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Original article

ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF ROYAL JELLY COLLECTED FROM GEOGRAPHICAL REGIONS WITH DIFFERENT CLIMATES IN THE NORTH OF IRAN

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Summary

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Royal jelly is one of the most important bee products. The biological activities of royal jelly can be influenced by various factors such as geographic origin, climatic conditions, vegetation. This study was carried out to evaluate the antibacterial and antioxidant activity of royal jelly samples collected from beehives from different geographical regions including mountain, coastal and plain regions in northern Iran. Antibacterial activity of royal jelly samples against ten bacteria was determined using agar well diffusion method. The MIC and MBC of royal jelly samples were determined by the broth microdilution method. Folin-Ciocâlteu reagent and reaction with DPPH were used to determine the total phenolic content and antioxidant potential of royal jelly samples, respectively. The MIC of samples ranged from 0.78 to 12.5% and MBC – from 3.12 to 50%. Samples collected from mountain regions showed the highest antibacterial activity with MIC for Gram-positive bacteria from 0.78 to 1.56% and for Gram-negative bacteria: from 1.56 to 3.12%. The total phenolic content and DPPH radical scavenging activity in royal jelly samples of the mountain region was significantly higher than those from the two regions with other climates. The results of this study indicated that the climate of the geographic region of sampling location had an effect on the antibacterial and antioxidant activity of royal jelly which may be due to differences in plant vegetation and the origin of the flowers of bees.

Key words: antibacterial activity, antioxidant activity, geographical region, royal jelly

INTRODUCTION

Today, due to population growth and the limitations of modern medicine, the necessity of research into introducing new antimicrobial agents of natural origin is strongly needed to reduce antibiotic resistance and unwanted side effects of chemical agents.

The honeybee is one of the most useful and most developed insects. Honeybee, on the one hand, performs pollination of

crops, gardens, and pasture plants increasing the crop yields and vegetation residues in rangelands, and on the other hand produces honey. Besides honey, other products such as royal jelly, propolis, pollen, wax have many benefits to humans.

Royal jelly is one of the most important bee products, also called King Angbin and King Jelly, which is attributed to numerous biological activities, including antibacterial and antioxidant ones. Royal jelly is a result of the secretion of the hypopharyngeal glands of the bees, produced at the age of 6 to 16 days. As age increases and activity changes, the secretion of these glands changes as well. All larvae are fed this substance in the first three days and after 3 days, those who become worker bees are fed honey and pollen, and those to be converted into a queen are fed only royal jelly. Royal jelly plays an important role in the growth of infants, completing the special sexual traits and the long life of the queen. Digestive and milky glands in the Honeybees by the production of royal jelly plays an important role in the evolution of the hive larvae, especially the queen. It seems that this kind of food causes the fundamental differences between the working bees and the queen, so that the life of the queen is more than fifty-two times the length of life of the worker bees, and in term of size the queen is 2–3 times larger than the workers (Fujita *et al.,* 2013).

In terms of physical properties, royal jelly is a concentrated and milky substance with a pungent smell and specific sour taste (pH =3.4–4.5), with a density of 1.1 g/cm^3, slightly soluble in water (Fratini *et al.,* 2016; Sadredini *et al.,* 2016).

Royal jelly contains 60–70% water, 11–23% carbohydrates, 9–18% protein, 4–8% lipids, and 0.8–3% vitamins, mineral salts and low molecular weight compounds. Royal jelly composition varies greatly according to the season, weather conditions, bee nutrition, and climatic conditions of the geographical region (Sabatini *et al.,* 2009; Fratini *et al.,* 2016).

Carbohydrates make up about 30% of the dry weight of the royal jelly. The most abundant sugars include fructose, glucose, and sucrose. Other oligosaccharides such as maltose, trehalose, melibiose, ribose, and other sugars are also found in small amounts (Fratini *et al.,* 2016).

Proteins are up to 50% of the dry weight of the royal jelly. The most important royal jelly protein compositions include the family of major royal jelly proteins (MRJPs), antimicrobial peptides (AMPs) including royalisin, apismin, jelleines, royalactin, apolipophorin III-like protein, and glucose oxidase (Fratini *et al.,* 2016).

