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Research Article

Content and Antimicrobial Activities of Bingol Royal Jelly

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Abstract

Royal jelly (RJ) is the special nutriment of the larva of queen honeybee (Apis mellifera) which is composed of several bioactive substances that include amino acids, proteins, carbohydrates, lipids, mineral salts, and vitamins. The content of RJ varies depending on genotype of the bees according to the flora species and climatic conditions and this affects biological activities of RJ. In recent years, literature has perceived an exponential growth in amount of drug (antibiotic)-resistant pathogenic bacteria. The main reasons of growing antibiotic resistance might be credited to the abuse of the antibiotic usage demonstrating prominence of examining other choices other than the communal antibiotics, such as bee products), to avoid a additional build-up in antibiotic resistance. To our knowledge, chemical content and antimicrobial activity of Bingol RJ (BRJ) has not been investigated to date. Therefore, the purpose of this study was to study composition and antimicrobial activities of BRJ. The results demonstrated that BRJ contains major flavonoids and phenolics such as apigenin, quercetin, naringenin, gallic acid, caffeic acid that contribute antimicrobial and antioxidant properties of BRJ. We have also shown that there are some middle and short chain fatty acids that include Linoleic acid and Propionic acid. BRJ also contain majority of trace elements and mineral. In addition to chemical content, antimicrobial activity of BRJ was also investigated towards pathogens. BRJ showed antimicrobial activity against *Salmonella typhimurium* (8.64 mm), *Escherichia coli* (9.1 mm) and *Staphylococcus aureus* (10.73 mm).

Keywords: Bingol royal jelly, antimicrobial, flavonoids, fatty acids, propionic acid

Bingöl Arı Sütünün İçeriği ve Antimikrobiyal Aktivitesi

Özet

Arı sütü (AS), amino asitler, proteinler, karbohidratlar, lipitler, mineral tuzları ve vitaminleri içeren çeşitli biyoaktif maddelerden oluşan kraliçe bal arısı larvalarının (*Apis mellifera*) özel besleyicisidir. AS içeriği, flora türlerine ve iklim koşullarına göre arıların genotipine bağlı olarak değişir ve bu, AS'nin biyolojik aktivitelerini etkiler. Son yıllarda, literatürde ilaca (antibiyotik) dirençli patojenik bakteri miktarında aşırı miktarda artışa rastlanmaktadır. Antibiyotik direncinin artmasının ana nedenleri, antibiyotik kullanımının kötüye kullanılmasına bağlı olabilir. Bu durum antibiyotik direncinde ek bir birikmeyi önlemek için arı ürünleri gibi antibiyotik özellik taşıyan maddelerin diğer seçenekler olarak incelenmesinin önemini ortaya koymaktadır. Bingol AS'nin (BAS) kimyasal içeriği ve antimikrobiyal aktivitesi bugüne kadar araştırılmamıştır. Bu çalışma ile BAS'ın içeriği ve antimikrobiyal aktivitelerinin araştırılması amaçlanmıştır. Sonuçlar BAS'ın, antimikrobial ve antioksidan özelliklerine katkıda bulunan apigenin, quercetin, naringenin, gallik asit, kafeik asit gibi majör flavonoidler ve fenolikler içerdiğini göstermiştir. Aynı zamanda BAS'ın içeriğinde linoleik asit ve propionik asit gibi bazı orta ve kısa zincirli yağ asitlerinin varlığı da gösterilmiştir. BAS ayrıca eser elementlerin ve mineralin birçoğunu içermektedir. Kimyasal içeriğe ek olarak, BAS'ın antimikrobiyal aktivitesi de patojenlere karşı araştırılmış ve *Salmonella typhimurium* (8.64 mm), *Escherichia coli* (9.1 mm) ve *Staphylococcus aureus*'a (10.73 mm) karşı antimikrobiyal aktivite göstermiştir.

