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ORIGINAL RESEARCH ARTICLE

Turkish royal jelly: amino acid, physicochemical, antioxidant, multi-elemental, antibacterial and fingerprint profiles by analytical techniques combined with chemometrics

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Royal jelly is one of the most remarkable commercial bee products used in cosmetics, medicine, and as a dietary supplement. This study aimed to determine some quality parameters (amino acid, multi-elemental, total phenolic-flavonoid, 10-HDA, sugar, and moisture content) as well as antioxidant and antimicrobial activities of different royal jelly samples (domestic and non-domestic samples). In this study, royal jelly samples exhibited high antioxidant and antimicrobial activity. 10-HDA values were found to be higher in domestic royal jelly samples (2.17–2.71%) compared to non-domestic samples (0.31–1.71%). Also, it was found that the acidity value varied between 2.57–3.37 mL 0.1 N NaOH/g and 3.87–4.97 mL 0.1 N NaOH/g in non-domestic and domestic royal jelly samples, respectively. L-Proline was the amino acid with the highest rate of occurrence in both domestic (21972.39–35053.00 nmol/g) and non-domestic (19688.31–21711.50 nmol/g) samples. It was determined that the new method developed for amino acid analysis has many advantages over other methods. Chemometrics of principal component analysis (PCA) showed that amino acid profile, elemental profile, and chemical parameters were capable of presenting characteristic and descriptive properties of royal jelly samples. In addition to these findings, Raman spectroscopy combined with chemometrics of hierarchical cluster analysis (HCA) was successfully used to distinguish domestic and non-domestic royal jelly samples. The obtained results will provide an important basis for subsequent research on the evaluation of the amino acid content, physicochemical properties, phenolic content, and the antioxidant and elemental profile of royal jelly.

Keywords: royal jelly; amino acids; antibacterial activity; 10-HDA; antioxidants; fatty acid; acidity

Introduction

Turkey is one of the countries with high beekeeping potential, economically important honey bee races, different geographic features, and rich floral resources. Although different bee products such as propolis, apilarnil (drone brood), honey, royal jelly, pollen, and bee bread are produced in Turkey, the production potential of these products varies depending on variables such as the climate characteristics of the region, bee diseases, the age of queen bees, winter colony losses, and anthropogenic factors (Güler & Demir, 2005). One of these products, royal jelly, is a yellowish or milky white, highly viscous acidic secretion produced by honey bees (*Apis mellifera*). This nutritive secretion, which is essential for the development of the queen bee, is produced by the hypopharyngeal and mandibular glands in the head of the young nurse worker honey bees and, at the beginning of the larval period, the female larvae can differentiate into queens or workers owing to this essential larval diet (Fratini et al., 2016; Ramadan & Al-Ghamdi, 2012; Ramanathan et al., 2018).

Royal jelly has a slightly sour taste, a pungent odor and it contains water, proteins, carbohydrates, lipids, free amino acids, minerals, and small amounts of polyphenols and vitamins (Kamyab et al., 2020). The composition of royal jelly is quite complex and its major component is water, ranging from 60–70%. Carbohydrate fraction is about 30 percent of the dry matter and the most abundant carbohydrates are glucose, fructose, and sucrose similar to honey. But it is also possible to find a small amount of different types of carbohydrates such as Maltose, Melibiose, Trehalose, Ribose, and other sugars (Fratini et al., 2016). One of the important criteria for determining the nutritional value and quality of royal jelly, a functional food with high protein content, is amino acid concentration (Jie et al., 2016; Liming et al., 2009; Matloubi et al., 2004).

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Proline, Lysine, β-alanine, Glutamic Acid, Aspartate, Phenylalanine, and Serine are amino acids with quantitatively highest percentage in royal jelly (Sabatı nı et al., 2009).

Ash content represents 0.8-3% of royal jelly (fresh matter) (Garcia-Amoedo & Almeida-Muradian, 2007; Messı a et al., 2003) while Potassium, Calcium, Sodium, Zinc, Magnesium, Copper, Iron and Manganese are the most abundant elements that are present in royal jelly. In addition, elements such as Nickel, Chromium, Tin, Tungsten, Antimony, Bismuth and Titanium are found in trace amounts (Bărnuțiu et al., 2011; Fratini et al., 2016). Another component of royal jelly is lipids consisting of sterols and fatty acids. The percentage of these lipids ranges between 4–8% of the fresh product or 15–30% of the lyophilized product. Approximately 90% of these lipids constitute fatty acids that have 8–10 carbon atoms unlike organic acids of most animal and plant tissues (Li et al., 2013; Ramadan & Al-Ghamdi, 2012). The major fatty acid in royal jelly is 10-HDA and this fatty acid is not present in any other natural product, including other bee products. Therefore, the presence of 10-HDA, a chemically unique bioactive component for royal jelly, can be used as a marker to distinguish royal jelly from other honey bee products (Barker et al.,1959; Kamyab et al., 2020; Yavuz & Gürel, 2017).

Royal jelly has many activities such as antibacterial, antioxidant, anti-inflammatory, disinfectant action, antitumor and antihypercholesterolemic activity (Viuda-Martos et al., 2008). Due to its biological properties, royal jelly has considerable commercial and economic importance and is nowadays utilized in many sectors such as food, pharmaceutical, cosmetic and manufacturing (Sabatını et al., 2009). Despite its wide usage in various fields, there is no internationally accepted standard for royal jelly in the world, although, some countries have mapped out their own standards for honey bee products. Therefore, this study was carried out to determine the characteristics of Turkish royal jelly by using domestic and non-domestic royal jelly samples.

Materials and methods

Royal jelly samples

In this study, 5 fresh royal jelly samples (RJ-1, RJ-2, RJ-3, RJ-4, RJ-5) from abroad (non-domestic) and 9 fresh royal jelly samples (RJ-6, RJ-7, RJ-8, RJ-9, RJ-10, RJ-11, RJ-12, RJ-13, RJ-14) from Turkey (domestic) were used. Domestic royal jelly samples were also obtained pure from beekeepers. Non-domestic royal jelly samples were purchased from different import companies. All royal jelly samples were kept refrigerated at −20 °C in dark bottles until use.

Estimation of total phenolic content (TPC)

The content analysis of total phenolic compounds was performed according to the Folin-Ciocalteu method proposed by Singleton et al. (1999) using Gallic acid as a reference standard. 0.5 g of each royal jelly sample was diluted to 5 mL with distilled water. 0.2 mL of this solution was then mixed with 1.5 mL of 0.2 N Folin–Ciocalteu reagent for 5 min and 1.2 mL of 7.5% sodium carbonate (Na_2CO_3) was then added. After incubation at room temperature for 90 min, the absorbance of the reaction mixture was measured at 760 nm. The results were expressed as gallic acid equivalent (mg GAE/g).

Estimation of total flavonoid contents (TFC)

Total flavonoid analysis of the royal jelly was performed using a modified version of the method by Dewanto et al. (2002). 0.5 g of each royal jelly sample was diluted to 5 mL with distilled water. Accordingly, 1 mL extract was mixed with 0.3 mL 10% $AlCl_3.6H_2O$ solution after the addition of 0.3 mL 5% $NaNO_2$ solution. 2 mL of 1 M NaOH solution was added and 2.4 mL of water was added, and the mixture was stirred. The absorbance at 510 nm was measured against the prepared reagent blank. Total flavonoid content was expressed as mg catechine equivalent (mg CAE/g).

Antioxidant capacity (AC)

Cupric ion reducing antioxidant capacity (CUPRAC) assay was used to determine the antioxidant capacity of royal jelly samples. 0.5 g of each royal jelly sample was diluted to 5 mL with distilled water. Accordingly, 0.1 mL extract was mixed with 1 mL of 1.0×10^{-2} M Cu (II) chloride solution, 1 mL of 7.5×10^{-3} M neocuproin solution, 1 mL of 1 M ammonium acetate buffer (pH 7.0), respectively. Finally, the total volume was completed to 4 mL with distilled water. The tube was kept at room temperature for 60 min. At the end of the period, the absorbance value of the solution at 450 nm was measured against the non-including antioxidant solution. Total antioxidant capacity was expressed as mg trolox equivalent (mg TE/g) (Apak et al., 2004).