Lipids make up 3 to 19% of royal jelly's dry weight. Almost 90% is made up of fatty acids, and the rest is from neutral fats, steroids, hydrocarbons, and phenols. The most important fatty acid in the structure of royal jelly is the 10 hydroxy-2 decanoic acid (10-HDA) which is an unsaturated fatty acid involved in the antibacterial activity of the product. This fatty acid is the most important marker of royal jelly quality. Also, octanoic acid at amounts lower than those of 10-HDA has nutritional roles (Fratini *et al.,* 2016).

Various studies have been reported the differences and diversity of antibacterial activities of royal jelly samples collected from different geographical regions (Garcia *et al.,* 2010; 2013; Garcia-Amoedo & de Almeida-Muradian, 2007).

The antioxidant activity of royal jelly can be attributed to phenolic compounds – phenolic acid, cinnamic acid, coumarin, isocoumarin, naphthoquinone, xanthan, anthraquinone, flavonoids, and lignin.

Numerous studies have pointed to the diversity of phenolic compounds and antioxidant activity of royal jelly samples collected from geographical regions with different climates in different parts of the world (Nabas *et al.,* 2014; Ceksteryte *et al.,* 2016; Balkanska *et al.,* 2017; Ozkok & Silici, 2017; Balkanska, 2018). The difference in the amount of phenolic compounds in the royal jelly depends on factors such as the weather, the herbal source used by the bee, the season, the genetic factors, environmental factors such as the type of soil (Perez-Perez *et al.,* 2013; Sadredini *et al.,* 2016).

The present study aimed to investigate the antibacterial and antioxidant effects of royal jelly collected from beehives from three geographical regions with different climates in the north of Iran.

MATERIALS AND METHODS

Royal jelly samples

Royal jelly samples were collected from beehives in three geographical regions with a different climate in the north of Iran including mountain, coastal, and plain. Until the experiments samples were kept at −18 °C in the freezer of the Microbiology Laboratory at Islamic Azad University of Azadshahr branch. Samples from beehives in the mountain region of the Shahkuh-e Sofla in Gorgan township (mountain), coastal regions of the Farahabad in Sari township (coastal), and plain regions of the Daland in Ramian township (plain) were collected. The geographical and vegetation characteristics of the regions are presented in Table 1.

Serial dilution of royal jelly samples was carried out in sterilised distilled water and dilutions were prepared, resulting in final concentrations of 100%, 50%, 25%, 12.5%, 6.3%, 3.12%, 1.56%, 0.78% and 0.39% v/v of each of the royal jelly samples.

Bacterial strains

In this study, the antibacterial activity of royal jelly samples was evaluated against 4 bacterial native isolates and 6 bacterial reference strains. The reference strains used in this study comprised three Gram-

Table 1. Characteristics of royal jelly samples and collection regions

Sample name and collection region	Climatic, geographical and vegetation characteristics
Shahkuh-e Sofla (mountain)	Shahkuh-e Sofla village is 65 km south-east of Gorgan in Golestan province. The village is 2540 meters above sea level with an average annual rainfall of 584 mm. It is a mountainous region with cold climate and snowy winters. The area's vegetation includes broad-leaved trees, shrubs and bush. Its plant species are yew, aras, juniper, pyro, maze, beech, hornbeam, sloe, barberry, lily, blackbird, and herbaceous and bush species of oxtongue, alfalfa, sage, and artemisia.
Farahabad (coastal)	Farahabad is a coastal town 25 km north of Sari in Mazandaran Province. It is 26 meters below sea level. The climate in this region is temperate with abundant annual rainfall. The vegetation of the region includes agricultural lands paddy and gardens contains citrus fruits such as oranges, tangerine, and orange.
Daland (plain)	Daland city of Ramian environs is located 60 km east of Gorgan in Golestan province. The city is 65 meters above sea level and has fertile plains. The climate in this region is temperate. The vegetation of the region is covered with oak, free, maple and evergreen trees, and agricultural lands.

negative bacteria included *Escherichia coli* (PTCC 1338), *Pseudomonas aeruginosa* (PTCC 1811) and *Shigella dysenteriae* (Persian Type Culture Collection, PTCC 1188) and three Gram-positive species included *Staphylococcus aureus* (PTCC 1112), *Bacillus cereus* (PTCC 1154) and *Enterococcus faecalis* (PTCC 1778) which were provided from the Iranian Research Organization for Science and Technology (IROST) in a lyophilised form. The reference strains were recovered in the Brain Heart Infusion (BHI) medium (Merck, Germany) for 24 h at 37 °C.

