Inhibition of Pathogenic Bacteria and Antioxidant Activity of Royal Jelly

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Abstract

This study aimed to determine the antibacterial and antioxidant activities of royal jelly. Effects of royal jelly were investigated on growth inhibition against pathogenic bacteria, including *Corynebacterium* spp., methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *S. aureus* and *S. epidermidis* by agar well diffusion and broth dilution methods. The results showed that royal jelly could inhibit tested bacteria, except *P. aeruginosa*, with diameters of inhibition zone ranging between 9.33±0.58 to 21.67±2.08 mm. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of royal jelly were determined. As a result, royal jelly sample number 1 and 2 had the highest activity against *Corynebacterium* spp. with MIC and MBC of 18.75 and 18.75 mg/ml, respectively. The study of antioxidant activity by ABTS assay showed that royal jelly sample number 3 had the highest antioxidant activity by 1.20±0.12 mg Trolox equivalent antioxidant capacity/g royal jelly. Furthermore, royal jelly sample number 1 showed the highest phenolic compound content by 1.65±0.34 mg gallic acid equivalent/g royal jelly, and royal jelly sample number 3 showed the highest flavonoid compound content by 0.58±0.14 mg quercetin equivalent/g royal jelly.

Keywords**:** *Antibacterial activity, Antioxidant activity, Pathogenic bacteria, Royal jelly*

1. Introduction

Royal jelly is white-yellowish and viscous substance, sour taste with the characteristic pungent odor of phenol which produced by the young worker bees (*Apis mellifera*) (Fujita et al., 2013). It was fed to the developing larvae during the first three days and the queen bee for the entire life. Royal jelly also has a significant impact on the bee lifespan. The worker bee lives around 45 days while the queen bee could live up to five years. Royal jelly contains amino acids, carbohydrates, lipids, minerals, vitamins, and a bioactive compound; trans-10-hydroxy-2-decenoic acid (10-HDA) (Garcia-Amoedo & Almeida-Muradian, 2003; Marghitas et al., 2013; Fratini, Cilia, Mancini, & Felicioli, 2016). Proteins are the most important component of royal jelly. Major royal jelly proteins (MRJPs), which are the family of proteins in royal jelly, play an important role in queen bee development. MRJP 1, 4, and 5 are considered essential amino acids sources, while MRJP 2, 3, and 5 provided nitrogen sources for queen bee (Tamura et al., 2009). 10-HDA is the major lipid component of royal jelly (Garcia-Amoedo & Almeida-Muradian, 2003). It presents only in royal jelly and possesses several pharmacological properties (Okamoto et al., 2003; Isidorov, Czyżewska, Jankowska, & Bakier, 2011; Ramadan & Al-Ghamdi, 2012; Wytrychowski et al., 2013; Peng, Sun, Lin, Kuo, & Li, 2017). Moreover, 10-HDA is also considered as the most important compound for investigating the quality and authenticity of royal jelly (Kolayli et al., 2016). The compositions of royal jelly are affected by various factors such as seasons, collector type, floral variety, geographical and environmental conditions (Ramadan & Al-Ghamdi, 2012; Seven et al., 2014).

Royal jelly has many beneficial properties such as anti-inflammation, anti-bacteria, antioxidation, immune-activating, and anti-tumoral activity (Bincoletto, Eberlin, Figueiredo, Luengo, & Queiroz, 2005; Ramadan & Al-Ghamdi, 2012; Seven et al., 2014; Ramanathan, Nair, & Sugunan, 2018). The bioactive proteins of royal jelly are known to have immune regulatory and antibacterial effects in several studies (Bíliková et al., 2002). They may be involved in an active defense system against bacterial infection in the honeybee (Fujiwara et al., 1990). Besides, royal jelly is believed to exert similar effects in humans as it does in bees (Ramadan & Al-Ghamdi, 2012).

Microbial infections are protected by the human immune system but, if the immune system is impaired, the pathogenic bacteria can adhere to host cells, invade, and cause of diseases. Bacteria also possess

numerous virulent genes that allow bacterial growth in an unfavorable environment. Resident bacteria such as *Corynebacterium* spp. *Staphylococcus aureus*, *S. epidermidis,* and *Pseudomonas aeruginosa* can also cause skin infection (Chiller, Selkin, & Murakawa, 2001). They can access to tissues via wounds and cause diseases. Methicillin-resistant *S. aureus* (MRSA) contains *mecA* gene that encodes penicillin-binding protein 2a (PBP-2a), which can reduce the affinity of PBP to β-lactamase antibiotics (Choi, Kang, & Kim, 2015).

Oxidation is a chemical reaction that produces free radicals. They lead to chain reactions that can be harmful to the cells of microbes. Moreover, they can link to oxidative stress resulting in many diseases. Antioxidants are chemical compounds that can prevent or delay oxidation reaction by inhibiting the oxidative chain reactions of other molecules (Velioglu, Mazza, Gao, & Oomah, 1998).

