

## Full Length Research Paper

# Antibacterial effect of Royal gelly, mix from Royal jelly and rape honey (1:100), rape and oak honeydew honeys against *Escherichia coli* (ATCC 25922)

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Received 22 May, 2012; Accepted 28 March, 2014

The Royal jelly (RJ), RJ and rape honey (HJ), rape (RH) and oak honeydew honey (HH) at concentrations of 10 to 45% were contaminated with 50 to 100 CFU/ml *Escherichia coli* ATCC 25922. After plating onto ChromoCult® TBX agar up to 30 min from contamination and after enrichment in Tryptic soy broth (TSB) at 37°C for 24 h, it revealed no visible colonies. These concentrations were defined as real bactericidal concentrations (RBC). Judging by results from spectrophotometric method, percent of inhibition and pH, it was observed, follow succession from drop of antibacterial activity for tested substances that RJ > HH > HJ > RH.

**Key words:** Royal jelly, honey, antibacterial, *Esherichia coli*.

## INTRODUCTION

The use of honey as a traditional remedy for microbial infections dates back to ancient times (Molan, 1992). The difference in antimicrobial potency among the different types of honeys can be more than 100-fold, depending on its geographical, seasonal and botanical source as well as through harvesting, processing and storage conditions (Molan and Cooper, 2000).

The antimicrobial activity of honey is attributed largely to osmolarity, pH, hydrogen peroxide production and the presence of other phytochemical components. *In vivo*, such activity may occur due to a synergistic relationship between any of these components rather than a single entity (Mavric et al., 2008). It was found that the honey acids and low pH exert the main antibacterial factors

(Bogdanov, 1997). Manuka honeys from New Zealand, which originates from the manuka tree (*Leptospermum scoparium*), is sold as a therapeutic antibacterial agent world wide. The presence of methylglyoxal (MGO) in this type of honey has been termed as unique manuka factor (UMF®) (Willix et al., 1992; Taormina et al., 2001).

Usually in spectrophotometrical studies for detection of antibacterial effect of honey, turbidity of solutions are connected, contaminated with microorganisms with high count of bacteria and as a result, there are no counted microorganisms from them. The minimum inhibitory concentration (MIC) described the concentration before the tubes with turbid solutions and all concentrations greater than MIC are described as bactericidal concentrations

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(Wahdan, 1998). Pathogen strains of *Escherichia coli* are often causative agents of more intestinal infections for animals and humans. According to spectrophotometric studies, the MIC<sub>100</sub> (the lowest concentration of test material which results in 100% inhibition of growth) value for *E. coli* to manuka honey was 12.5% (v/v) (Patton et al., 2006).

Some authors found that growth of *E. coli* was completely inhibited by 30 to 100% honey concentrations (Noori and Al-Waili, 2004). In other study, antibacterial activity of 13 types of honey were tested at four concentrations (10, 5, 2.5 and 1% w/v) against *E. coli*. It was found that several honeys can inhibit *E. coli* and may have potential as therapeutic honeys (Wilkinson and Cavanagh, 2005). As the potential role for honey as a topical agent to manage surgical site or infections is increasingly acknowledged, other types of honeys need to be assessed and evaluated (Gethin and Cowman, 2008).