Native isolates included *S. aureus* isolated from the nasal cavity of the carrier, *E. coli* isolated from the stool, *P. aeruginosa* isolated from water, and *B. cereus* isolated from the soil. The isolates were identified using routine microbial laboratory tests for each (selective media culture, Gram stain, catalase and oxidase tests, biochemical tests).

The 24-hour cultures of bacteria were inoculated into Nutrient Broth culture medium (Merck, Germany) and incubated at 37 °C to obtain turbidity equal to 0.5 McFarland = 1.5×10^8 CFU/mL. When turbidity of the tube was equal to 0.5 McFarland standard, the absorbance value on 625 nm wavelength was between 0.08 to 0.1 (Weinstein *et al.,* 2018).

Evaluation of the antibacterial activity of royal jelly

The antibacterial activity of royal jelly samples was carried out based on agar diffusion and well method. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each specimen were tested by broth microdilution tube method.

Agar well diffusion method

In the well method, the uniform spread culture was prepared from 0.5 McFarland bacterial suspensions (1.5×10^8 CFU/mL) from all reference strains and native isolates on Muller Hinton Agar medium (Himedia, India) using sterile cotton swabs. Then, using a sterilised cork borer, wells with a diameter of 4.5 mm were created on the medium and 100 µL of each of the serial dilutions of royal jelly samples was poured into wells. The plates were incubated at 37 °C for 24 hours. After incubation, the diameter of the inhibition zone was measured using a millimeter ruler and recorded (Eshraghi *et al.,* 2003; Weinstein *et al.,* 2018).

Determination of MIC and MBC

The determination of MIC of royal jelly samples was carried out based on turbidimetric assay and by using the microdilution tube method in sterile 96 well microplate. For this purpose, serial dilutions of royal jelly samples were prepared in nutrient broth (Merck, Germany) into the wells of microplate. A suspension equivalent to 5×10^5 CFU/mL from each of bacteria was added to each of these wells and incubated for 24 h at 37 °C. There were also negative control tubes (serial dilutions of royal jelly prepared in nutrient broth without bacterial suspension) and positive control tubes (bacterial suspension of 5×10^5 CFU/mL without royal jelly). The results for visible microbial turbidity were recorded after 24 h of incubation. The last dilution (lowest concentration) in which microbial turbidity was not observed, was considered as MIC. For the determination of MBC, the tube that contained royal jelly with concentration higher than the MIC was cultured onto the nutrient agar medium (Merck, Germany). The MBC was defined as the lowest con-

centration at which no colony was observed on the agar (Weinstein *et al.,* 2018).

Determination of the total phenolic content of royal jelly

The total phenolic content (TPC) of royal jelly samples was analysed by using Folin-Ciocâlteu reagent. In this method, 0.5 mL royal jelly solution in distilled water (0.1 g/mL) was mixed with 2.5 mL of Folin-Ciocâlteu reagent (2N) and incubated for 5 minutes. Then 2 mL of sodium carbonate solution (75 g/L) was added into the royal jelly solution and incubated for 2 hours at 25 °C. After incubation for reaction and formation of blue colour, the absorbance of the solution was measured at 765 nm by using a UV-Visible spectrophotometer (Jenway-UK). (Folin-Ciocâlteu and from gallic acid within the concentration range from 0.1 to 0.9 mg/mL (R^2=0.9998, y=0.1254x–0.1424). The total phenolic content was reported as the mean value of triplicate assays and expressed as mg gallic acid equivalent per 100 g of royal jelly sample (mgGAE/100 g) (Singleton *et al.,* 1999; Ozkok & Silici, 2017).