Acidity and moisture content

The acidity and moisture content value of each fresh royal jelly sample were determined according to the method of Turkish Standards Institute (TS 6666, 2000).

Sugar analysis

Sugar content was determined by HPLC with a refractive index detector by the DIN 10758 method.

10-Hydroxy-2-decenoic acid (10-HDA) analysis

The 10-HDA analysis of each royal jelly sample was determined using the TS 6666 method (TS 6666, 2000).

Table 1. Results of total phenolic (TPC)-flavonoid (TFC), antioxidant capacity (AC) and physicochemical parameters analysed in RJ samples.

Sample Name	TPC (mg GAE/g)	TFC (mg CAE/g)	AC (mg TE/g)	Moisture (%)	Acidity (mL 0.1N NaOH/g)	10-HDA (%)	Fructose (%)	Glucose (%)	Saccharose (%)	Maltose (%)	Isomaltose (%)	Erlose (%)	Melezitose (%)	Maltotriose (%)
RJ-1	2.36 ± 0.05^{cde}	0.54 ± 0.02^{abc}	3.83 ± 0.06^{f}	67.92 ± 2.38^{a}	2.63 ± 0.05^{h}	0.31 ± 0.03^{g}	4.68 ± 0.19^{def}	5.23 ± 0.21^{a}	2.33 ± 0.24^{ab}	0.90 ± 0.19^{e}	0.34 ± 0.06^{abcd}	1.91 ± 0.08^{b}	0.69 ± 0.19^{a}	0.04 ± 0.030^{d}
RJ-2	2.12 ± 0.07^{e}	0.52 ± 0.02^{bcd}	4.42 ± 0.03^{e}	67.43 ± 2.70^{a}	2.57 ± 0.05^{h}	0.27 ± 0.04^{g}	4.84 ± 0.31^{cdef}	5.22 ± 0.07^{a}	2.24 ± 0.17^{abc}	0.95 ± 0.05^{e}	0.54 ± 0.06^{a}	2.25 ± 0.08^{ab}	0.04 ± 0.01^{c}	0.85 ± 0.07^{b}
RJ-3	2.38 ± 0.04^{cd}	0.64 ± 0.03^{a}	4.93 ± 0.03^{abc}	56.50 ± 1.57^{b}	3.37 ± 0.05^{f}	$1.71 \pm 0.04e$	6.29 ± 0.32^{a}	5.33 ± 0.21^{a}	2.10 ± 0.15^{abc}	2.01 ± 0.18^{bc}	0.34 ± 0.14^{abcd}	2.44 ± 0.23^{a}	0.04 ± 0.04^{c}	0.82 ± 0.08^{b}
RJ-4	2.11 ± 0.20^{e}	0.42 ± 0.02^{de}	3.81 ± 0.04^{f}	67.30 ± 1.98^{a}	2.57 ± 0.15^{h}	0.29 ± 0.07^{g}	4.25 ± 0.33^{f}	4.22 ± 0.13^{bc}	1.83 ± 0.19^{bc}	0.85 ± 0.15^{e}	0.44 ± 0.09^{abc}	1.90 ± 0.08^{b}	0.02 ± 0.01^{c}	0.93 ± 0.09^{b}
RJ-5	2.19 ± 0.04^{de}	0.44 ± 0.03^{cde}	5.08 ± 0.12^{ab}	62.14 ± 3.20^{ab}	3.10 ± 0.10^{g}	0.86 ± 0.05^{f}	5.90 ± 0.21^{ab}	5.31 ± 0.14^{a}	2.52 ± 0.02^{a}	1.34 ± 0.06^{d}	0.53 ± 0.01^{a}	2.32 ± 0.19^{a}	0.03 ± 0.02^{c}	1.13 ± 0.03^{a}
RJ-6	2.69 ± 0.06^{b}	0.53 ± 0.03^{abcd}	5.18 ± 0.05^{a}	63.25 ± 1.46^{ab}	4.97 ± 0.05^{a}	2.34 ± 0.06^{bcd}	4.19 ± 0.15^{f}	4.41 ± 0.17^{b}	2.53 ± 0.17^{a}	2.43 ± 0.26^{a}	0.13 ± 0.03^{d}	0.88 ± 0.15^{de}	0.43 ± 0.10^{b}	0.05 ± 0.03^{d}
RJ-7	2.12 ± 0.07^{e}	0.34 ± 0.01^{ef}	5.21 ± 0.02^{a}	64.02 ± 2.86^{a}	4.17 ± 0.05^{d}	2.17 ± 0.02^{d}	5.53 ± 0.16^{abc}	5.40 ± 0.19^{a}	1.22 ± 0.13^{de}	1.81 ± 0.13^{c}	0.24 ± 0.03^{bcd}	1.34 ± 0.18^{c}	0.03 ± 0.05^{c}	0.52 ± 0.12^{c}
RJ-8	1.86 ± 0.03^{f}	0.61 ± 0.02^{ab}	4.10 ± 0.07^{f}	65.55 ± 2.06^{a}	3.87 ± 0.05^{e}	2.51 ± 0.16^{abc}	5.43 ± 0.33^{bcd}	5.34 ± 0.17^{a}	1.12 ± 0.10^{e}	0.13 ± 0.05^{f}	0.20 ± 0.06^{d}	0.22 ± 0.08^{f}	0.05 ± 0.02^{c}	0.04 ± 0.02^{d}
RJ-9	3.12 ± 0.02^{a}	0.52 ± 0.06^{bcd}	5.04 ± 0.08^{ab}	61.69 ± 2.28^{ab}	4.60 ± 0.10^{bc}	2.68 ± 0.07^{a}	5.43 ± 0.18^{bcd}	4.60 ± 0.18^{b}	0.82 ± 0.21^{e}	0.35 ± 0.05^{f}	0.44 ± 0.08^{ab}	0.21 ± 0.04^{f}	0.83 ± 0.13^{a}	0.14 ± 0.02^{d}
RJ-10	2.57 ± 0.10^{bc}	0.43 ± 0.04^{cde}	4.63 ± 0.06^{de}	63.22 ± 1.83^{ab}	4.47 ± 0.05^{c}	2.71 ± 0.21^{a}	5.49 ± 0.21^{bc}	4.63 ± 0.35^{b}	2.33 ± 0.31^{ab}	0.14 ± 0.04^{f}	0.12 ± 0.01^{d}	0.42 ± 0.10^{f}	0.04 ± 0.01^{c}	0.05 ± 0.01^{d}
RJ-11	2.33 ± 0.08^{de}	0.50 ± 0.03^{bcd}	4.80 ± 0.06^{bcd}	65.23 ± 3.17^{a}	4.53 ± 0.05^{c}	2.63 ± 0.11^{ab}	5.04 ± 0.45^{cde}	4.50 ± 0.20^{b}	2.03 ± 0.25^{abc}	0.09 ± 0.04^{f}	0.22 ± 0.09^{bcd}	0.51 ± 0.08^{ef}	0.03 ± 0.04^{c}	0.04 ± 0.02^{d}
RJ-12	2.57 ± 0.10^{bc}	0.50 ± 0.07^{bcd}	4.71 ± 0.26^{cde}	64.15 ± 1.96^{a}	4.43 ± 0.15^{c}	2.57 ± 0.06^{abc}	5.10 ± 0.01^{cde}	4.50 ± 0.02^{b}	1.71 ± 0.06^{cd}	0.10 ± 0.01^{f}	0.20 ± 0.01^{d}	0.40 ± 0.01^{f}	0.21 ± 0.02^{bc}	0.04 ± 0.02^{d}
RJ-13	2.60 ± 0.02^{bc}	0.44 ± 0.03^{cde}	4.93 ± 0.03^{abcd}	64.23 ± 2.94^{a}	4.37 ± 0.05^{cd}	2.30 ± 0.14^{cd}	4.33 ± 0.13^{ef}	3.79 ± 0.05^{c}	2.50 ± 0.26^{a}	0.13 ± 0.02^{f}	0.20 ± 0.10^{d}	0.92 ± 0.04^{d}	0.03 ± 0.03^{c}	0.03 ± 0.01^{d}
RJ-14	2.19 ± 0.04^{de}	0.30 ± 0.01^{f}	5.19 ± 0.11^{a}	64.03 ± 1.10^{a}	4.80 ± 0.10^{ab}	2.59 ± 0.09^{abc}	5.20 ± 0.32^{bcd}	5.22 ± 0.23^{a}	1.03 ± 0.10^{e}	2.33 ± 0.21^{ab}	0.21 ± 0.09^{cd}	0.40 ± 0.18^{f}	0.13 ± 0.08^{c}	0.53 ± 0.15^{c}

*The results are presented as mean values ± standard error. Values in the same column that are followed by the same superscript letter such as a, b, c, d, etc. are not significantly different ($p < 0.05$).

