identified as important antibacterial compounds (Sesta, 2006; Adams et al., 2008; Mavric et al., 2008).

RJ has shown antimicrobial effects against a wide range of bacteria, viruses, yeast, and fungi (Alreshoodi & Sultanbawa, 2015). It has been reported that RJ has antibacterial activity against both Gram-positive and Gram-negative bacteria due mainly to fatty acids present in RJ, such as trans-10-hydroxydec-2-enoic acid, 3-hydroxydodecanoic acid, 11-oxododecanoic acid, and 11-S-hydroxydodecanoic acid (Melliou & Chinou, 2005; Alreshoodi & Sultanbawa, 2015). Furthermore, a series of short peptides (jelleines, royalisin) present in RJ have also been shown to possess strong antibacterial properties against Gram-positive and Gram-negative bacteria and yeasts (Fujiwara et al., 1990; Fontana et al., 2004; Alreshoodi & Sultanbawa, 2015; Bilikova et al., 2015).

Conclusion

As the development of antibiotic-resistant bacteria spreads and an increasing interest in the alternative use to antibiotics therapies has been developed, bee products may receive renewed recognition as healing agents. In general, honey and royal jelly, alone or in combination, appear to have a potential as alternative therapeutic agents against MRSA infections, but clinical studies are needed for confirmation.

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