Evaluation of the free radical scavenging activity

The free radical scavenging activity of royal jelly samples was determined using the 2,2-diphenyl-1-picrylhydrazyl hydrate radical (DPPH) (Brand-Williams *et al.,* 1995; Balkanska *et al.,* 2017). DPPH is a free radical, hydrophilic, stable, and purple that due to the presence of single electrons has a maximum absorption at 515–517 nm. The transfer of hydrogen atoms to DPPH radicals from reductive compounds such as phenols and their conversion to non-radical forms results in a decrease in absorption of DPPH solution at this wavelength and the change of the colour to yellow. One hundred µL of 1000 ppm methanolic royal jelly solution and 100 µL methanolic DPPH· solution (2 mM) were mixed and placed in a dark place. The absorbance was measured at 517 nm after 15 min of incubation at 25 °C. The control test was done with methanol instead of royal jelly against methanol as blank,. The experiments were performed in triplicate and the percent inhibition of DPPH free radicals was calculated by the equation:

$$\text{DPPH scavenging activity (\%)} = 1 - [(A_{\text{Control}} - (A_{\text{Sample}} / A_{\text{Control}})] \times 100$$

Statistical analysis

All data were expressed as mean±standard deviation (n=3). The data were analysed statistically with one-way ANOVA using SPSS version 18.0 software. Comparison of the means with Duncan's multi-domain test was performed at a level of significance $P \leq 0.05$.

RESULTS

Antibacterial activity of royal jelly

Results of the mean diameter of inhibition zone of 10 tested bacteria against royal jelly collected from beehives from three different geographical regions including mountain (Shahkuh-e Sofla), coastal (Farahabad), and plain (Daland) regions are presented in Table 2.

Royal jelly samples collected from the beehives in the Shahkuh-e Sofla (mountain) and Farahabad (coastal) areas showed significant antibacterial activity and in concentrations of 100 and 50% against all tested bacteria were effective ($P<0.05$). Among these, the antibacterial activity of royal jelly of mountain area

Table 2. Inhibition zone diameters (mm) in the presence of different concentrations of royal jelly samples, well method (Mean±SD)

Bacteria (reference strain/native isolate)	Royal jelly concentration (%)			
	12.5	25	50	100
Shahkuh-e Sofla (mountain)				
E. coli (N)	–	5 ± 0.5^{Bb}	9.5 ± 0.5^{Ad}	10.5 ± 0.7^{Ae}
E. coli (R)	–	–	5.5 ± 0^{Be}	7.5 ± 0.5^{Af}
B. cereus (N)	–	–	10 ± 1^{Bd}	14.5 ± 1^{Ad}
B. cereus (R)	–	–	13.5 ± 0.7^{Bc}	16.5 ± 0.6^{Ac}
P. aeruginosa (N)	–	–	–	5.5 ± 0^{Ag}
P. aeruginosa (R)	–	–	10 ± 0.5^{Ad}	10.5 ± 1.2^{Ae}
S. aureus (N)	–	8.5 ± 1^{Ba}	21.5 ± 1.5^{Aa}	23.5 ± 0.9^{Aa}
S. aureus (R)	–	9 ± 1^{Ba}	17 ± 0.8^{Ab}	18.5 ± 0.4^{Ab}
E. faecalis (R)	–	–	11 ± 1^{Bd}	13.5 ± 0.1^{Ad}
S. dysenteriae (R)	–	–	–	8.5 ± 0.5^{Af}
Farahabad (coastal)				
E. coli (N)	–	–	11.5 ± 1.5^{Ac}	12 ± 0.5^{Ab}
E. coli (R)	–	–	5 ± 0.5^{Bd}	6.5 ± 0.5^{Ad}
B. cereus (N)	–	–	10.5 ± 0.5^{Ac}	11 ± 1^{Ab}
B. cereus (R)	–	–	13 ± 1.2^{Ab}	14.5 ± 0.7^{Aa}
P. aeruginosa (N)	–	–	6.5 ± 0^{Ad}	7 ± 0^{Ad}
P. aeruginosa (R)	–	–	5.5 ± 0^{Ad}	6.5 ± 0^{Ad}
S. aureus (N)	–	–	7 ± 0^{Bd}	9.5 ± 0.9^{Ac}
S. aureus (R)	–	–	14.5 ± 0.9^{Aa}	15.5 ± 0.5^{Aa}
E. faecalis (R)	–	–	–	5.5 ± 0^{Ad}
S. dysenteriae (R)	–	–	5 ± 0^{Ad}	6 ± 0.5^{Ad}
Daland (plain)				
E. coli (N)	–	–	–	5 ± 0^{Ab}
E. coli (R)	–	–	–	5 ± 0.5^{Ab}
B. cereus (N)	–	–	–	5.5 ± 0.5^{Ab}
B. cereus (R)	–	–	6 ± 0.5^{Aa}	7 ± 1^{Aa}
P. aeruginosa (N)	–	–	5.5 ± 0.9^{Aa}	6 ± 0.5^{Aa}
P. aeruginosa (R)	–	–	5.5 ± 0^{Aa}	6 ± 1^{Aa}
S. aureus (N)	–	–	–	5.5 ± 0.7^{Ab}
S. aureus (R)	–	–	–	6 ± 0.5^{Aa}
E. faecalis (R)	–	–	6 ± 1^{Aa}	6.5 ± 0.5^{Aa}
S. dysenteriae (R)	–	–	–	6.5 ± 1^{Aa}