Anahtar Kelimeler: Bingöl arı sütü, antimikrobiyal, flavonoidler, yağ asitleri, propionik asit

Introduction

Royal jelly (RJ) is a yellow milky sticky bee product which is produced from the mandibular and hypopharyngeal glands of the worker honeybee and is the special nutriment of the larva of queen honeybee (*Apis mellifera*) (Pavel et al. 2011). It is composed of bioactive compounds such as amino acids, carbohydrates, proteins, lipids, minerals and vitamins (Nagai and Inoue 2004). Considering its potent antioxidant activity, major components of the RJ are flavonoids and phenolic compounds (Yang et al., 2019; Šedivá et al., 2018; Kocot et al., 2018).

In recent years, studies have motivated on the anti-microbial (Coutinho et al., 2018; Park et al., 2019), anti-inflammatory (Yang et al., 2018), anti-diabetic (Khazaei et al., 2018), anti-oxidant (Danis et al., 1994; Asadi et al., 2019; Gu et al., 2018), anti-tumor (Filipič et al., 2015) and anti-aging (Park et al., 2012) activities of RJ from different origins. The physical properties and chemical composition of the bee products vary with the genotype of the bees according to the flora species and climatic conditions and this affects the anticancer, antioxidant and antimicrobial activities of the bee products (Kocot et al., 2018). RJ has been utilised as an antique therapy with outstanding antimicrobial activities and is presently used as a pharmacological substance (Fratini et al. 2016; Cornara et al. 2017). Accordingly, an enhanced comprehension of the constituents of RJ might further develop the pharmacological and medical usages of RJ as an unconventional medication.

Recent years have witnessed an exponential increase in amount of drug (antibiotic)-resistant pathogenic bacteria (Nugent et al., 2010; WHO, 2012), that slowed down the labours to preserve pathogen-free therapeutic abilities. This augmented the harshness of bacterial diseases and infections. The main reasons of growing antibiotic resistance might be credited to the abuse of the antibiotic usage (Nugent et al., 2010) revealing importance of investigating other choices other than the communal antibiotics, such as bee products), to prevent a further build-up in antibiotic resistance (Noori et al., 2013). To our knowledge, chemical content and antimicrobial activity of Bingol RJ (BRJ) has not been investigated to date. Therefore, the main goal of this study was to study composition and antimicrobial activities of BRJ.

Material And Method

Mineral Content Analysis by ICP-MS

In the study, ICP-MS NexION® 2000 (PerkinElmer® Inc., USA) device with quartz nebulizer gasifier, cyclonic spray chember and integrated auto-sampler was used for elemental analysis of the samples. The ICP-MS method was prepared by using a washing solution containing 1% hydrochloric acid-ultra-pure water and approximately 0.2 grams of RJs samples were weighed in the microwave oven. 10 ml HNO_3 was added into the sample and burnt in microwave. The solution for ICP-MS calibration was made at the concentrations given in Table 1 by diluting them with commercially available multi-element standards of 1% (HNO_3-ultra-pure water). Additionally, ICP-MS calibration experiments were carried out prior to the measurements. 100 ppb 45Sc, 89Y, 209Bi internal standard were used for control of elemental analysis.

Table 1. Calibration standards used in ICP-MS

			Analyts		
1. Std	0,1	(ppb)	^{23}Na, ^{24}Mg, ^{27}Al,^{39}K, ^{43}Ca, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{59}Co, ^{63}Cu, ^{66}Zn	^{82}Se, ^{85}Rb, ^{107}Ag	^{202}Hg, ^{208}Pb
2. Std	1	(ppb)			
3. Std	10	(ppb)			
4. Std	50	(ppb)			
5. Std	125	(ppb)			
6. Std	250	(ppb)			
7. Std	500	(ppb)			
Internal Std			^{45}Sc	^{89}Y	^{209}Bi

