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Original Article

Reduction Effect of Royal Jelly and Rape Honey Alone and in Combination Against Methicillin-Resistant *Staphylococcus aureus* (MRSA) Strains

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Multidrug resistant and methicilin-resistant *Staphylococcus aureus* (MRSA) is involved in severe difficult to treat skin and soft tissue infections in humans. In the present study the antibacterial reduction effect of royal jelly (RJ), rape honey (RH), as well as in combination (RJ:RH, 1:100 w/w) against multidrug resistant MRSA strains was evaluated by means of a microbiological method "*in vitro*". Royal jelly and rape honey mixture possessed a higher antibacterial activity than rape honey. The concentrations of royal jelly (20 and 30% v/v) had a total inhibitory effect against tested MRSA strains. Royal jelly alone and in rape honey mix (RJ:RH, 1:100 w/w) have a potential as alternative therapeutics against MRSA strains, resistant for antibiotics.

Key Words: Bee honey, Royal jelly, Antibacterial activity, Methicilin-resistant *Staphylococcus aureus*

INTRODUCTION

The broad spectrum of antibacterial activity of honey is mainly against gram positive bacteria (1) gram-positive and gram-negative (2), and also fungi and yeasts (3) 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 (4, 5), the high osmolarity (honey consists of 80% w/v of sugars) (5), the presence of lysozyme and its high antimicrobial potential (6) are well characterized (7). Recently, methylglyoxal (MGO) in manuka honey and the antimicrobial peptide bee defensin-1 in revamil honey have been identified as important antibacterial compounds (8, 9).

The high antibacterial effect of RJ has also been reported (10, 11). The antibacterial activity of royal jelly, rape honey, individually and in combination has been reported against resistant strain of *Escherichia coli* (12) and *Aeromonas hydrophila* (ATCC 7965) (13).

Antibiotic-resistant bacteria represent a critical problem in modern medicine world-wide (14) and consequently; scientific efforts have been developed to control bacterial infections with alternative medicines beyond conventional antibiotic therapy. Among these alternative therapeutic agents are honey (15), propolis (16) and royal jelly (17). The difference between MRSA and other forms of *S. aureus* is that MRSA has become resistant to many kinds of antibiotics, making it more difficult to treat. Antimicrobial activities of

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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

royal jelly (RJ), rape honey (RH), as well as in combination (RJ:RH, 1:100 w/w) against MRSA strains with multidrug resistance not has been reported previously.

The aim of this study was to determine the antimicrobial effect of rape honey and royal jelly, individually or in combination (RJ:RH, 1:100 w/w) against MRSA strains with multidrug resistance.

MATERIALS AND METHODS

Test substances

Test substances were Bulgarian rape honey (RH), royal jelly (RJ) and mix of royal jelly and rape honey (RJ:RH), (1:100 w/w). The rape honey and royal jelly 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 supplemented with carbohydrate syrups or treated with antimicrobial drugs. Until the analysis, samples were stored in sterilized jars in refrigerator at 0~4℃. 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 (18). The botanical origin of the samples was established by their melissopalynological, organoleptic, physical and chemical characteristics (19). 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 queens cells. The following parameters of samples were determined: sugars (fructose, glucose, sucrose by HPLC according to Sesta (20); proteins by Folin-Ciocalteu reagent; water content by refractometry; dry matter of the sample by subtracting the water content from 100; pH values by potentiometrically by pH meter Mi 150 (1% water solution of royal jelly); total acidity by titration with 0.1 N NaOH; electrical conductivity of 1% water solution of royal jelly by conductometer (18, 21) (Table 2). Royal jelly was stored prior to analyses in a dark bottle in freezing conditions (-20℃). 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, associated with antimicrobial activity in honey (21) all test substances were stored in the dark and dilutions were prepared immediately prior to testing (22).

All data referring to physical and chemical parameters of used for experiments rape honey and royal jelly were statistically processed by the Student's *t*-test and presented as mean, standard deviation (SD), minimum and maximum values (Tables 1 and 2).

Bacterial strains and preparation of inoculum

Three MRSA isolates belonging to Dr. D. Sergelidis collection were used in our study. These isolates belonged to spa types t127 (isolated from goat carcass), t4038 (isolated

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

Table 3. Experimental data for dilutions of 0.5 McFarland standard and isolation rate of tested MRSA strains on Baird Parker agar with 0.01 and 0.0025% w/v potassium telluride