Amino acid analysis by liquid chromatography-tandem mass spectrometry (LC–MS/MS)

Extraction methods

1 g of royal jelly was taken into falcon and 10 mL of extra-pure water was added. The solution was vortexed for 1 min and it stayed in the sonicator for over 15 min at 45 °C. Samples were centrifuged for 5 min at 13500 RPM and 50 μL of clear supernatant was mixed with 50 μL of internal standard and 900 μL of extraction solution (mobile pahse A, methanol, acetonitril: v:v:v, 5:15:15). After being vortexed, the sample was injected in the LC-MS/MS system.

LC–MS/MS conditions

LC was performed using an LC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was carried out with a Jasem amino acid column and set at 35 °C with a mobile phase flow rate of 0.6 mL/min. Gradient elution mobile phases consisted of 5 mM of ammonium formate, 2% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient began initially at 80% B during 1.0 min, decreasing linearly to 25% at 4.0 min, this was maintained for 1.0 min and after 5.10 min gradient was back to the initial ratio. The total method was 10 min. Sample temperature was maintained at 4 °C in the autosampler prior to analysis. A sample volume of 5 μL was injected into the analytical column for analysis.

MS/MS analyses were conducted on an Agilent 6460 triple quadruple LC-MS equipped with an electrospray ionization interface. Electrospray ionization was performed in the positive ionization mode. The mass spectrometer operated with the cycle time of 500 ms. To find the optimal parameters of ion path and ion source of the studied compound, the quantitative optimization was done by direct injection of standards using a HPLC Agilent 1260. Multiple reaction monitoring (MRM) mode of the dominant product ion for each solution was realized using the optimal conditions. The ion source parameters were as follows: Gas Temp.: 275 °C; Gas Flow: 10 L/min; Nebulizer: 45 psi; Sheat Gas Heater: 375 °C; Sheat Gas Flow: 10 l/min; Capillary (positive): 2500 V. MRM mode consisted of comparing the ion pair (precursor and product ion m/z values) and LC retention times with standards served to confirm the identification of analyte in the samples. Data acquisition and processing were accomplished by using MassHunter (Agilent LC-MS software).

Calibration curve and quantification

All calibration curves were prepared with different amino acid standard concentrations (Table S1) and all points were injected 3 times. All the amino acids curve linearity is $R^2 \geq 0.995$. LOD and LOQ values of the amino acids (calculated over S/N ratio) are in Table S1.

Because of the absence of the blank sample, amino acid standards were spiked with extra-pure water and recovery values were calculated. All values are in the range of 98–102%. In this method amino acids deuterium-changed internal standards were used. Owing to the usage of internal standards, the recovery values were that high.

Determination of elemental profiles of royal jelly samples by inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS analysis was carried out by modifying the method proposed by Oroian et al. (2016). The elements Potassium (K), Lithium (Li), Boron (B), Beryllium (Be), Sodium (Na), Magnesium (Mg), Aluminum (Al), Ruthenium (Ru), Silicon (Si), Phosphorus (P), Calcium (Ca), Vanadium (V), Chromium (Cr), Cesium (Cs), Nickel (Ni), Rhodium (Rh), Palladium (Pd), Platinum (Pt), Selenium (Se), Thallium (Tl), Gallium (Ga), Copper (Cu), Cobalt (Co), Iron (Fe), Strontium (Sr), Rubidium (Rb), Cadmium (Cd), Tellurium (Te), Manganese (Mn), Silver (Ag), Lead (Pb), Tin (Sn), Barium (Ba), Iridium (Ir), Gold (Au), Mercury (Hg), Zinc (Zn), Arsenic (As), Antimony (Sb), Indium (In), Hafnium (Hf), and Bismuth (Bi) were determined using ICP-MS in the royal jelly samples.

Antibacterial activity

Preparation of royal jelly samples and microorganisms for antibacterial activity tests

Each royal jelly sample was lyophilized and weighed (1 g) using previously sterilized wooden applicators (Yamaoki et al., 2014). Following the lyophilization process, the total volume of these samples was completed to 2 mL by adding sterile distilled water and mixed thoroughly by vortex. These royal jelly samples (50%, w/v) were used to determine the antibacterial activity (Osés et al., 2016). Then, antibacterial properties of royal jelly samples were tested against five Gram-positive bacteria (*Bacillus cereus* BC 6830, *Enterococcus faecalis* NCTC 12697, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* BC 7231, *Staphylococcus aureus* NCTC 10788) and five Gram-negative bacteria (*Escherichia coli* NCTC 9001, *Escherichia coli* BC 1402, *Pseudomonas aeruginosa* NCTC 12924, *Salmonella Typhimurium* RSSK 95091, *Yersinia enterocolitica* ATCC 27729). The bacteria used in this study were obtained from Bayburt University, Vocational School of Health Services. The selected pathogenic microorganisms were seeded on Mueller Hinton Agar (MHA) media by streak plate method and bacterial samples taken from discrete colonies at the end of 24 h incubation period were transferred to Mueller Hinton Broth (MHB) media. At the end of 18 hours of incubation period in the MHB medium, bacterial cultures were adjusted to 0.5 McFarland turbidity standard and used as an inoculum (Osés et al., 2016).

Screening for antibacterial activity

In vitro inhibitory activities of royal jelly samples were investigated by the agar well diffusion (AWD) method and the inhibitory activity of the samples was detected as a clear zone around the wells (Osés et al., 2016). Antibacterial activity tests were performed using MHA (Merck). Autoclaved-sterilized MHA mediums were cooled to 50 °C at room temperature and transferred to sterile petri dishes by taking 25 mL volumes immediately. After the addition of the 500 μL inoculum (0.5 McFarland turbidity standard), the petri dishes were cooled at room temperature for 1 h (Osés et al., 2016). At the end of this period, 5 mm diameter wells were cut into the surface of the solidified mediums using a sterile cork borer. 50 μL of the previously prepared royal jelly samples [50% (w/v)] was transferred to these wells and then the petri dishes were incubated at 37 °C for 24 h. At the end of the incubation period, observed inhibition zones were measured with a ruler and recorded. All tests were performed in duplicate (Sherlock et al., 2010). In addition to these processes, to make a comparison with inoculated MHA mediums, non-inoculated MHA mediums were seeded using sterile swabs and agar well diffusion tests were repeated as described above in these media (Naik et al., 2010).

Determination of minimum inhibition concentration (MIC)

MIC values of royal jelly samples were determined by microbroth dilution method using 96-well microplates. For this purpose, first of all, royal jelly samples were adjusted to 256 mg/mL with sterile distilled water as described above. Then, 95 µL of sterile MHB was distributed in each well of the 96 well microtiter plates. After these processes, overnight grown pathogenic microorganisms were adjusted to 0.5 McFarland turbidity and 5 µL of inoculums were added to each well. Thus, totally 100 µL of MHB media + inoculum mixture was prepared in each well. Then, 100 μL of royal jelly samples (256 mg/mL) were added to each of the first wells and they were gently mixed at least three times with a multichannel micropipette. Afterwards, half of the mixture (100 μL) in the first well was taken via a multichannel micropipette and were transferred to the second well. These procedures were repeated successively up to the eighth well. In this manner, the starting concentration of the royal jelly sample (128 mg/mL) was diluted in half at each step. Immediately after these procedures, the absorbance values of the suspensions in the wells were measured and recorded at 600 nm wavelength via a microplate reader (Thermo, Multiskan Go). After these processes, the microplates were incubated at 37 °C for 24 h and the absorbance values were again measured and recorded at the end of the incubation

period. After the 24 h incubation period, the first wells in which the absorbance values increased were considered as non-bactericidal or non-bacteriostatic concentrations and the concentrations in the upper wells were accepted as the minimum inhibition concentration (Şahin et al., 2003; Zgoda & Porter, 2001).