The hypopharyngeal glands of the honeybee (*Apis mellifera* L.) produces royal jelly (RJ) that is essential to feed and raise broods and queens (Li et al., 2010). RJ may cause allergic reactions in humans, asthma and even fatal anaphylaxis; thus this product remains unaffordable in most countries (Leung et al., 1997; Lombardi et al., 1998; Takahama and Shimazu, 2006). From another perspective, it was found to have more positive effect of RJ: immunostimulating, activating vegetative and central neural systems etc. The main RJ acid, 10-hydroxy-2-decenoic acid (10-HDA) is known to have high antibiotic effect (Blum et al., 1959; Melliou and Chinou, 2005). Research suggests that the 10-HDA found in RJ may inhibit the vascularization of tumors (Izuta et al., 2007). Recently, it was found to have specific antibacterial peptide Royalisin in RJ and displayed certain antibacterial activities against Gram-positive bacteria (Shen et al., 2010). Some authors work on peptides originally isolated from the RJ and on their analogs. They found synergy with peptides belonging to the family of temporins (temporin A and temporin B) against *Staphylococcus aureus* A170 and *Listeria monocytogenes* (Romanelli et al., 2011). Few studies found out about antibacterial effect of RJ on Gram-negative microorganisms (Shirzad et al., 2007). Recently, *E. coli* have been used to determine the minimum inhibitory concentration (MIC) of a freshly reaped RJ. The MIC of RJ against *E. coli* was 2% (v/v) (Boukraa et al., 2009). To avoid acid taste and allergic reactions after consumption of royal jelly, many producers recommend mixing of this product with honey, mainly in proportion of 1:100. In available references not cited, studies about exact degrees of antibacterial activity from this mix have been conducted. There are no comparative investigations between spectrophotometrical and microbiological methods for MIC determination for RJ and mix from RJ, with different types of honey.

Thus, the aim of our study was to investigate, by microbiological method, the real bactericidal concentration (RBC) or 100% inhibition (0 CFU/ml), after 24 h incubation by 37°C, of royal jelly (RJ), mix from royal jelly and rape honey (HJ), independent used rape (RH) and honeydew (HH) honeys to referent strain of *E. coli* (ATCC 25922). Also, to compare calculated % of inhibition by parallel implemented microbiological and spectrophotometrical method.

## MATERIALS AND METHODS

### Test substances

Used in the experiment, RJ was obtained from "Beekeeping Centre-South", town Stara Zagora, Bulgaria. Investigated RH was obtained in the period of May to June, 2011 from the village Kozarevec, region of Stara Zagora, Bulgaria. Mixtures from RJ and RH (HJ) in proportion 1:100 w/w (1.0 royal jelly: 100.0 rape honey) have been done experimentally. Used in the study, HH was obtained in a period of July to August, 2008 from apiary near Madzharovo, Haskovo region, Bulgaria. Prior to analyses, all honey samples were stored in dark place, with storehouse conditions (up to 18°C). The samples (bee honey and royal jelly) were from bee families not treated with sugars or antibiotics. Samples (bee honeys) were received by unsealing of combs, centrifugation and filtration. As used in the study, RJ was pipette directly from queens cells. The analysis of RJ samples was published in Balkanska et al. (2012). In brief, the following parameters were determined: proteins by Folin-Ciocalteu reagent; sugars (fructose, glucose, sucrose) by high performance liquid chromatography (HPLC); water content by refractometer; pH, potentiometrically; total acidity by titration with 0.1 N NaOH; electrical conductivity by conductimeter.

pH and free acidity were applied by the European requirements (Bogdanov et al., 1997; Codex Alimentarius Committee on sugars, 2001; European Commission, 2002). The botanical origin of the samples was established by their melissopalynological, organoleptic, physical and chemical characteristics (Table 2) (von der Ohe et al., 2004; Oddo et al., 2004). From some authors, only storage of RJ in frozen state prevents decomposition of biologically active proteins and thus RJ should be frozen as soon as it is harvested (Li et al., 2007). For our experiments, RJ was stored prior to analysis in the dark bottle in frozen conditions (-10°C). Immediately before conducting microbiological assays in order to aid pipetting during preparation of diluted honey solutions, all test substances were adjusted to 40°C in a water bath. Solutions containing 10, 20, 30, 40 and 45% (v/v) from each of test substances were prepared in sterile TSB. To prevent photodegradation of glucose oxidase, connected with antimicrobial activity in honey (Bogdanov, 1997), all honey samples and HJ were stored at room temperature in the dark and dilutions were prepared immediately prior to testing (Sherlock et al., 2010).