Dilution of 0.5 McFarland standard	% potassium telluride	MRSA t127 CFU (0.1 ml)	MRSA t548 CFU (0.1 ml)	MRSA t4038 CFU (0.1 ml)
10^{-1}	0.0025%	$> 10^7$	$> 10^7$	$> 10^7$
	0.01%	$> 10^7$	$> 10^7$	$> 10^7$
10^{-2}	0.0025%	$> 10^6$	$> 10^6$	$> 10^6$
	0.01%	$> 10^6$	$> 10^6$	$> 10^6$
10^{-3}	0.0025%	$> 10^5$	$> 10^5$	$> 10^5$
	0.01%	284 / 125	$> 10^5$	$> 10^5$
10^{-4}	**0.0025%**	**165 / 185**	**388 / 369**	**349 / 336**
	0.01%	98 / 82	176 / 173	3 / 3
10^{-5}	0.0025%	45 / 36	59 / 31	45 / 33
	0.01%	6 / 4	18 / 17	2 / 0
10^{-6}	0.0025%	8 / 3	7 / 4	6 / 2
	0.01%	0 / 0	1 / 0	0 / 0
10^{-7}	0.0025%	1 / 0	1 / 0	0 / 0
	0.01%	0 / 0	0 / 0	0 / 0

from unpasteurized goat's milk) and t548 (isolated from marinated anchovies). They were multidrug resistant exhibiting resistance to three or more antibiotic classes. Only strain belonged to spa type t127 was found to carry the specific mecA gene (20). Strains were stored in cryo-tubes containing Tryptone Soy broth (Merck, Darmstadt, Germany) supplemented with 15% glycerol at -80℃. Prior to experiments the MRSA strains were incubated for 35℃ in TSB (Merck, Darmstadt, Germany) for 24 h and then a loopfull was streaked onto Blood agar and incubated for 24 h at 35℃. 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 (1.5×10^8 CFU/ml). Decimal dilutions to 10^{-4} in 9 ml sterile TSB were prepared from the initial suspension.

Experimental design

Because of possibility that potassium tellurite in Baird Parker agar could have inhibitory effect for *Staphylococcus aureus* and it should be reduced to maximize the isolation rate it was made initial experiment with MRSA strains. It was found that dilutions of bacterial suspension adjusted to the 0.5 McFarland standard (1.5×10^8 CFU/ml) from all MRSA strains possessed different isolation rate in Baird-Parker agar with 0.01 and 0.0025% w/v after 24 h incubation at 35℃. The high isolation rate in Baird Parker agar with 0.0025% w/v potassium telluride explain using of this percent for next experiments with RH, RJ:RH and RJ (Table 3).

Dilutions of test substances

In sterile glass were weigh 20 g of rape honey (RH) and add 20 ml sterile TSB to prepare 50% (w/v). For preparing of mix from rape honey and royal jelly (RJ:RH, 1:100 w/w) in sterile glass were weigh 0.2 g of royal jelly and add 20 g of rape honey. To prepare 50% (w/v) solution from RJ:RH (1:100 w/w mix) then add 20.2 ml of sterile TSB. To prepare 50% (w/v) RJ solution with sterile stick were weigh equal parts royal jelly and sterile TSB. All initial solutions were shake well with glass sticks. From initial 50% (w/v) solutions were made 5.7 ml 10, 20, 30 (RJ) and 40% (v/v) (RH and RJ:RH) solutions.

The tubes were inoculated with the bacterial cultures from each MRSA isolate according to the method described by Patton *et al*. (23). After contamination all solutions were incubated at 35℃ for 24 h for first and respectively for 48 h for second experiment. In order to the determination of survived staphylococci after 24 and 48 h, serial 10-fold

Table 4. Calculation and convertion to logarithmic CFU /ml for positive controls

Dilution	Mean values from 2 Petri dishes		Mean from two experiments	CFU/ml	log CFU/ml
	1st experiment	2nd experiment			
MRSA spa type t127					
Positive control (contamination)					
10^{-4}	216	229	222.5	2.2×10^3	3.34
Positive control (10^{-4}) after incubation for 24 h					
10^{-5}	146	169	157.5	1.6×10^8	8.2
10^{-6}	Uncountable	–	–	–	–
MRSA spa type t548					
Positive control (contamination)					
10^{-4}	148	152	150	1.5×10^3	3.17
Positive control (10^{-4}) after incubation for 24 h					
10^{-5}	62	78	70	0.7×10^8	7.85
10^{-6}	Uncountable	–	–	–	–
MRSA spa type t4038					
Positive control (contamination)					
10^{-4}	155	170	162.5	1.6×10^3	3.2
Positive control (10^{-4}) after incubation for 24 h					
10^{-5}	91	96	93.5	9.35×10^7	7.97
10^{-6}	Uncountable	–	–	–	–

dilutions in 0.1% peptone water supplemented with 2.5% NaCl were prepared. Thereafter, 0.1 ml from each tube was streaked onto Baird Parker agar (Merck, Darmstadt, Germany) containing 0.0025% w/v potassium telluride and rabbit plasma fibrinogen. Typical *S. aureus* colonies were counted after incubation at 35℃ for 24 h. For the detection of survivors at populations lower than 10 CFU/g, the first dilution was incubated for enrichment at 35℃ for 24 h and then 10 µl were spread plated on Baird Parker agar.