Determination of minimum bactericidal concentration (MBC)

After determining the minimum inhibition concentrations of the royal jelly samples, to determine the minimum bactericidal concentration, 5 μL suspension was taken from each well of the MIC test plates and transferred to the MHA medium. These petri dishes were incubated at 37 °C for 24 h and, at the end of the incubation period, the lowest concentration where bacterial growth was not observed was accepted as MBC. All these assays were performed two times (Rakholiya et al., 2014).

Raman analysis

Raman Progeny X2 spectrometer (Analytical Devices, Inc. Wilmington, MA) was used for spectral acquisition. All measurements were obtained in the 200-2000 cm-1 spectral range. Instrument control and data acquisition were accomplished by using Xantus V3.0.0.0 software. Benzonitrile standard solution was used for verification prior to the Raman measurements. Raman measurements were performed using a portable Raman spectrometer, Progeny (Rigaku Raman Technologies, Wilmington, MA, USA), equipped with 1064 nm YAG (yttrium aluminium garnet) laser. Peltier cooled InGaAs (indium gallium arsenide) detector was used for detection of scattered light of Raman. All spectra were recorded in 2000–200 cm^{-1} spectral range. Laser power and exposure time were selected as 0.25 watts and 0.851 seconds, respectively. Spectrum acquisition of each sample was repeated three times in the same condition, and an average spectrum was obtained. Measurements were obtained through glass vials.

Chemometrics and data analysis

Chemometrics of hierarchical cluster analysis (HCA) was applied to the Raman data of royal jelly samples. Hierarchical cluster analysis was performed by using OPUS 7.2 (Bruker, Germany). HCA was applied with the aim of revealing an intrinsic relationship between domestic and non-domestic royal jelly samples. Clusters of royal jelly samples were visualized by OPUS Version 7.2 (Bruker, Germany). First-derivative and vector normalized Raman spectra of royal jelly samples were subjected to the hierarchical cluster analysis. Ward's algorithm was applied and normal to reprolevel spectral distance was selected for calculation of spectral distances. The spectral range of 1995-300 cm-1 was used for HCA analysis.

Additionally, multivariate analysis of principal component analysis (PCA) was applied to the total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity (AC), moisture content, acidity, 10-HDA and total sugar content properties of royal jelly samples. Separately from this, PCA analysis was performed for amino acid profiles which were obtained by LC-MS/MS analysis of royal jelly samples. Also, a distinct PCA analysis was performed for elemental profiles which were obtained by ICP-MS analysis of royal jelly samples. All of the PCA analyses of royal jelly samples were performed by using JMP (JMP 15, SAS Inc) statistical software.

One-way analysis of variance (ANOVA) was used to assess statistical contrast of royal jelly samples on antioxidant capacity, total flavonoid content, total phenolic content, moisture, acidity, 10-HDA, Fructose Glucose, Sucrose, Maltose, Isomaltose, Erlose, Melezitose and Maltotriose using Tukey's test with Minitab 17.0 software.

Results

Total phenolic-flavonoid content and antioxidant activity

The total phenolic-flavonoid content of royal jelly samples were 1.86 ± 0.03-3.12 ± 0.02 mg GAE/g and 0.30 ± 0.01-0.64 ± 0.03 mg CAE/g, respectively (Table 1). In this study, royal jelly samples in general exhibited high antioxidant activity (3.81 ± 0.06 – 5.21 ± 0.02 mg TE/g) (Table 1). The lowest value for TPC was 1.86 ± 0.03 mg GAE/g in RJ-8 sample and the highest value was 3.12 ± 0.02 mg GAE/g in RJ-9. TFC was determined at the lowest rate in the RJ-14 (0.30 ± 0.01 mg CAE/g) and the highest rate in RJ-3.

Moisture content and acidity

The moisture ratio (%) ranged from 56.50 to 67.92%, all of which were within nationally recommended parameters for quality of royal jelly (TS 6666, 2000) except for three samples (RJ-3, RJ-9, RJ-5). In this study, the acidity was between 3.87–4.80 mL 0.1 N NaOH/g in domestic royal jellies (RJ6-RJ14) and 2.57–3.37 mL 0.1 N NaOH/g in non-domestic royal jellies (RJ1-RJ5) (Table 1).

Sugar content

Glucose, Fructose, Saccharose, Maltose, Isomaltose, and Erlose were identified and quantified in all the samples within the scope of this research. Fructose (4.19–6.29%) and Glucose (3.79–5.40%) were the dominant sugars in all samples (Table 1). Saccharose ratio in all investigated royal jelly samples ranged from 0.82 to 2.52%.

10-HDA

The values of 10-HDA in 14 samples that we analyzed ranged from 0.27 ± 0.04 to 2.71 ± 0.21% (Table 1). In

Table 2. Concentrations of amino acids in RJ samples (nmol/g).

Amino acids	RJ-1	RJ-2	RJ-3	RJ-4	RJ-5	RJ–6	RJ- 7	RJ-8	RJ-9	RJ-10	RJ- 11	RJ-12	RJ-13	RJ-14
1-Methyl-L-Histidine	23.77	21.72	25.78	22.27	24.40	14.83	18.317	5.35	6.52	42.38	10.10	17.75	9.73	18.07
3-Amino isobutyric acid	179.60	179.23	207.17	184.23	179.86	204.55	168.94	209.61	159.76	138.45	126.24	162.01	133.20	189.24
3-Methyl-L-Histidine	6.80	7.15	3.43	7.42	6.87	6.47	5.94	6.54	4.43	14.74	4.15	6.48	4.08	6.52
Argininosuccinic acid	7.70	5.27	nd	nd	nd	nd	nd	nd	10.12	nd	nd	nd	6.26	7.45
Beta-Alanine	2694.35	2952.73	3172.35	2814.33	3615.66	4650.11	4494.73	2556.80	2968.53	1808.60	1970.72	2158.02	2247.90	4760.13
DL-5-Hydroxy lysine	9.03	9.63	14.15	7.83	9.24	7.01	7.34	2.18	3.58	2.25	1.39	8.67	2.86	7.78
DL-Homocystine	nd	nd	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethanolamine	75.60	94.52	46.77	74.43	58.24	95.79	91.46	187.67	34.26	77.01	44.45	70.23	23.89	44.77
Gamma-aminobutyric acid	448.13	455.73	557.89	465.81	440.13	508.80	407.11	470.44	360.55	317.44	269.91	319.47	284.05	442.41
L-2-Aminoadipic acid	2.613	2.46	2.58	2.46	2.30	4.96	3.55	6.80	4.93	9.12	2.09	5.66	2.49	1.97
L-2-aminobutyric acid	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd
L-Alanine	184.85	188.97	159.32	186.89	204.02	427.91	201.79	469.78	364.24	260.49	192.07	213.76	252.14	204.94
L-Anserine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Arginine	1317.08	1298.69	1642.73	1362.85	1403.50	2113.07	1582.09	2727.88	1772.79	2175.54	1560.90	2070.21	1985.05	1420.98
L-Asparagine	106.97	105.79	123.57	112.49	138.56	172.94	146.40	51.34	136.33	108.49	86.33	95.00	122.78	109.13
L-Aspartic acid	1170.83	1184.07	1398.11	1206.36	1424.45	1009.95	1421.89	1179.07	724.82	828.20	718.45	710.85	725.30	1307.06
L-Carnosine	nd	nd	nd	Nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	Nd
L-Citrulline	28.40	28.33	27.22	28.47	27.88	33.64	28.69	28.40	29.07	29.00	27.15	29.37	30.56	31.82
L-Cystathionine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Cystine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
L-Glutamic acid	2565.68	2526.98	3099.96	2635.05	3002.34	2933.24	3092.78	3169.00	2604.82	2324.13	2008.80	2091.97	2151.15	2913.06
L-Glutamine	190.28	160.16	202.85	188.32	212.31	536.54	137.86	480.91	473.81	394.47	324.27	275.19	388.30	201.19
L-Glycin	360.57	359.59	283.44	349.62	296.19	334.38	375.47	454.73	269.21	292.73	196.59	233.41	244.94	302.92
L-Histidine	582.23	627.09	631.25	595.49	614.64	655.37	606.12	633.88	663.95	532.20	577.07	542.81	770.71	543.15
L-Homocitrulline	600.52	600.72	600.09	600.88	600.79	600.10	600.29	599.64	601.46	599.97	599.94	600.44	600.49	600.76
L-Isoleucine	114.05	113.64	114.69	112.00	130.12	207.65	138.79	301.30	230.32	163.15	137.03	167.44	156.87	173.82
L-Leucine	78.65	77.11	86.91	80.48	106.71	174.21	117.63	284.52	129.74	156.27	118.80	129.22	115.06	134.37
L-Lysine	14389.94	13868.23	16508.85	14216.24	13407.141	18043.05	15093.65	30740.86	20615.57	18052.67	14501.89	17723.43	13246.82	13563.47
L-Methionine	8.76	8.24	11.97	8.30	9.88	11.60	12.52	11.83	7.21	8.43	6.98	8.02	6.38	9.53
L-Ornithine	199.46	199.29	227.67	200.00	202.64	328.15	248.25	378.59	215.01	403.13	168.48	266.28	256.13	219.17
L-Phenylalanine	274.15	269.12	117.57	294.04	734.26	241.28	409.10	225.94	190.02	149.64	114.75	176.69	101.26	671.98
L-Proline	20342.53	19688.31	21576.28	20535.39	21711.50	28497.09	23119.17	35053.00	24219.90	23654.54	23698.77	23569.55	22542.77	21972.39
L-Serine	111.08	106.32	118.39	112.03	112.33	245.91	134.69	279.04	263.42	201.08	113.42	111.41	135.22	118.97
L-Threonine	82.34	70.33	83.50	79.29	79.54	152.74	93.62	232.84	140.27	146.76	86.98	106.38	90.62	101.38
L-Tryptophan	2.53	2.53	2.48	2.589	2.59	20.83	2.67	37.95	42.19	44.58	29.66	3.00	38.59	7.95
L-Tyrosine	103.20	100.19	80.71	101.34	140.01	113.76	120.52	146.51	74.35	79.96	60.93	85.47	71.12	162.76
L-Valine	258.08	245.77	176.43	252.32	223.75	362.58	215.98	388.19	251.46	280.32	155.93	193.82	256.30	235.57
O-Phospho-L-Serine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
O-Phosphoryl Ethanolamine	108.92	114.43	144.11	126.64	159.20	178.79	146.35	149.91	177.28	125.76	127.38	137.99	142.67	161.27
Sarcosine	101.39	96.21	73.84	98.01	87.46	212.28	101.85	128.05	121.61	135.88	100.44	140.84	152.19	126.20
Taurine	5066.53	5245.01	6112.78	5282.22	5797.61	4387.37	6968.42	9276.24	3472.17	3922.04	3287.30	4690.55	3191.44	4457.98
Trans-4-hydroxy L-proline	533.73	525.05	543.05	536.78	791.68	902.13	532.74	454.27	849.44	296.17	536.77	676.95	516.57	1342.01