### Bacterial strain and preparation of bacterial suspension

Bacterial suspension was with density 0.5 McFarland and prepared from 24 h bacterial culture of referent strain *Escherichia coli* ATCC 25922, by taking 3 to 4 colonies and dissolving in 0.85% sterile saline solution. Received bacterial suspension was with approximate concentration  $1.5 \times 10^8$  CFU/ml. From suspension were prepared tenfold dilutions with sterile Tryptic soy broth (TSB), (Merck) at to  $10^{-7}$ . For detection of exact count of *E. coli* from each of

dilutions (1 ml) was made cultivation with ChromoCult® TBX Agar (Merck), followed by incubation with 37°C for 24 h. By microbiological examination from bacterial suspension and dilutions it was found that in dilution used for contamination of test substances, *E. coli* were in concentration of  $1.1 \times 10^3$  CFU/ml.

#### Determination of percent inhibition (%) by spectrophotometric assay

A spectrophotometric assay through Vis Spectrophotometer (Model: SP-870 Plus) method described by Patton et al. (2006), with some differences, was performed. Briefly, 0.6 ml of start bacterial suspension ( $1.1 \times 10^3$  CFU/ml) was added to 11.4 ml from each dilution of the concentrations stated (10 to 45% v/v) from all the test substances. Test-tubes were incubated in the dark at 37°C for 24 h. The optical density was determined just prior to incubation ( $T_0$ ) and again after 24 h incubation ( $T_{24}$ ) at 620 nm. The optical density (OD) from each of solutions at  $T_0$  was subtracted from the OD for each replicate at  $T_{24}$ . The percent inhibition of growth was determined using the formula:

$$\text{Percent inhibition} = 1 - (\text{OD test tube} / \text{OD of corresponding control tube}) \times 100$$

#### Determination of percent inhibition (%) and real bactericidal concentration (RBC) by microbiological assay

Used quantity of 0.6 ml for test suspension of *E. coli* in each test solution (11.4 ml) maintained the mean final concentration of 55 CFU/ml from all dilutions in TSB contaminated with bacterial suspense test substances and possessed different acidity (Table 1). By parallel microbiological assay it was found that in positive control (0.6 ml from used dilution from bacterial suspense to 11.4 ml TSB), count of *E. coli* increased after 24 h incubation from initial 55 CFU/ml to  $1.1 \times 10^8$  CFU/ml. To determine whether the antibacterial activity of all tested substances was bacteriostatic or bactericidal, 1 ml from each test-tube with each concentration of test substances was plated in 2 Petri dishes, poured with about 15 ml selective agar (ChromoCult® TBX Agar) (Merck), followed by incubation at 37°C for 24 h and counting of visible colonies (Table 2).

To compare with spectrophotometric method and to establish time depending influence from test substances to the growth of *E. coli*, this microbiological survey was done parallel with spectrophotometric assay twice, up to 30 min after inoculation (without incubation), and after 24 h incubation at 37°C from all dilutions in TSB of contamination with bacterial suspense test substances. Similar methodology was used in previous study but with *Aeromonas hydrophila* as test microorganism (Stratev et al., 2012). The concentration of test substances with used dilution from bacterial suspension in TSB, which causes lack of visible colonies onto selective TBX agar up to 30 min from contamination and after incubation for 24 h at 37°C, was defined as the real bactericidal concentration (RBC) (Stratev et al., 2012).

With a view to calculate percent of inhibition by microbiological method we adopted 100% as the initial (55 CFU/ml up to 30 min) and final ( $1.1 \times 10^8$  CFU/ml after 24 h incubation) bacterial count in positive control without antibacterial substances. Having in mind the exact counts of colonies before and after incubation, from each of test substances was calculated the percent of inhibition. RBC and percent of inhibition by microbiological method were compared with percent of inhibition found by spectrophotometric method (Table 2). All experiments were done in the "Department of Hygiene, Technology and Control of Animal Foodstuffs, Veterinary Legislation and Management", Trakia University, Stara Zagora, Bulgaria.

**Table 1.** pH and free acidity of royal jelly, honey samples and positive control in TSB before inoculation.

Parameter	RJ	HJ	RH	HH	TSB
pH	3.98	5.23	5.41	4.99	7.3
Free acidity	62	55	32	40	-

RJ – Royal jelly; HJ –Royal jelly mixed in rape honey 1:100 w/w; RH – rape honey; HH – oak honeydew honey; TSB – Triptic Soy Broth.