The use of royal jelly has increased interestingly because of its attractive properties and health benefits (Kolayli et al., 2016). Generally, the royal jelly is used in cosmetics, healthy foods, food supplements, medical products, and pharmaceutical ingredients (Guo, Kouzuma, & Yonekura, 2009; Fratini et al., 2016). Accordingly, a better understanding of royal jelly components may improve the clinical and pharmaceutical uses of royal jelly as alternative medicine. Therefore, this research aimed to evaluate the biological activities of royal jelly from a bee farm in Thailand in order to develop the product which has antibacterial and antioxidant activities for alternative therapy in the future.

2. Objectives

1. To investigate the antibacterial activity against pathogenic bacteria of royal jelly
2. To determine the antioxidant activity and phenolic and flavonoid compounds content of royal jelly

3. Materials and Methods

3.1. Materials

3.1.1 Royal jelly

Six fresh royal jelly samples were obtained from bee farms in Thailand. Royal jelly samples 1, 2, 5, and 6 were from Phrae Province, sample 3 was from Nan Province, and sample 4 was Chiang Rai Province. The samples were kept frozen at -20 °C and protected from light. All samples were dissolved in distilled water before use.

3.1.2 Bacteria strains and culture

The tested Gram-positive bacteria, including *Corynebacterium* spp., methicillin-resistant *S. aureus* (MRSA) DMST 20625, *S. aureus* ATCC 25923, and *S. epidermidis* ATCC14990, and Gram-negative bacteria; *P. aeruginosa* ATCC 27853 were cultured on Mueller Hinton agar (MHA) at 37°C for 18-24 hr.

3.2. Antibacterial activity

3.2.1 Agar well diffusion

The effects of royal jelly were investigated on growth inhibition against pathogenic bacteria by agar well diffusion method. Bacterial cultures were adjusted by comparing to McFarland standards No. 0.5 and swabbed on an agar plate. The agar plates with microbial inoculum were punched aseptically with 6-8 mm diameter sterile cork borer. Next, royal jelly was diluted with distilled water to 300 mg/ml and added into the wells. Gentamicin and doxycycline 1 mg/ml concentration were used as positive controls, and distilled water was a negative control. Then, agar plates were incubated at 37°C for 18-24 hr. After that, the diameters of the inhibition zone was determined (Balouiri, Sadiki, & Ibnsouda, 2016). The experiment was performed triplicates independently.

3.2.2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) analysis

The MIC value of royal jelly was evaluated by the broth dilution method. Royal jelly was prepared by serial two-fold dilutions in Mueller Hinton broth (MHB). Then, inoculated with microbial inoculum after dilution to standardized microbial suspension, McFarland No. 0.5. After well-mixing, the inoculated tubes were incubated at 37°C for 18-24 hr. Then, the tubes were examined for growth or turbidity using unaided eyes. Then, MBC was determined by streak plate from tubes which no visible bacterial growth, and the plates were incubated at 37°C for 24 hr. The MBC endpoint was defined as the lowest concentration of royal jelly

that killed bacteria more than 99.9% (Balouiri et al., 2016). The experiment was performed triplicates independently.

3.3. Antioxidant activity

3.3.1 ABTS radical cation decolorization assay

Antioxidant activity of royal jelly samples was determined using ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) radical cation decolorization assay (Re et al., 1999). Briefly, an equal volume of 7 mM ABTS was mixed with 2.45 mM potassium persulfate ($K_2S_2O_8$) to produce ABTS radical cation ($ABTS^{\bullet+}$) mixture. The mixture was kept in the dark for 12-16 hr at room temperature to reach a stable oxidative state. The working solution was prepared by diluting the mixture with deionized water to achieve the absorbance of 0.700±0.020 at 734 nm. Royal jelly at various concentrations was mixed with $ABTS^{\bullet+}$ solution and incubated in dark condition for 10 minutes. After that, the absorbance was measured at a wavelength of 734 nm. Antioxidant activity will be presented as the ability to scavenge 50% of free radicals (IC50) and compared to TEAC (Trolox equivalent antioxidant capacity).

3.3.2. Phenolic compound analysis

The total polyphenols content of royal jelly was evaluated by the Folin-Ciocalteu method according to the protocol of Singleton, Orthofer, & Lamuela-Raventos (1999) with slight modifications using gallic acid as a calibration standard. Royal jelly was mixed with the Folin-Ciocalteu reagent. The absorbance was measured at 765 nm by a spectrophotometer. Total phenolic content was calculated and reported in milligrams of gallic acid equivalents per gram of royal jelly samples (mg GAE/g royal jelly).