nd: not detected.

Table 3. Elemental composition of RJ samples (mg/kg).

Elements	RJ-1	RJ-2	RJ-3	RJ-4	RJ-5	RJ-6	RJ-7	RJ-8	RJ-9	RJ-10	RJ-11	RJ-12	RJ-13	RJ-14
Li	nd	nd	nd	nd	nd	nd	nd	nd	0.01	0.01	0.01	nd	nd	nd
Be	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
B	nd	nd	nd	nd	nd	nd	nd	0.14	0.62	0.53	0.34	0.66	0.01	nd
Na	158.19	173.82	120.23	156.04	139.03	133.44	111.00	158.56	89.99	86.35	102.40	355.17	23.02	122.51
Mg	258.68	270.61	286.19	207.64	289.17	315.94	286.48	273.45	286.92	256.95	306.10	1032.89	28.42	300.55
Al	0.55	0.71	0.80	1.10	0.65	0.90	0.67	0.83	0.75	0.48	1.59	1.19	1.92	0.86
Si	8.57	10.63	7.69	13.56	9.67	8.51	4.81	6.96	10.91	6.55	7.48	17.34	1.29	5.49
P	1126.98	1234.01	1394.31	1035.09	1404.64	1585.19	1342.40	1331.06	1325.45	1136.49	1406.87	4796.58	137.68	1429.97
K	2689.76	2739.69	2877.10	1942.90	2912.63	3102.99	2852.37	2693.66	3228.42	2906.28	3254.28	11212.51	294.09	3018.02
Ca	25.67	26.76	22.57	19.43	22.93	23.18	21.49	20.88	23.10	21.36	22.67	77.56	8.43	21.96
V	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cr	0.02	0.03	0.18	0.03	0.02	0.02	0.04	0.03	0.02	0.02	0.02	0.03	0.01	0.02
Mn	0.63	0.67	1.22	0.52	0.75	0.54	0.52	0.44	0.55	1.19	1.16	1.71	0.10	0.55
Fe	7.24	8.26	9.70	8.35	7.97	8.44	6.58	5.96	6.93	5.77	7.32	23.37	0.94	6.21
Co	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ni	nd	nd	0.02	0.04	0.02	0.07	0.17	0.05	0.28	nd	0.06	nd	0.72	0.04
Cu	3.19	3.27	3.67	2.81	3.58	2.76	3.94	3.96	2.51	2.97	3.72	12.78	0.43	4.08
Zn	18.12	18.65	19.12	14.95	19.51	20.06	17.07	17.74	20.05	15.12	19.02	66.44	6.36	18.78
Ga	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
As	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Se	0.04	nd	nd	nd	nd	0.07	nd	nd	0.03	0.03	0.02	0.14	0.01	nd
Rb	1.74	1.76	3.03	1.42	2.43	0.86	1.55	2.79	2.78	1.35	2.62	5.99	0.26	3.84
Sr	0.24	0.26	0.05	0.15	0.07	0.07	0.14	0.06	0.05	0.04	0.06	0.08	0.02	0.03
Ru	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	nd
Rh	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pd	0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ag	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd
Cd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	nd	nd	nd
In	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Sn	nd	nd	nd	nd	0.12	nd	nd	nd	0.21	nd	nd	nd	nd	nd
Sb	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	nd
Te	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Cs	nd	nd	nd	nd	nd	nd	nd	nd	0.01	nd	0.01	0.01	nd	nd
Ba	0.03	0.07	0.02	0.02	0.02	0.03	0.01	0.05	0.03	0.04	0.02	0.04	0.01	0.02
Hf	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ir	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pt	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Au	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Hg	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Tl	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pb	nd	nd	nd	nd	nd	0.01	nd	nd	0.01	nd	nd	0.01	nd	0.01
Bi	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd: not detected (<0.00).

the TSE 6666 (2000), it has been reported that the 10-HDA value of royal jelly should be a minimum of 1.40%. Accordingly, except for the four royal jellies used in this study, it was found that the others complied with the standards.

Amino acid profile

The content of 42 amino acids studied using LC–MS/MS from royal jelly samples was summarized in Table 2. While amino acids DL-Homocysteine, L-Carnosine, L-Cystathionine, L-Cystine, and O-Phospho-L-Serine cannot be detected in any royal jelly sample, argininosuccinic acid was detected only in RJ-1, RJ-2, RJ-9, and R-13 samples. The other remaining 36 amino acids were detected in royal jelly samples at different concentrations. RJ-8 had a higher concentration of L-Proline (35053.00 nmol/g) and L-Lysine (30740.86 nmol/g), L-Glutamic Acid (3169.00 nmol/g), L-Arginine (2727.88 nmol/g). The highest total concentration of Beta-Alanine and L-Aspartic acid was found to be in RJ-14 and RJ-5, respectively.

Elemental composition

In this study, concentrations of major and trace elements were investigated in RJ samples. As seen in Table 3, the major elements in royal jelly samples were found to be K, P, Mg and Na. The two major elements in the royal jelly samples were the K macro element with a concentration of 294.09–13005 mg/kg followed by the P macro element with a concentration of 137.68–4796 mg/kg. Significantly the highest concentrations of K were found in RJ-12 samples.