Diameters are given in (mm) including well (4.5 mm); N: native isolate; R: reference strain; (–): no zone of inhibition. Different lowercase letters in each column indicate significant difference at the 5% probability level; different uppercase letters in each row indicate significant difference at the 5% probability level.

against native isolate of *S. aureus* was interesting with mean inhibition zone diameter of 23.5, 21.5, and 8.5 mm, in concentrations of 100, 50 and 25%, respectively (Table 2).

Royal jelly samples collected from beehive in the plain area (Daland) showed lower antibacterial activity compared to other samples (P<0.05). This royal jelly sample with mean inhibition zone diameters of 7, 6.5, and 6.5 mm in pure concentration, was effective against reference strains of *B. cereus*, *E., faecalis*, and *S. dysenteriae* respectively (Table 2).

In general, in comparison with the mean diameter of the inhibition zone of the tested bacteria, royal jelly samples collected from beehives in the mountain (Shahkuh-e Sofla) and plain (Daland) regions showed the highest and lowest diameter of inhibition zone, respectively (P<0.05). Therefore, the geographic region climate of samples collecting site was found to influence significantly the antibacterial activity of royal jelly samples from the mountain, coastal, and plain sites (P<0.05).

As can be seen from the results of Table 2, Gram-positive bacteria were significantly more sensitive to different concentrations of royal jelly samples than Gram-negative bacteria (P<0.05).

Statistical analysis of inhibition zone diameters showed that *S. aureus* (reference strain and native isolate) and *B. cereus* (reference strain) were the most sensitive to different concentrations of royal jelly and Gram-negative bacteria *E. coli* (reference strain), *P. aeruginosa* (native isolate) and *S. dysenteriae* were the most resistant to different concentrations of royal jelly samples (P<0.05).

MIC and MBC of royal jelly

Results of the MIC and MBC of royal jelly samples collected from beehives of three geographical regions with different climates including mountain (Shahkuh-e Sofla), coastal (Farahabad), and plain (Daland) regions are presented in Table 3. MIC values of royal jelly samples were within the range from 0.78 to 12.5%, and MBC – from 3.12 to 50% (Table 3). In this method, like the well method, the royal jelly sample collected from the mountain region (Shahkuh-e Sofla) showed the highest antibacterial activity against the tested bacteria compared with the other two regions. MICs of royal jelly sample collected from mountain region

Table 3. Minimum inhibitory concentration (MIC, %) and minimum bactericidal concentration (MBC, %) of royal jelly samples

Bacteria (reference strain/native isolate)	Shahkuh-e Sofla (mountain)		Farahabad (coastal)		Daland (plain)	
	MIC	MBC	MIC	MBC	MIC	MBC
E. coli (N)	3.12	12.5	6.25	25	12.5	50
E. coli (R)	1.56	12.5	3.12	25	6.25	25
B. cereus (N)	0.78	3.12	3.12	6.25	6.25	12.5
B. cereus (R)	0.78	3.12	1.56	3.12	3.12	12.5
P. aeruginosa (N)	3.12	12.5	12.5	50	12.5	50
P. aeruginosa (R)	3.12	12.5	6.25	25	6.25	50
S. aureus (N)	1.56	12.5	3.12	12.5	3.12	12.5
S. aureus (R)	0.78	6.25	1.56	12.5	3.12	12.5
E. faecalis (R)	1.56	12.5	3.12	12.5	3.12	12.5
S. dysentriae (R)	3.12	12.5	6.25	25	6.25	25

N: native isolate; R: reference strain.