Fatty Acids Analysis by GC-MS

Hara and Radin (1978) method was used for lipid extraction from RJ. For this purpose, 5 g of RJ was dissolved in 10 mL of hexane/isopropanol (with as ratio of 3:2) for 30 sec at 10 krpm in the homogenizer and centrifuged at 5 krpm for 10 minutes. The supernatant was taken and filtered and put in tubes. Fatty acids require to be derivatised in order to look at GC. Derivation with methyl esters is often preferred. For this aim, Christie (1990) method was preferred because it was practical and highly efficient. According to this method: the above-prepared lipid extract was taken into 30 mL capped tubes to prepare the methyl ester. 5 mL of 2% methanolic sulfuric acid was added and vortexed. This mixture was allowed to methylate in a 50 ^oC oven for 15 hours. After 15 hours the tubes were removed and chilled to ambient temperature and vortexed by adding 5 mL of 5% NaCl. The fatty acid methyl esters (FAME) formed in the tubes were extracted with 5 mL of hexane and the hexane phase was removed from the top with a pastor pipette and treated with 5 mL of 2% $KHCO_3$ and allowed to stand for 1-2 hours. The solvent of the mixture containing the methyl esters was then evaporated under nitrogen at 45 °C and the fatty acids under the flasks were dissolved with 1 mL of hexane and analyzed on GC-MS by capping amber color GC vials.

Agilent 7890A / 5970 C model GC-MS apparatus (USA) and SGE Analytical BPX90 100m x 0.25mm x 0.25 column (Australia) were used. The temperature program was heated gradually from 120 °C to 250 ° C and the total time was set to 45 minutes. The temperature program is as follows; Heats up to 120 °C to 250 °C at 5 °C / min and waits at this temperature for 19 min and the total time is 45 min. The autosampler washed with hexane 5 times before taking the sample and after giving it to the column. Injection volume 1 LL and split ratio 10: 1, solvent delay time 12 minutes, carrier gas was selected as He and the flow was adjusted to constant gas flow at 1 mL / min. H_2 flow 35mL / min, dry air flow 350 mL / min, N / min is automatically set by the program.

Phenolics and Flavonoids Analysis by HPLC

Chromatographic analysis were performed using SIL-20A HT autosamplers, CTO-10AS column oven and SPD-20A UVVIS detector using HPLC (LC-20AT, Shimadzu, Japan) system. For the chromatographic analysis, isocrotic system was set to mobile phase A: 0.5% acetic acid, B: 95% acetonitrile + 4.5% ultra pure water + 0.5% acetic acid, column flow rate 1 ml/min, injection volume 5 µl, column temperature 30 °C and detection wavelength 280 and 326 nm (Ruch et al., 1989; Prieto et al., 1999)

Anti-microbial Activity Test

The different concentrations of RJ were tested for their anti-microbial activity against *Salmonella typhimurium* NRRL 4413 (Gr-), *Escherichia coli* ATCC 25922 (Gr -), *Staphylococcus aureus* ATCC 6538 P (Gr +), *Sacharomyces cerevisiae* (baker's yeast)

The anti-microbial activity was tested using the spread plate agar method, in which 6 mm discs of the samples were placed on plates based on the number of trial agents (Kaya et al., 2018). Using a micropipette, 10 µl each of the RJ at 0.25, 0.5 and 1 mg/mL concentrations and other agents were absorbed into the discs on all plates. During this test, a negative control (sterile pure water), a positive control (penicillin-streptomycin), different concentrations of RJ were tested. After incubation at 37°C for 24 h, the diameter of the inhibition zone was measured. The activity of RJ was tested for antifungal activity against *Saccharomyces cerevisiae* and antibacterial capability against various pathogenic organisms; *Salmonella typhimurium, Escherichia coli*, *Staphylococcus aureus*.

Statistical Analyses

All measurements were repeated three times, and statistical analysis was performed with GraphPad Prism 5.01 software and comparable data sets were evaluated and the analyses were conducted using by one-way ANOVA (Analysis of variance) by Tukey's multiple comparison test and one-way ANOVA Newman-Keuls Post-Hoc Test with; $p<0.05$ was considered as significant.