Table 4. Inhibition zone diameters (mm) and minimum inhibitory concentrations (mg/mL) and minimum bactericidal concentration (mg/mL) of RJ samples.

	RJ-1			RJ-2			RJ-3			RJ-4			RJ-5			RJ-6			RJ-7		
Microorganism	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC
1	17	16	32	17	4	8	19	4	8	15	8	16	19	4	8	18	16	16	21	4	8
2	18	8	16	21	2	4	18	8	8	20	4	4	20	2	4	18	8	8	19	8	16
3	10	16	16	11	16	32	13	8	32	12	32	32	13	32	32	15	16	32	16	8	32
4	14	8	16	13	32	32	12	16	32	17	4	8	13	8	16	16	8	16	17	8	32
5	9	32	64	9	32	64	12	16	16	9	16	64	10	16	32	13	16	32	9	32	64
6	10	16	32	10	16	32	11	16	32	10	32	32	14	16	32	9	32	32	9	16	32
7	9	16	16	9	16	32	8	32	64	9	32	64	9	32	64	11	16	32	10	32	64
8	15	32	32	14	4	16	17	4	8	14	8	32	13	8	32	18	4	8	23	2	2
9	18	4	4	18	2	8	20	4	4	18	2	4	22	2	4	21	4	4	22	2	4
10	12	32	32	12	16	32	14	8	32	12	32	32	13	16	32	13	16	32	15	8	16
	RJ-8			RJ-9			RJ-10			RJ-11			RJ-12			RJ-13			RJ-14		
Microorganism	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC	IZD	MIC	MBC
1	22	2	4	22	2	2	23	2	2	19	4	8	21	4	8	20	4	4	18	4	16
2	22	4	8	18	8	8	20	2	4	19	4	4	22	4	4	19	4	8	19	6.12	16
3	14	16	32	20	4	4	18	4	8	15	8	8	17	16	16	14	8	16	19	4	8
4	18	8	16	20	2	8	20	2	4	18	16	32	12	16	32	19	2	4	19	4	16
5	10	32	32	12	16	32	12	16	32	10	16	32	12	16	32	10	32	32	13	8	16
6	9	32	64	10	16	32	10	16	32	11	16	16	10	32	32	10	16	32	8	32	64
7	11	16	32	9	16	64	9	32	64	10	32	64	9	32	64	10	32	64	10	32	32
8	24	2	2	21	2	2	23	2	2	19	8	16	23	2	4	22	2	2	21	2	4
9	24	2	2	20	2	4	17	4	8	24	4	8	23	2	2	8	64	64	19	4	8
10	17	8	8	19	4	4	19	4	4	17	8	16	19	8	16	16	4	16	14	16	32

1: *Bacillus cereus* BC 6830, 2: *Enterococcus faecalis* NCTC 12697, 3: *Staphylococcus aureus* NCTC 10788, 4: *Staphylococcus aureus* BC7231, 5: *Staphylococcus aureus* ATCC 25923, 6: *Escherichia coli* NCTC 9001, 7: *Escherichia coli* BC 1402, 8: *Pseudomonas aeruginosa* NCTC12924, 9: *Salmonella typhimurium* RSSK 95091, 10: *Yersinia enterocolitica* ATCC 27729, IZD: Inhibition zone diameters; MIC: minimum inhibitory concentrations; MBC: minimum bactericidal concentration.

Antibacterial activity

Table 4 shows the results of the antibacterial activity of royal jelly samples. The results obtained in this study showed that the royal jelly samples at 500 mg/mL concentration had an antibacterial effect against all Gram-positive and Gram-negative bacteria. Among the Gram-negative bacteria, *Escherichia coli* NCTC 9001 and *Escherichia coli* BC 1402 strains were less sensitive against royal jelly samples compared to other strains, and the inhibition zones observed around them were narrower. But, on the other hand, it was observed that *Bacillus cereus* BC 6830 and *Enterococcus faecalis* NCTC 12697 strains were the most sensitive strains against royal jelly samples. In addition to this, it was observed that *Staphylococcus aureus* ATCC 25923 strain was more resistant compared to *Staphylococcus aureus* NCTC 10788 and *Staphylococcus aureus* BC7231 strains.

Chemometrics

HCA analyses results of domestic and non-domestic royal jelly samples are presented in Figure 1. As it can be seen in Figure 1 well-separated two clusters (numbered as 1 and 2) were observed on the HCA dendrogram with quite a high heterogeneity value of 300. While all of the non-domestic royal jelly samples were clustered on the right side of the HCA dendrogram, all of the domestic royal jelly samples were clustered on the left side of the HCA dendrogram.

The principal component analysis (PCA) biplot for the royal jelly samples is presented in Figure 2. PCA was performed on the basis of TPC (mg GAE/g), TFC (mg CAE/g), AC (mg TE/g), water content (%), acidity (ml 0.1 N NaOH/g), 10-HDA (%), and total sugar content properties. PCA was performed with the aim of obtaining a small number of factors that contain the maximum variability among samples. PC1 revealed the most variation, the differences among samples along PC1 axis explained more compared to the similar distances along PC2 axis. Three principal components (PCs) with eigenvalues $>$ 1 represent 87% of total variance, while PC1, PC2 and PC3 describe 46.6%, 23.9%, and 17.4% of total variance, respectively. According to the bi-plot in Figure 1, the royal jelly samples RJ-6, RJ-7, RJ-9, RJ-10, RJ-11, RJ-12, RJ-13, and RJ-14 were located on the right side of the plot while RJ-1, RJ-2, RJ-3, RJ-4 and RJ-5 were located on the left side of the biplot which illustrated that they possess approximately opposite responses. The biplot presented that RJ-6, RJ-7, RJ-9, RJ-10, RJ-11, RJ-12, RJ-13 and RJ-14 were closely related to each other since all of them were domestic royal jelly samples. The non-domestic royal jelly samples RJ-1, RJ-2, and RJ-4 seemed to be closely related to each other.

Principal component analysis (PCA) score plot for the royal jelly samples is presented in Figure 3. The score plot was built on the basis of amino acid profile of royal jelly samples. PC1 revealed the most variation,

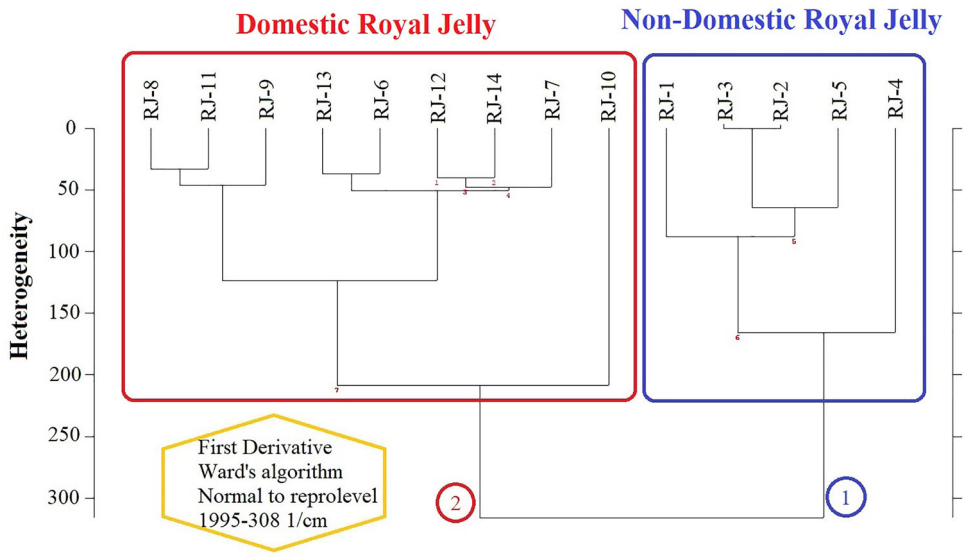

Figure 1. Dendrogram of HCA (Ward's Algorithm) of RJ samples.

the differences among samples along PC1 axis explained more compared to the similar distances along PC2 axis. Three principal components (PCs) with eigenvalues >1 represent 93% of total variance, PC1, PC2 and PC3, PC4, PC5, PC6, PC7 described 36.0%, 23.1%, 13.96%, 7.77%, 5.37%, 4.22% and 3.13% of total variance, respectively. According to the score plot in Figure 2, the royal jelly samples RJ-1, RJ-2, RJ-3, RJ-4, RJ-5, RJ-7, RJ-11, RJ-12, RJ-13 and RJ-14 were located on the left side of the score plot while RJ-6, RJ-8, RJ-9 and RJ-10 were located on the right side of the score plot. Score pot represented that RJ-1, RJ-2, RJ-3, RJ-4, RJ-5, RJ-7 and RJ-14 were closely related to each other.