Table 4. Total phenolic content (TPC) and free radical scavenging activity of royal jelly samples

Sample	Total phenolic content (mg GAE/100 g)	Free radical scavenging activity (%)
Shahkuh-e Sofla (mountain)	10.10 ± 1.35^{a}	7.35 ± 1.05^{a}
Farahabad (coastal)	7.81 ± 0.15^{b}	5.40 ± 0.08^{b}
Daland (plain)	5.56 ± 0.95^{c}	3.80 ± 0.11^{c}

Different lowercase alphabet letters in each column indicate significant difference at the 5% probability level.

(Shahkuh-e Sofla) for Gram-positive *B. cereus* (native isolate), *B. cereus* (reference strain), *S. aureus* (reference strain), *S. aureus* (native isolate) and *E. faecalis* were 0.78, 0.78, 0.78, 1.56 and 1.56% respectively. These values were 3.12% for inhibiting the growth of Gram-negative bacteria except for the native *E. coli* isolate (1.56%). These results indicated the significant antibacterial activity of this bee product (Table 3).

On the other hand, royal jelly samples collected from plain region (Daland) showed the lowest antibacterial activity against the tested bacteria. MBC of this royal jelly sample was 50% for *E. coli* (native isolate) and *P. aeruginosa* (reference strain and native isolate). All tested Gram-positive bacteria including native isolates and reference strains were killed with a minimum concentration of 12.5% of this royal jelly sample (Table 3).

The Gram-positive bacteria such as *S. aureus* and *B. cereus* were more sensitive compared to Gram-negative bacteria such as *E. coli* and *P. aeruginosa* with this method, as the MBC of royal jelly samples were in the range of 3.12 to 12.5% for Gram-positive bacteria but within the range 12.5–50% for Gram-negative bacteria (Table 3).

Total phenolic content and free radical scavenging activity of royal jelly

Table 4 shows the total phenolic content and percentage of free radical scavenging of DPPH in royal jelly samples collected from beehives from three geographical regions with different climates. The sample collected from the beehives in the mountain region (Shahkuh-e Sofla) had the highest total phenolic content and the highest free radical scavenging activity compared to samples of other two climates The royal jelly sample had the highest total phenolic content – 10.1 mg gallic acid per 100 g (mg GAE/100 g). Also, this sample had the highest DPPH free radical inhibition at 1000 ppm compared to other samples: 7.35% ($P<0.05$).

DISCUSSION

The royal jelly sample collected from the mountain region (Shahkuh-e Sofla), showed the most pronounced antibacterial activity against the tested bacteria ($P<0.05$). Garcia *et al.* (2013) have reported difference in antibacterial activity of four royal jelly samples collected from different geographical regions of Argentina, showing that the geographic origin of royal jelly collecting location was effective in its antibacterial activity. The results of their research indicated that Gram-positive bacteria were more sensitive to different concentrations of royal jelly samples than Gram-negative bacteria, which is consistent with the results of our study. They also reported that *Enterococcus* bacteria were more resistant than other Gram-positive genera, which is one

of the results of the present study (Garcia *et al.,* 2013).

Antibacterial activity of royal jelly can be attributed to major royal jelly proteins (MRJPs), royal jelly antimicrobial peptides (AMPs) which include royalisin, apisimine, jelleines, and royalactin, apolipophorin III-like protein and glucose oxidase. In addition to proteins, 10-hydroxy-2-decanoic acid (10-HDA) is the most important fatty acid found in royal jelly, which plays a key role in its antibacterial activity (Fratini *et al.,* 2016).

Most protein constituents of royal jelly belong to a family called major royal jelly proteins (MRJPs), also called apalbumin, that make up 83–90% of royal jelly protein constituents. In this protein family, eight proteins with molecular weights of 49 to 87 kDa have been identified (MRJP 1–8). These proteins play a key role in feeding the queen. They can also play an important role in the production of other bee compounds, especially pollen grains. In addition to these proteins, there are other proteins in lower amounts that have antimicrobial activity and the antimicrobial properties of royal jelly are related to these proteins. These antimicrobial peptides (AMPs) include royalisin, apisimine, and jelleines, royalactin (Fratini *et al.,* 2016).