Results and Discussion

Analysis of BRJ Content

The physical properties and chemical composition of the RJ vary with the genotype of the bees according to the flora species, climate and geography (Kocot et al., 2018). RJ produced by *Apis mellifera* is a highly active biological compound and is likely one of the most interesting bee products (Stocker et al., 2005). The mineral content of Bingol RJ was determined by ICP-MS. The minerals in Bingol RJ and their concentration are given in Table 2. Na (98.525 ppm), Mg (287.237 ppm), Al (8773 ppm), K (2579.161 ppm), Ca (102.488 ppm), Cr (701 ppm), Mn (636 ppm), Fe (20.456 ppm) , Ca (144 ppm), Cu (3.188 ppm), Zn (14.145 ppm), Se (76 ppm), Rb (2,960 ppm), Ag (588 ppm). Hg and Pb residues were not found in the contents of Bingol RJ both of which are heavy metals and produce toxicity in human cells and tissues. Trace elements possess a very important role in the biological properties associated with RJ, owing to their numerous biological activities. Detailed literature search revealed that concentrations of 28 trace (Mo, Tl, W, Sb, Sr, Bi, Cr, Al, Ba, Cd, Hg, Pb, Sn, Te, Ni, Ti, V and Co) and mineral (Cu, Mn, Ca, Mg, K, Na, Zn, Fe, P and S) elements were methodically explored in botanically and geographically distinct RJ samples (Stocker et al., 2005).

The vast amount of bee-derived compounds have been investigated in several variety of cancer cell lines and preclinical studies (Kocot et al., 2018). Flavonoids are a diverse chemical class having a widespread range of pharmacological properties some of which include anti-inflammatory, antidiabetic, antimicrobial and anticancer activities. A number of flavonoids particularly chrysin, hesperidin, quercetin, myricetin, rutin hydrate and kaempherol have been widely studied in cancer cell lines and animal models of tumorigenesis (Turk et al., 2019; Taslimi et al., 2019; Caglayan et al., 2019; Arshi et al., 2019; Imran et al., 2019; Demirel Sezer et al., 2019). The flavonoid content of RJ was analysed by HPLC. The major flavonoids determined in RJ are as follow: luteic acid, apigenin, gallic acid, ellagic acid, quercetin, vanillin, caffeic acid, rutin hydrate, catechin, kaempferol and chlorogenic acid (Table 3). In another study RJ was shown to contain

hesperetin, isosakuranetin, naringenin, acacetin, apigenin, and its glucoside, chrysin, luteolin glucoside, isorhamnetin and kaempferol (López-Gutiérrez et al., 2014).

Table 2. Mineral content of BRJ. Concentrations are given as ppm. ND: not detected.

Na	98.525
Mg	287.237
Al	8773
K	2.579.161
Ca	102.488
Cr	701
Mn	636
Fe	20.456
Ca	144
Cu	3.188
Zn	14.145
Se	76
Rb	2.96
Ag	588
Hg	ND
Pb	ND

In literature, fatty acids (FAs) have been categorised as long-chain (contain more than 12 C), medium-chain (between 6–12 C), and short-chain (less than 6 C) fatty acids, of which medium-chain fatty acids (MCFAs) exist mostly in the free form. Intracellular fatty acid metabolism possess an main role in regulation of inflammation and excessive amount of reactive oxygen species as oxidative stress causing agents might imitate a pathogen triggered inflammation and promote lipid peroxidation when antioxidant levels are decreased (Wang et al., 2016).