Finally, PCA score plot which was built on the basis of the elemental analysis profile of domestic and non-domestic RJ samples is presented in Figure 4. The differences among samples along PC1 axis explained more compared to the similar distances along PC2 axis. Three principal components (PCs) with eigenvalues >1 represent 98% of total variance, PC1, PC2 and PC3, PC4, PC5, PC6, PC7, PC8, PC9, PC10 described 49.7%, 13.1%, 10.3%, 6.3%, 5.8%, 3.8%, 3.4%, 2.34%, 1.74% and 1.61% of total variance, respectively. According to the score plot in Figure 4, the royal jelly samples RJ-1, RJ-4, RJ-5, RJ-7, RJ-8, RJ-10 and RJ-13 were located on the left side of the score plot while RJ-2, RJ-6, RJ-9, RJ-11, RJ-12 and RJ-14 were located on the right side of the score plot. Score plot represented that RJ-4, RJ-5, RJ-6, RJ-7, RJ-10 and RJ-14 were closely related to each other. RJ-13 and RJ-12 exhibited quite different locations on the PCA score plot.

Discussion

In this study, the aim was to present detailed data on the chemical properties and some pharmacological activities of royal jelly. The phenolic compounds and flavonoids are responsible for antioxidant activity. These phenolic compounds interfere with propagation reactions, or inhibit the enzymatic systems involved in initiation reactions (Kausar & More, 2019). Considering that RJ is produced after the digestion of honey bee pollen by natural enzymes (Kostić et al., 2020), and that all pollen phenolic compounds are also found in royal jelly, it can be reported that the antioxidant effect of royal jelly is related to phenolic compounds, proteins and peptides (Maqsoudlou et al., 2019). Total phenolic content of Bulgarian and China royal jelly was detected to range between 11.82-26.07 mg GAE/g (Balkanska, 2018) and 21.2 mg CAE/g (Nagaı & Inoue, 2004), respectively. Phenolic content of royal jelly samples was very similar to those found for Turkish royal jelly by Özkök and Silici (2017) which were 0.59 mg GAE/g in royal jelly. The types and amounts of phenolic compounds containing cinnamic acids and flavonoids in royal jelly vary depending on the plant species visited by honey bees, plant health, environmental and seasonal factors (Ramadan & Al-Ghamdi, 2012). Royal jelly may be used as functional food thanks to its naturally high antioxidant potential (Viuda-Martos et al., 2008).

The water content of foods is an important value that determines what types of microorganisms can spoil the food (Doyle & Sperber, 2009). The water content of royal jelly plays a key quality parameter and its quantitative analysis is always a part of the quality control on royal jelly (Kausar & More, 2019). The water content of royal jelly is affected by the season of production, processing techniques, storage conditions and shelf life. Kanelis et al. (2015) reported that the water content of 176 royal jelly samples collected from 34 major beekeeping regions in Greece was between 60.0% and 70.0%.

Royal jelly is soluble (to some extent) in water and acidic with a Ph of 3.4–4.5 (Lercker, 2003; Ramadan & Al-

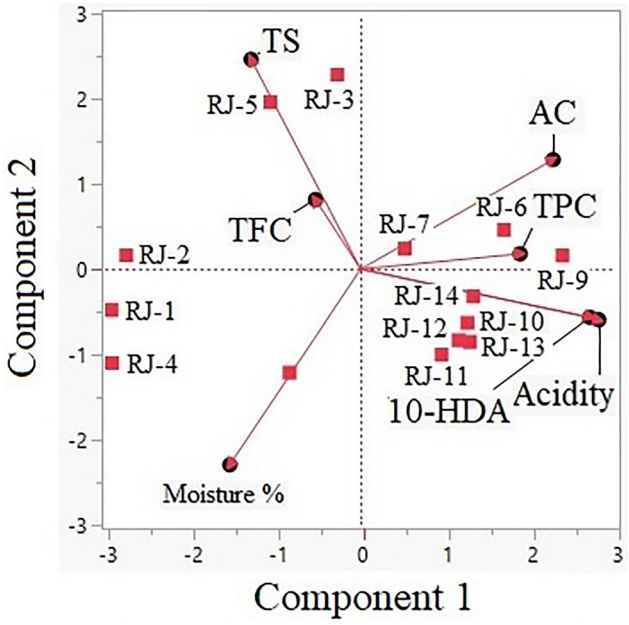

Figure 2. Principal component analysis (PCA) biplot for RJ samples (variables; total phenolic content (TPC), total flavonoid content (TFC), antioxidant capacity (AC), moisture content (%), acidity (mL 0.1 N NaOH/g), 10-HDA (%) and total sugar content properties).

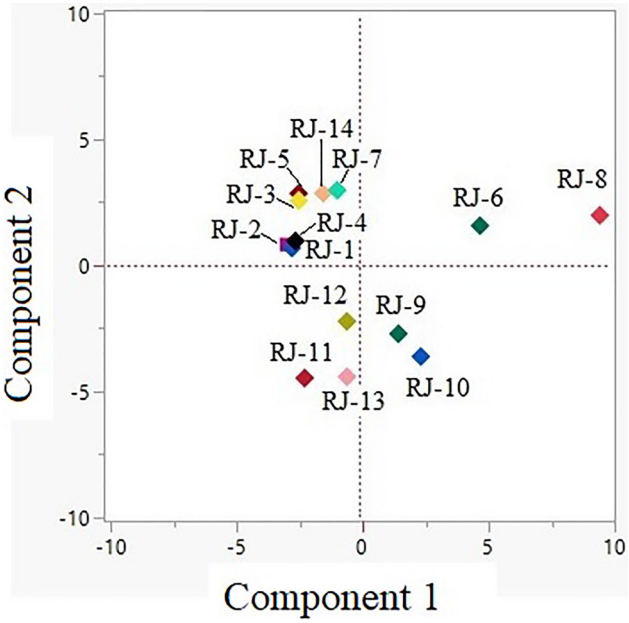

Figure 3. Principal component analysis (PCA) score plot based on the amino acid profile of RJ samples.

Ghamdi, 2012). Generally, our results remain within the values of Dınkov et al. (2016), who stated that royal jelly was typically acidic with values ranging between 3.31 and 4.42 mL 0.1 N NaOH/g. However, it was determined that the acidity degree of royal jelly from different regions varies between 3.687–4.42 mL 0.1 N NaOH/g (Balkanska et al., 2012). As the geographic origins of royal jelly differ; changes in its color, taste, aroma, and texture may have caused variability in acidity.

Carbohydrates represent 30% of the dry matter in royal jelly. However, just like honey, Glucose and Fructose are the dominant sugars in royal jelly. It also contains sugars such as Gentiobiose, Maltose, Sucrose, Isomaltose, Trehalose, Erlose, Raffinose and Melezitose in different concentrations, and the proportion of these sugars may be useful in determining the authenticity of the product in adulterated conditions (Sabatı nı et al., 2009). Concentrations of sugars detected in this research varied, while Fructose and Glucose were the most abundant sugars among others determined. Sugar profiles could help toward distinguishing between royal jellies. The sugar content of royal jelly could be linked not only to the geographical origin, but also to the method and the time of harvest, the ratio of honey present in royal jelly, and the larval age (Lercker et al., 1986; Zhu et al., 2019).