The antimicrobial peptides of the royal jelly are relatively small polypeptides with 10 to 50 amino acids, due to the presence of 2 to 9 alkaline amino acid sequences such as lysine, arginine, and histidine with a positive charge, whose electrostatic reaction with the surface of the cell membrane of the bacterium that has a negative charge, results in penetration of the cell membrane and, by creating holes in the cell membrane of the bacterium, affects the permeability of the membrane. In other words, the amino acid sequence of peptides plays a very important role for antibacterial activity, and the presence and displacement of an amino acid can affect this antibacterial activity (Brogden, 2005; Pandey *et al.,* 2011; Ebenhan *et al.,* 2014).

In addition to reacting with the bacterial cell membrane, the antimicrobial peptides of royal jelly can bind to other targets such as DNA, RNA, and proteins within the cell, and prevent the synthesis of essential constituents of bacterial cells such as DNA, RNA, and proteins (Li *et al.,* 2012; Fratini *et al.,* 2016).

Recently, another protein called apolipophorin III, which can contribute to the antibacterial activity of royal jelly with other antimicrobial peptides has been identified (Han *et al.,* 2011).

The glucose oxidase enzyme is one of the other proteins found in the royal jelly, which plays a role in its antibacterial activity by catalysing glucose conversion to hydrogen peroxide (Sagona *et al.,* 2015).

The most important fatty acid in the structure of the royal jelly is 10-hydroxy 2 decanoic acid (10-HDA), an unsaturated fatty acid involved in its antibacterial activity. This fatty acid is the most important indicator of the quality of the royal jelly. However, some researchers have also pointed to the antibacterial role of other fatty acids in royal jelly such as 3-hydroxydodecanoic acid, 11-oxododecanoic acid, and 11-hydroxydodecanoic acid (Melliou & Chinou, 2005).

In addition, Parveen & Rao (2012) suggested that secondary metabolites in the royal jelly such as phenols, flavonoids, glycosides, terpenoids, sterols, lignin, and saponins can show significant antibacterial activity against Gram-positive bacteria and Gram-negative bacteria. Andreas *et al.* (2005) also found that the biological activity of the royal jelly was very diverse

and presented great variations and that it was linked to other microelements. In general, the observed difference in antibacterial activity of various samples of royal jelly collected from different regions with different climate is related to royal jelly compounds.

Royal jelly composition could vary with seasonal and regional conditions of feeding (Chen & Chen, 1995; Antinelli *et al.,* 2003; Attalla *et al.,* 2007; Sabatini *et al.,* 2009), with the type of plants from which nectar and pollen are supplied (Ozkok & Silici, 2017), with metabolites and changes in the physiology of nurse bees as well as with the larval age (Brouwers *et al.,* 1987; Abd-alla *et al.,* 1995), with bees' genetics and race (Liu *et al.,* 2008; Zheng *et al.,* 2011), and above all could be modified from the storage conditions postharvest (Ragab & Ibrahim, 1999; Li *et al.,* 2008; Liu *et al.,* 2008; Zheng *et al.,* 2011). Several research studies correlated these variations of royal jelly composition to its antimicrobial activity (Abd-alla *et al.,*1995; Ragab & Ibrahim, 1999; Li *et al.,* 2008; Liu *et al.,* 2008; Zheng *et al.,* 2011).

One of the results of this study was the higher sensitivity of Gram-positive bacteria compared to that of Gram-negative bacteria against different concentrations of royal jelly samples. This sensitivity is also reported in other studies (Fujiwara *et al.,* 2004; Garcia *et al.,* 2010, 2013; Moselhy *et al.,* 2013). Studies have shown that the cell wall of Gram-negative bacteria was more resistant than that of Gram-positive bacteria against many antibiotics, antimicrobial chemicals, and even many herbal drugs. This resistance can be due to the lower permeability of the outer membrane of these bacteria and the presence of the lipopolysaccharide layer of the cell wall and as well as the periplasmic space that limits the entry of antimicrobial agents into the bacterial cell (Nikaido, 2003).