In this study, fatty acid content of the RJ was analysed by GC-MS. Methyl octanoate, Methyl tetradeconoate, Methyl hexadecanoate, Methyl octadecanoate, Methyl 7-octadecenoate, Linoleic acid and Propionic acid are some of the fatty acids present in BRJ (Table 4). The total content of fats and fatty acids in the RJ has been predicted to be between 7–18%. Instead of carboxylic acids between 14–20 C atoms generally identified in animals and plants, the RJ includes short hydroxy FA with 8–12 carbon atoms in the chain and dicarboxylic acids (Nabas et al., 2014; Kocot et al., 2018).

Table 3. *Flavonoid and phenolic content of BRJ.*

Luteic Acid
Apigenin
Gallic Acid
Ellagic Acid
Epicatechin
Quercetin
Vanillin
Caffeic acid
Rutin hydrate
Catechin
Kaempferol
Chlorogenic acid

Table 4. Fatty acid content of BRJ

Methyl octanoate
Methyl tetradecanoate
Methyl hexadecanoate
Methyl octadecanoate
Methyl 7-octadecenoate
Linoleic acid
Propionic acid

Antimicrobial Properties of BRJ

Protein and peptides found in RJ has been reported to contribute in defence machinery of honeybee towards pathogenic microorganisms through straight inactivation of microorganism occurring RJ, as well as through stimulation of cytokines involving in regulation of transcription of defensive proteins and/or peptides (Bărnuţiu et al. 2011; Al-Abbadi 2019). In addition to peptides, flavonoids also contribute microbiological avtivity of RJ. In one study, some flavonoids that include apigenin, gallic acid, quercetin (which is also found in BRJ) were demonstrated to have antimicrobial effects towards *Enterobacter cloaceae, E. aerogenes* and *Pseudomonas aeruginosa* (Basile et al. 1999; Chanwitheesuk et al., 2007; Nitiema et al., 2012). To evaluate antimicrobial activity of BRJ, different concentrations of it were assessed toward different Gram negative/positive bacteria and yeast by disc diffusion method. 1 mg/mL BRJ showed antimicrobial activity against *Salmonella typhimurium* (8.64 mm), *Escherichia coli* (9.1 mm) and *Staphylococcus aureus* (10.73 mm) while antibiotic mixture revealed an inhibition zone of approximately 20 mm for each bacteria (Table 5).

Table 5. Antimicrobial activity of BRJ toward different Gram negative/positive bacteria and yeast by disc diffusion method. Antibiotic zone was measured as cm.

Bacteria	BRJ (1 mg/ml)	BRJ (0.5 mg/ml)	BRJ (0.25 mg/ml)	Negative control (Water) (4)	Antibiotic zone diameter (cm) (5)
Salmonella typhimurium NRRL 4413 (Gr -)	0.864	0.784	0.638	0	1.918
Escherichia coli ATCC 25922 (Gr -)	0.91	0.884	0.774	0	2.024
Staphylococcus aureus ATCC 6538 P (Gr +)	1.073	1.06	0.934	0	2.072
Sacharomyces cerevisiae (baker's yeast)	0.658	0.685	0	0	1.803

Royal jelly, secreted from the salivary glands of worker bees, is a special food that influences the development of female bee larvae, where a diet low in royal jelly allows the development of larvae into worker bee adults, but larvae feed sufficient royal jelly instead develop into queen bees. It has been reported that royal jelly has a potential antitumor activity in mice. Studies have demonstrated that RJ possesses anticancer activity by inducing apoptotic and anti-proliferative pathways (Kocot et al., 2018).

Conclusion

Best of our knowledge, chemical content and antimicrobial activity of BRJ has not been studied to date. The aim of this study was to examine composition and antimicrobial activities of BRJ. The results demonstrated that BRJ contains major flavonoids and phenolics such as apigenin, quercetin, naringenin, gallic acid, caffeic acid that contribute antioxidant properties of BRJ. We have also shown that there are some middle and short chain fatty acids that include Linoleic acid and Propionic acid. BRJ also contain majority of trace elements and mineral. In addition to chemical content, antimicrobial activity of BRJ was also investigated towards pathogens indicating antimicrobial properties of BRJ.

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