3.3.3. Flavonoid compound analysis

The flavonoid compound content was evaluated by the aluminum chloride colorimetric method according to the protocol of Singleton et al. (1999) with slight modifications using quercetin as a calibration standard. Royal jelly was mixed with aluminum chloride and potassium acetate. The absorbance was measured at 415 nm by a spectrophotometer. Total flavonoid content was calculated and reported in milligrams of quercetin per gram of royal jelly samples (mg quercetin equivalent /g royal jelly).

3.4. Statistical analysis

The data were evaluated by the statistical program by One-Way analysis of variance (ANOVA) in SPSS program (version 24.0) with a significant difference at $P \leq 0.05$.

4. Results and Discussion

4.1 Antibacterial activity

Antibacterial activity of six royal jelly samples on pathogenic bacteria was determined using the agar well diffusion method by measuring the diameter of the inhibition zone. The results showed that royal jelly samples except sample number 3 had antibacterial activity against pathogenic bacteria with inhibition zone ranging between 9.33±0.58 to 21.67±2.08 mm (Table 1). All samples could not inhibit Gram-negative bacteria; *P. aeruginosa* by agar well diffusion assay and sample number 3 could not diffuse through the agar due to its viscosity. Susilowati et al. (2017) also reported that royal jelly might have low antimicrobial activity against *P. aeruginosa*. Royal jelly sample number 1, 2, and 6 showed high inhibition zone against *S. aureus,* whereas sample number 4, 5, and 6 showed high antibacterial efficacy against *Corynebacterium* spp. Followed by MRSA, Minimum inhibitory concentration, MIC, and minimum bactericidal concentration, MBC of royal jelly was also determined. The MBC corresponded to the concentration that kills 99.9% of the microorganisms (Bílikova, Huang, Lin, Šimuth, & Peng, 2015). The results of MIC and MBC were reported in Table 2. Royal jelly samples had MIC and MBC ranging between 18.75 to 150 mg/ml. Royal jelly sample number 1 and 2 had the highest activity against *Corynebacterium* spp. with MIC and MBC of 18.75 mg/ml. The royal jelly samples were tested comparing to the negative control; distilled water and the positive control antibiotic drug; gentamicin and doxycycline (for MRSA) at 1 mg/ml.

Table 1 Inhibition of pathogenic bacteria by royal jelly

Royal jelly samples (300 mg/ml)	Inhibition zone (mm)				
	Pathogenic bacteria				
	Corynebacterium spp.	MRSA	*P. aeruginosa*	*S. aureus*	*S. epidermidis*
1	0	10.00±1.00	0	12.33±1.15	11.00±1.41
2	0	10.00±1.00	0	13.00±1.00	10.67±0.58
3	0	0	0	0	0
4	20.33±1.53	11.00±1.00	0	10.33±0.58	9.33±0.58
5	21.00±1.73	12.00±1.00	0	10.33±0.58	0
6	21.67±2.08	11.00±1.00	0	12.67±0.58	9.33±0.58
Control					
Gentamicin	31.00±1.73	ND	31.00±1.00	29.67±0.58	31.00±1.00
Doxycycline	ND	31.67±0.58	ND	ND	ND
Distilled water	0	0	0	0	0

*ND = Not determine, Data represented mean values of three replicates±SD

Table 2 Minimum inhibitory concentration and minimum bactericidal concentration of royal jelly

Royal jelly samples	MIC and MBC (mg/ml)									
	Pathogenic bacteria									
	Corynebacterium spp.		MRSA		*P. aeruginosa*		*S. aureus*		*S. epidermidis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	18.75	18.75	75.00	75.00	150.00	150.00	75.00	75.00	75.00	75.00
2	18.75	18.75	75.00	75.00	150.00	150.00	75.00	75.00	75.00	75.00
3	37.50	37.50	75.00	75.00	150.00	150.00	75.00	75.00	75.00	75.00
4	37.50	37.50	75.00	75.00	150.00	150.00	75.00	75.00	75.00	75.00
5	37.50	37.50	75.00	75.00	150.00	>150.00	75.00	75.00	75.00	75.00
6	37.50	37.50	75.00	75.00	150.00	150.00	75.00	75.00	75.00	75.00
Control										
Gentamicin	0.0039	0.0039	ND	ND	0.0039	0.0039	0.0078	0.0156	0.0039	0.0078
Doxycycline	ND	ND	0.0039	0.0039	ND	ND	ND	ND	ND	ND

*ND = Not determine

Royal jelly has antimicrobial activity against microorganisms, including bacteria, fungi, yeast, and viruses. It has been reported that the royal jelly has antibacterial activity due to fatty acids present in royal jelly, in particular 10-HDA. Moreover, short peptides: jelleines and royalisin, also showed strong antibacterial properties (Alreshoodi & Sultanbawa, 2015). The main factors which provide the antibacterial activity of royal jelly, especially against Gram-positive bacteria, were MRJPs and royalactin (Fratini et al., 2016). The results from this study showed that Gram-positive bacteria were more sensitive to royal jelly than Gram-negative bacteria. Besides, many researchers also reported that royal jelly showed higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Gómez-Caravaca et al., 2006; Garcia, Finola, & Marioli, 2013).