The experiments were performed twice and the results

Table 5. Antibacterial activity of Rape Honey (RP), Royal Jelly (RJ) and mix RJ:RH (1:100) at several concentrations in Tryptone Soy broth (TSB) against MRSA t127

Substance	Positive control		Concentration	Counts after first experiment (24 h)	Counts after second experiment (48 h)
	Initial inoculum	Count after 24 h			
RH	3.34 log CFU/ml	8.2 log CFU/ml	10%	>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	3.34 log CFU/ml	8.2 log CFU/ml	10%	>8 log CFU/ml	>8 log CFU/ml
			20%	0	0
			30%	0	0
RJ:RH (1:100)	3.34 log CFU/ml	8.2 log CFU/ml	10%	>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 6. Antibacterial activity of Rape Honey (RP), Royal Jelly (RJ) and mix RJ:RH (1:100) at several concentrations in Tryptone Soy broth (TSB) against MRSA t548

Substance	Positive control		Concentration	Counts after first experiment (24 h)	Counts after second experiment (48 h)
	Initial inoculum	Count after 24 h			
RH	3.17 log CFU/ml	7.85 log CFU/ml	10%	>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	3.17 log CFU/ml	7.85 log CFU/ml	10%	0	0
			20%	0	0
			30%	0	0
RJ:RH (1:100)	3.17 log CFU/ml	7.85 log CFU/ml	10%	>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 7. Antibacterial activity of Rape Honey (RP), Royal Jelly (RJ) and mix RJ:RH (1:100) at several concentrations in Tryptone Soy broth (TSB) against MRSA t4038

Substance	Positive control		Concentration	Counts after first experiment (24 h)	Counts after second experiment (48 h)
	Initial inoculum	Count after 24 h			
RH	3.2 log CFU/ml	7.97 log CFU/ml	10%	>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	3.2 log CFU/ml	7.97 log CFU/ml	10%	0	0
			20%	0	0
			30%	0	0
RJ:RH (1:100)	3.2 log CFU/ml	7.97 log CFU/ml	10%	>7 log CFU/ml	>7 log CFU/ml
			20%	0	0
			30%	0	0
			40%	0	0

are presented as mean values. To calculate the reduction rate the counts of MRSA cells in the positive controls after 24 h incubation in TSB (Table 4) was compared with results from two experiments (Tables 5, 6, and 7).

All analyses were done in Department of "Food Hygiene and Control, Veterinary Legislation and Management", Trakia University, Faculty of Veterinary Medicine, Stara Zagora, Bulgaria.

RESULTS

There were not survived cells of MRSA t127 (3.34 log10 reduction) after 24 h of 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 log10 and 1.23 log10 was observed in TSB with 30% RH and 20% RJ:RH (1:100) after 48 h of incubation (Table 5). The counts in the other concentrations of all substances were more than 8 log10 after 48 h.

A reduction of 3.17 log10 of MRSA t548 was observed after 24 h of incubation in TSA with 10, 20 and 30% RJ and with 40% RJ:RH (1:100) (Table 6). Although a reduction almost 3.17 log10 was observed after 24 h in TSA with 40% RJ:RH (1:100), count of MRSA t548 reached 3.54 log10 at 48 h. The counts in the other concentrations of all substances were more than 7 log10 after 48 h.

The staphylococcal cell count of MRSA t4038 was reduced by 3.2 log10 after 24 h in TSB with 40% RH, 10, 20 and 30% RJ, 20~40% RJ:RH (1:100), and after 48 hours in TSB with 30% RH and again with 20, 30 and 40% RJ:RH (1:100) (Table 7). The population reached 7 log10 after 24 h incubation in TSB with 10 and 20% RH and then after 48 h of incubation it declined to 3.47 and 3.26 log10, respectively. In all other cases the population was grown by at least 7 log10.

DISCUSSION

There are not many references in the literature on the antimicrobial activity of royal jelly and honey, and particularly for the MRSA. RJ has shown antimicrobial effects against a wide range of bacteria, viruses, yeast, and fungi (24). 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 (11, 24). 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 (17, 24~26).

In study from Algeria, the minimum inhibitory concentration (MIC) of RJ was 1.7% (v/v) against *S. aureus* and 2% against *E. coli* (27). When starch was added in RJ, a MIC decrease of 61% and 30% against *S. aureus* and *E. coli*, respectively. 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 (28). Manuka honey showed a MIC of 6% and 7% against methicillin-resistant and methicillin-sensitive *S. aureus* (29).

In general from our results, by means of a microbiological method independent use of rape honey (10~30%) not have total antibacterial effect on MRSA strains. Royal jelly and rape honey mixes possessed a higher antibacterial activity than rape honey. Royal jelly alone and in rape honey mix (1:100 w/w) have a potential for alternative therapy against MRSA strains, resistant for antibiotics.

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