The interesting point about comparing the well method to broth microdilution method for determination of MIC and MBC in this study is that in the microdilution method, royal jelly samples were able to inhibit the tested bacteria at very low concentrations, whereas in the well method antibacterial effect was observed only in very high concentrations – 100% and 50%. This finding was also mentioned in other studies (Garcia *et al.,* 2013). The reason for this can be the high molecular weight of the antimicrobial compounds of royal jelly samples, the hydrophobicity of the compounds, and their non-solubility in water. Given that the well method is based on agar diffusion, low molecular weight compounds can spread more and with better speed and display higher antibacterial activity, while in the microdilution method, the bacterial suspension is mixed with the desired concentration of royal jelly in the tube. On the other hand, many studies attribute the antibacterial activity of royal jelly to 10-HDA, a water-insoluble fatty acid and one of the most important constituents in royal jelly that contributes to its numerous biological activities (Garcia-Amoedo & Almedia-Muradian, 2003; Fratini *et al.,* 2016). The lower antibacterial activity in well method can be related to poor propagation of this substance in agar. However, the direct proximity of royal jelly with bacterial suspension in the microdilution method of broth solves this problem.

The well method is used as a popular and validated technique for testing antibacterial activity. The disadvantage of this method is that it is not a quantitative technique. Another important point in this technique is that hydrophobic compounds are hardly propagated in agar. So the well

method is still an appropriate technique for determining antibacterial activity, at least as an estimate method (Kalemba & Kunicka, 2003; Wilkinson, 2006). On the other hand, the microdilution broth method is a quantitative and accurate method for assessing the sensitivity of microorganisms to different compounds (Finegold & Baron, 1992).

The biological activity of royal jelly is mainly related to active bio fatty acids, proteins, and phenolic compounds (Jamnik *et al.,* 2012). Dezmirean *et al.* (2012) analysed the total phenolic content of the royal jelly samples in the range of 148 to 436 mg GAE/kg. Balkanska *et al.* (2017) have reported total phenolic content of 20 samples of royal jelly from different regions of Bulgaria in the range of 11.66 to 36.63 mg GAE/g while the amount of DPPH free radical inhibition of royal jelly samples was reported at 11.7 to 39.39%. Ceksteryte *et al.* (2016) reported total phenolic content of royal jelly samples from Lithuania of 10.7 mg GAE/g.

To investigate the relationship between physicochemical parameters, antioxidant activity and total phenolic content of 10 fresh royal jelly samples, Balkanska (2018) reported total phenolic content of the samples ranging from 11.82 to 26.07 mg GAE/g, and amount of DPPH free radical inhibition of royal jelly from 15.07 to 35.59%. In another study, Ozkok & Silici (2017) reported an average total phenolic content of royal jelly samples from different provinces of Turkey of 59.16 mg GAE/100 g. The total phenolic content of royal jelly samples collected from Jordan was 23.3 µg GAE/mg (Nabas *et al.,* 2014). As can be seen in the above studies, the difference in the total phenolic content of royal jelly samples in different parts of the world is quite evident. The difference in the amount of phenolic compounds in royal jelly samples depends on factors such as the climate, the origin of the flowers and plants used by the honey bee, the season, the genetic factors and environmental factors such as the type of soil (Perez-Perez *et al.,* 2013; Sadredini *et al.,* 2016). Other reasons for these differences are the conditions for the preservation of royal jelly samples. They are very important for its biological properties. Royal jelly is extremely sensitive to light and heat and its oxidation rapidly happens in direct contact with air (Buttstedt *et al.,* 2013; Fratini *et al.,* 2016). Pavel *et al.* (2014) have compared commercial samples with fresh and native royal jelly samples from different geographical regions of Romania and concluded that freshly harvested samples had higher total phenolic content, DPPH free radical inhibition, and 10 hydroxy-2 decanoic acid content compared to commercial samples (Pavel *et al.,* 2014). The results indicated a close relationship between antibacterial activity and antioxidant activity of royal jelly samples. This relationship can be attributed to the high content of total phenolic compounds in royal jelly which were the highest and lowest in mountain and plain regions, respectively.

CONCLUSION

Royal jelly produced by honey bees is a natural product with great potential for use in the food, pharmaceutical, and medical industries. Samples of royal jelly collected from the mountain region showed the highest antibacterial activity against the tested bacteria with the MBC in the range of 3.12 to 12.5% v/v. The results of this study indicate that the climate of the geographic region of sampling location influenced the antibacterial and antioxidant activity of the royal jelly, that

may be due to differences in plant vegetation and flower origin of the worker bees. However, study on the identification of royal jelly compounds in different regions is recommended to identify the active ingredient and its association with other environmental factors such as season, weather, soil composition, etc.

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