4.2 Antioxidant activity

Antioxidant activity of royal jelly samples was studied *in vitro* by the ABTS assay, which is commonly used to analyze the antioxidant capacity due to their accuracy, simplicity, and stability (Reddy, Sreeramulu, & Raghunath, 2010). The 50% inhibitory concentration (IC_{50}) values and antioxidant activity of royal jelly were determined. The highest antioxidant activity was found from royal jelly sample number 3 (Table 3).

Table 3 Antioxidant activity of royal jelly by ABTS assay

Royal jelly samples	IC_{50} (mg/ml)	Antioxidant activity (mg TEAC/g royal jelly)
1	281.73±12.59	0.93±0.05^{c}
2	289.60±19.07	0.91±0.08^{c}
3	218.88±10.07	1.20±0.12^{a}
4	264.83±30.15	1.00±0.15^{bc}
5	250.06±38.48	1.08±0.28^{ab}
6	248.07±13.79	1.07±0.14^{ab}

*Different letters; a, b, and c indicate a significant difference according to Duncan at P<0.05

The total phenolic and flavonoid compound contents of royal jelly samples were shown in Table 4. The values varied in the range of 1.39±0.35 to 1.65±0.34 mg gallic acid equivalent/g royal jelly and 0.29±0.09 to 0.58±0.14 mg quercetin equivalent/g royal jelly, respectively.

Table 4 Phenolic and Flavonoid compounds content of royal jelly

Royal jelly samples	Phenolic compound content (mg gallic acid equivalent/g royal jelly)	Flavonoid compound content (mg quercetin equivalent/g royal jelly)
1	1.65±0.34^{a}	0.29±0.09^{d}
2	1.63±0.38^{ab}	0.34±0.11^{cb}
3	1.45±0.31^{c}	0.58±0.14^{a}
4	1.64±0.29^{a}	0.41±0.08^{b}
5	1.50±0.35^{bc}	0.48±0.09^{a}
6	1.39±0.35^{c}	0.33±0.07^{c}

*Different letters; a, b, c, and d indicate a significant difference according to Duncan at P<0.05

The antioxidant activity of royal jelly may obtain from the phenolic compounds, amino acid, carboxylic fatty acids, short-chain hydroxyl, and vitamins A and E (Guo, Kouzuma, & Yonekura, 2009). Royal jelly contained phenolic compounds that worker bees collected from plants where they gather nectar (Marcucci et al., 2001; Fiorani, Accorsi, Blasa, Diamantini, & Piatti, 2006). The main groups of plants phenolic compounds are derivatives of flavonoids, cinnamic acid and cumarins (Manthey & Grohmann, 2001). The phenolic compounds or polyphenols are reported to show anti-inflammatory, anti-carcinogenic, analgesic activities, and immune-modulating and exert these functions as antioxidants (Gómez-Caravaca et al., 2006). The phenolic and flavonoid compounds in royal jelly are known to exhibit antioxidant properties which play a key role in the pharmacological activities of royal jelly. The redox properties of phenolic compounds allow them to act as hydrogen donors, reducing agents and singlet oxygen quenchers (Balkanska, Marghitas, & Pavel, 2017). Moreover, 10-HDA also known to possess various biological properties, including antioxidant activity (Nagai, Sakai, Inoue, Inoue, & Suzuki, 2001; Isidorov et al., 2011). Park et al. (2019) also reported that MRJP in royal jelly showed antimicrobial and antioxidant activities.

The composition of royal jelly and its antioxidant capacity can be influenced by various factors such as the environmental factors, flower source of the nectar, time for harvest, seasons, and processing methods (Balkanska et al., 2017). However, antioxidant activity should be performed by other methods for understanding various mechanisms of antioxidant action.

5. Conclusion

The results showed that royal jelly samples from a bee farm in Thailand showed antibacterial activity against Gram-positive pathogenic bacteria ranging between 18.75 to 150 mg/ml, while the effectiveness decreased against Gram-negative bacteria. Moreover, they were also effective against drug-resistant bacteria,

MRSA. Furthermore, royal jelly samples also showed antioxidant activity from phenolic and flavonoid compounds found in the royal jelly. Therefore, effective activities of royal jelly could be useful for the development of the product, which has antibacterial and antioxidant activities. However, active constituents and the percentage of each chemical constituent in royal jelly should be further investigated.

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