## RESULTS

Results found by microbiological method showed that after a period from 30 min for adaptation of bacterial cells, their count by 10% concentration from all test substances remained almost constant (Table 2). Concentrations above 20% had influence on the count of *E. coli* and this effect was great for RJ, where count was 3 CFU/ml (94.6% of inhibition) and by high concentrations (30, 40 and 45%), cultivated forms of bacteria was not found (100% of inhibition). Despite this, we have in mind a short time for influence on microorganisms; in this case we cannot consider this effect as bactericidal. For HJ, RH and HH, cultivated forms of *E. coli* was found from 20 to 40%, but its count was lower than 10% concentrations. After a period for incubation of test substances by 37°C for 24 h was count bacterial growth, but with different intensity from each test substances and concentrations (Table 2).

Received results from microbiological assay for 10% concentrations from all tested substances showed high quantity of bacteria above  $10^5$  CFU/ml (< 40% inhibition). Similar results were found by 20% RJ and HH. For RJ and HJ, 20% concentration lead to inhibition of bacterial growth and this was more intensive for RJ. Detected count of bacteria for RJ was only 7 CFU/ml (93.6% inhibition), and for HJ it was  $2.0 \times 10^4$  CFU/ml (46.5% inhibition). For concentrations 30, 40 and 45%, RJ had lack of bacterial multiplication and 100% inhibition of bacterial cells, which demonstrated the real bactericidal effect from substance (RBC). Similar tendency was found for same concentrations of HH.

For 20%, HJ found increased bacterial count but not with normal speed and with long generation time which lead to low bacterial count in comparison with 20% concentration of tested types of honey and 10% HJ. Low bacterial increasing was found by 30% (97.9% inhibition), HJ. These data was close to percent of inhibition found by spectrophotometrical method, respectively (99.8 and 99.9%) by 30 and 40% HJ. We can note 100% of inhibition by 45% HJ, as well as microbiological and spectrophotometrical method (Table 2). In concentrations of 30, 40 and 45% RH and 30, 40% HJ was found high % of inhibition and low count cultivated forms of *E. coli* whose number remained almost constant in comparison with this before incubation. This meant that bacterial cells

**Table 2.** Antibacterial effect of different substances on *E. coli* ATCC 25922.

Test substance	% (v/v)	Spectrophotometrical method	Microbiological method			
			30 min		24 h	
			% inhibition	CFU/ml	% inhibition*	CFU/ml
RJ	10	74.2	57	0	>10 <sup>5</sup>	<40.0
	20	95	3	94.6	7	99.4
	30	97.2	0	100	0	RBC
	40	96.9	0	100	0	RBC
	45	96.3	0	100	0	RBC
HJ	10	83.9	32	41.8	>10 <sup>5</sup>	<40.0
	20	98.7	3	94.6	2.0×10 <sup>4</sup>	46.5
	30	99.8	8	85.5	23	97.9
	40	99.9	9	83.6	12	98.9
	45	100	14	74.6	0	RBC
RH	10	51	11	80.0	>10 <sup>5</sup>	<40.0
	20	66.4	5	90.9	>10 <sup>5</sup>	<40.0
	30	99.9	11	80.0	8	99.3
	40	99.9	7	87.3	11	99.0
	45	99.9	11	80.0	1	99.9
HH	10	61.2	47	14.6	>10 <sup>5</sup>	<40.0
	20	82	2	96.4	>10 <sup>5</sup>	<40.0
	30	97.7	4	92.7	0	RBC
	40	94.3	3	94.6	0	RBC
	45	94.4	2	96.4	0	RBC
<b>Positive control (TSB)</b>			55	0	1.1×10 <sup>8</sup>	0

RJ – Royal jelly; HJ –Royal jelly mixed in rape honey 1:100 w/w; RH – rape honey; HH – oak honeydew honey; TSB – Tryptic Soy Broth; RBC - Real Bactericidal Concentration; \* - inhibitory effect calculated in percent (%) as a value difference on a basis CFU/ml in positive control.

were alive and vital but not in condition to breeding by these concentrations.