10-HDA is the major fatty acid of royal jelly. This fatty acid shows various pharmacological and biological activities and is a marker compound for royal jelly (Šedivá et al., 2018). It is also responsible for important biological activities in colony development (Wu et al., 1991). Therefore, 10-HDA is the most important quality parameter for royal jelly adulteration and decreases with the storage of royal jelly (Sabatını et al., 2009). It was determined that the 10-HDA value of non-domestic royal jelly samples except for one sample is below the limit value recommended by TSE 6666 (2000). Our results showed that domestic royal jelly samples had a significantly higher 10-HDA level (2.17 ± 0.02–2.71 ± 0.21%) than those non-domestic samples (0.27 ± 0.04–1.71 ± 0.04%). Similarly, Wei et al. (2013) stated that the 10-HDA content of royal jelly varied by geographical origin. They reported that western China had a higher 10-HDA level (2.01 ± 0.05%) than northeastern (1.87 ± 0.05%) and eastern (1.75 ± 0.03%). Western China is characterized by cold and drought climates. The northeastern region has a high latitude and extremely cold weather. In addition, the eastern region is much warmer and more humid than that of the northeastern region (Wei et al., 2013). However, Kausar and More (2019) determined the 10-HDA content in fresh and lyophilized royal jelly samples as 3.22% and 2.31%, respectively, different from our study. The 10-HDA value of fresh royal jelly samples from Brazil was determined between 1.58-3.39%; the value of domestic royal jelly samples used in our study was determined also in accordance with this range (Garcia-Amoedo & Almeida-Muradian, 2007). On the contrary, El-Guendouz et al. (2020) reported that the content of 10- HDA in 6 royal jelly samples from Morocco, Portugal and Spain was represented with a lower rate (0.9%-1.2%) compared to our results. Chemical content of royal jelly is quite complex and in addition to 10-HDA, which is one of the important fatty acid components of royal jelly, peptides such as Royalisin, Jelleines have been reported to have antimicrobial effect (Kim et al., 2019; Kim & Jin, 2019; Yang et al., 2018). Similarly, Al-Abbadi (2019) investigated antimicrobial activity of frozen and

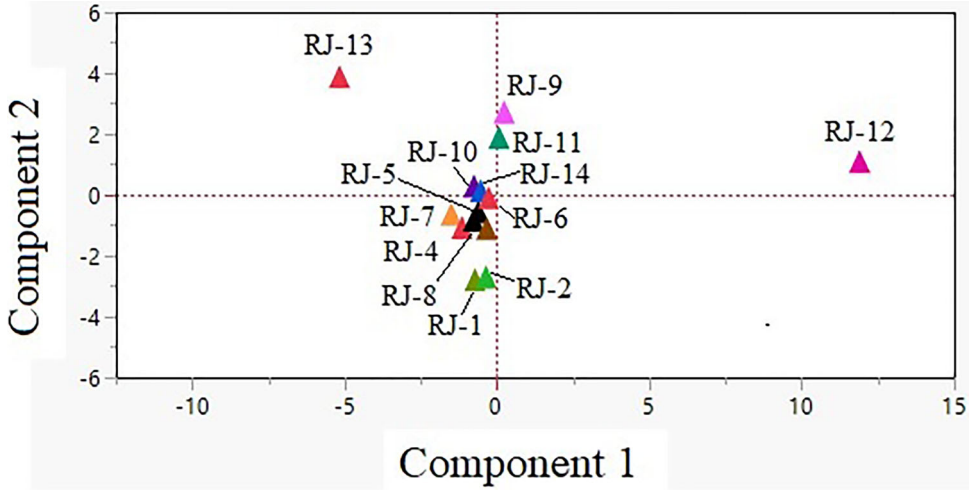

Figure 4. Principal component analysis (PCA) score plot based elemental analysis profile of RJ samples.

powdered royal jelly samples from Chinese and fresh royal jelly samples from Jordanian against human pathogenic bacteria and fungi by the well diffusion method. They stated that the frozen Chinese royal jelly samples showed the best effect against *Salmonella Typhimurium* (14028), but was significantly inactive against *Escherichia coli* (25922). These researchers reported that fresh Jordanian royal jelly samples inhibited the growth of *E. coli* (0157), *E. coli* (25922), *S. aureus* (25923), *K. oxytoca* (13182), *K. pneumoniae* (7700) and E. *aerogenes* (35029), but were not active against *P. aeruginosa* (27253). In a study published by Altuntas et al. (2020), MBC value of royal jelly against *Listeria monocytogenes* pathogen was determined by broth microdilution method. Compatible with our results, they concluded that royal jelly had a bactericidal effect for *Listeria monocytogenes* at a concentration of 41.67 mg/mL (Altuntas et al., 2020).

The accuracy of the test was revealed by verifying the method we developed to detect the concentration of free amino acids in royal jelly. Firstly, calibration curves for five different concentrations were created. Since there was no royal jelly that contained free amino acids, the measurement was made by adding the standard substance to the desired concentration and the rate of recovery was determined. LOQ and LOD values were found from calibration curve approaches and signal-to-noise ratio. As a result of the suitability of these parameters obtained, analyses were made to determine the concentration of free amino acids in RJ. This analysis method has some important aspects. All amino acids were analyzed with high sensitivity and some of the isomeric (Leucine and Isoleucine etc.) and isobaric (Glutamine and Lysine, Alanine and Sarcosine, GABA and 3-Aminoisobutyric Acid, etc.) amino acids were separated under chromatography conditions. Chromatographic separation is particularly important because many amino acids have the same product of ion (44.2 Da). The amino acids with the highest concentration in the samples were L-Proline, L-Lysine, Taurine, B-Alanine, L-Glutamic Acid, L-Arginine, and L-Aspartic Acid, respectively. Similarly, Liming et al. (2009) detected 26 amino acids by the fast method of UPLC in royal jelly. They reported that the mean content of total amino acid and free amino acids in fresh royal jelly was 111.27 mg/g and 9.21 mg/g, respectively. As a result, differences in amino acid concentration can be an important parameter in evaluating the quality of royal jelly.

The presence-variability of minerals in royal jelly is related to the source of the feed, the time of harvest, biological and environmental factors of honey bees (Fratini et al., 2016). Trace elements have an important role in the pharmacological activities associated with royal jelly, as these elements have many biological functions (Ramadan & Al-Ghamdi, 2012). The elemental composition of royal jelly varied between samples, but the highest rate in all samples was K (294.09–13005 mg/kg), followed by P (137.68–4796 mg/kg), Mg (28.42–1032.89 mg/kg), Na (23.02–355.17 mg/kg), Ca (8.43–77.56 mg/kg) and Zn (3.36–66.44 mg/kg) (Table 4). When these results are evaluated, it can be said that geographical, botanical, and processing factors affect the elementary composition of royal jelly.

According to the HCA (ward's Algorithm) dendrogram, it can be interpreted that domestic and non-domestic royal jelly samples were explicitly distinguished from each other related to their geographical origin. As a result, Raman spectroscopy combined chemometrics of HCA analysis could be effectively used for discrimination of domestic and non-domestic royal jelly samples. Results from the current research are quite compatible with previous reports in which vibrational spectroscopy was successfully applied to the detection of the origin of selected foods or supplements (Cebi et al., 2016; 2019). The acidity and 10-HDA were closely related and presented similar information on PC1 (Figure 2). The variables, including total phenolic and antioxidant

capacity, were closely correlated with each other and showed a similar information on principal component 1 (PC1). A positive correlation was observed between total sugar content and total flavonoid content. Moisture (%) was not closely related with any of the variables since none of the evaluated variables was in the same location with moisture on the PCA biplot.

Conclusions

This new method developed for amino acid analysis has many advantages over other methods. The lack of derivatization, the use of internal standards, simple sample preparation, and the separation of isomer amino acids are the outstanding points of the method. Chemometrics of PCA analysis showed that amino acid profile, elemental profile, and chemical parameters were suitable for the presentation of the characteristic and descriptive properties of royal jelly samples since chemometrics revealed the hidden relationship between variables. Additionally, Raman spectroscopy combined with chemometrics of HCA analysis presented a high potential for classification and discrimination of domestic and non-domestic royal jelly samples. A further study may light the way for quality and adulteration problems and geographical origin issues in the royal jelly and related apicultural products. The obtained results will provide an important basis for subsequent research for the evaluation of the amino acid content, physicochemical content, phenolic content, the antioxidant and elemental profile of royal jelly.

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Supplementary material

Supplementary Table S1 is available for this article at: https://doi.org/10.1080/00218839.2021.1889222.

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