## DISCUSSION

Our findings for RBC dependence from pH for tested types of honeys were in agreement with pH as the main antibacterial factor (Bogdanov, 1997). The high inhibition effect from 30, 40 and 45% RH and 20, 30 and 40% HJ could be connected with high concentrations of solutions, and for HJ also from additional antimicrobial substances from RJ (Shirzad et al., 2007). Our data for comparisons between percent of inhibition, found by microbiological and spectrophotometrical method, was not proven for all tested types of honey and RJ opinion from some authors for strong dependence between turbidity of contaminated honey solutions and high count of live microorganisms (Wahdan, 1998).

Comparison with RBC for 30 to 45% RJ and HH with high turbidity and calculated high percent of inhibition, found by spectrophotometrical method, proved our opinion (Table 2). This lead to the conclusion that RJ and HH turbidity of contaminated test solutions with high quantity of *E. coli* could not be connected with lack of real bactericidal activity. On the other hand 40 to 45% RH turbidity of contaminated test solutions could be connected with high quantity of fast crystallized glucose in this type of honey (Devillers et al., 2004). These findings could be connected also with data found by spectrophotometrical method for Manuka Care 18+ (UMF) honey, contaminated with *E. coli* NCIMB 8545. According to this study, the plots obtained for the bacterial species using the spectrophotometric method have a distinct nonlinear relationship (Patton et al., 2006). According to some authors, there is evidence of a two-stage process of inhibition, something that requires further investigation (Snow and Manley-Harris, 2004).

Recent studies for quality of RJ showed that in this product found high quantity of proteins (9 to 18%) (Sabatini et al., 2009). In connection with these amino acids, particularly L-proline, were with high concentrations in RJ (369 to 1930 µg/g) (Saito et al., 2011). It is well known that honeydew honeys contain more proline than blossom honeys (Bogdanov et al., 1997). This could be connected with findings that precipitate formation in honeys with high amount of protein can be explained by a well known interaction of hydrogen bonding between hydroxyl groups of phenolic compounds and peptide bonds of protein in forming strong insoluble polyphenol-protein complex in aqueous solution, resulting to high turbidity (Hategekimana et al., 2011). RJ shows high antibacterial activity toward *E.coli* ATCC 25922, which could be connected with its high acidity mainly from 10-hydroxy-2-decenoic acid (10-HDA), with high antibiotic characteristics (Blum et al., 1959; Melliou and Chinou, 2005).

Small differences between percent of inhibition, found by microbiological and spectrophotometric method for 30 to 45% RJ and HH, could be explained from data obtained by other studies. It was found that proteins with high affinity for binding polyphenols are those containing proline and from this the relative haze-forming activity depends on its proline content (Siebert, 1999, 2006). This findings lead to an hypothesis that small differences for percent of inhibition between RJ and HH by two used methods could be explained with high quantity of proline such that binding polyphenols lead to high turbidity and consequently resulted in the calculated low percent of inhibition by spectrophotometric method.

Considering the great potential of royal jelly and honey for treatments of different bacterial diseases, in the future, it is essential that research should continue and statistical comparisons between data obtained with microbiological and spectrophotometrical methods for detection of real antibacterial activity be made.

## Conclusion

The antibacterial activity from RH with added RJ (1:100) increased and for 20% solution reached 46.5% inhibition ( $2.0 \times 10^4$  CFU/ml). Small differences between percent of inhibition, found by microbiological and spectrophotometric method, for 30 to 45% RJ and HH could be explained by findings that proteins containing proline have high affinity for binding polyphenols and depending on proline content, which is the relative haze-forming activity (Siebert, 1999, 2006). On the basis of our results it was observed follow succession from drop of antibacterial activity for tested substances that: RJ > HH > HJ > RH. From this we could conclude that the basic factor which determined the antibacterial activity from test substances to *Escherichia coli* ATCC 25922 come from

acidity (pH or free acidity). Future investigations to determine if the main antibacterial factor is pH or free acidity needs to be done.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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