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Original article

Evaluation of bioactive compounds and proliferation properties of different royal jelly samples

Aslı Özkök[1], Esin Akbay[2], Aslı Elif Tanuğur Samancı[3], Nazlı Mayda[2], Mehmet Ali Onur[2]

[1]Hacettepe University, Bee and Bee Products Research and Application Center (HARUM), 06800, Beytepe, Ankara, Turkey. [2]Hacettepe University, Science Faculty, Biology Department, 06800, Beytepe, Ankara, Turkey. [3]SBS Scientific Bio Solutions Co Bee&You Propolis, 34775, İstanbul, Turkey.

Summary In this study cell proliferation potentials were evaluated of different royal jelly. Physical, chemical and palynological analysis were done at the royal jelly samples, which are collected 5 different regions (RJ1: Balıkesir 1, RJ2: Bursa 1, RJ3: Çanakkale 1, RJ4: Yalova 1, RJ5: Bursa 2) of Turkey. In addition to this, cell proliferation potentials of these different royal jelly samples were evaluated by 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All analysis results were found to vary from region to region. MTT results indicated that after 24h incubation, RJ2 and RJ4 dilutions significantly different from control. However, light microscopy analysis showed same groups caused less viability potential in adipose derived mesencymal stem cells after 72h incubation. On the other hand, the highest dilutions of RJ1 and RJ3 cause more cell death with IC50 potential after 72h incubation. Among all groups, the lowest concentration of RJ5 has been shown to have the best growth-stimulating activity on adipose derived mesenchymal stem cells. The results of cell viability test highlight the potential differences of royal jelly samples on stem cell growth stimulation.

Keywords: Cell viability, Royal jelly, Mesencymal stem cells, MTT assay, growth-stimulating activity.

Introduction

Royal Jelly is one of the important bee products and secreted from the mandibular and hypopharyngeal glands of young worker nurse honeybees (*Apis mellifera* L.) (1,2). It has milky-white to yellowish colour and sour-sharp taste. It is an essential food for the honeybees especially for the queen bee. Queen bees have long life and fertility because they have been fed with royal jelly throughout their lives (3). Since royal jelly is believed to have the same effect on humans as bees, royal jelly is widely used as a cosmetic or dietary supplement. Thus, royal jelly has many biological activities in humans. Researchers showed that antiaging (4), antioxidant (5), antibacterial (6,7), antitumoral (4,8,9), antihypertensive (10), immunomodulator (11), anti-inflammatory (12) and liver protector (13) properties of royal jelly and many studies still continue about this product.

The physical, chemical and palynological contents of royal jelly are changing from royal jelly to royal jelly. This may be due to the different of *Apis mellifera* subtypes and nutritional status from different plants. But generally, it contains: water (60-70%), proteins (12-15%), carbohydrates (7-18%), fatty acids and lipids (3-8%), and small amounts of vitamins (B-complex vitamins, vitamin C, vitamin E), free amino acids and minerals (copper, zinc, iron, calcium, manganese,

potassium, sodium) (3). Water, sugar (fructose and glucose), protein and 10-hydroxy-2-decenoic acid (10-HDA) contents are the most common criteria used for royal jelly quality authenticity (3,14).

In this study, palynological analysis were done at the royal jelly samples, which are collected different regions of Turkey, and stem cell proliferation potentials of these different royal jelly samples was evaluated by 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Materials and Methods

Collection of Materials

Royal jelly samples were collected from Balıkesir, Bursa, Çanakkale and Yalova regions by the experienced beekeepers. It is very perishable product so it has to be stored in the deep-frozen immediately after collection. Therefore, the royal jelly samples were brought to the laboratory with cold chain immediately after collection and stored in the freezer.

Palynological Analysis

Palynological analysis of royal jelly samples were done according to Piana et al., (15). 200 pollen were counted and pollen spectra (%) were created. Accordingly, the percentage of pollen was greater than 45 is determined as dominant pollen, the percentage of pollen was between 16 and 44 is determined as secondary pollen, the percentage of pollen was between 3 and 15 is determined as minor pollen and the percentage of pollen was less than 3 is determined as trace pollen. However, there may be exceptions between some plant types. For example, chestnut (*Castanea sativa*) plants have overrepresented pollen content and should be 70-90% for dominance (16-20).

Cell line determination

The adipose tissue derived mesenchymal stem cells (AdMSCs) were preferred to use in this in vitro experiment. These cells are widely used in differentiation and proliferation tests because they possess stem cell properties and have the potential to react quickly to many different molecules (21).

Isolation, Culture and Characterization of Cells

Mesenchymal stem cells used in this study and primary explant cell culture technique developed by our group was used for the isolation of AdMSCs (22). Adipose tissue fragments were transferred to well plates and incubated with primary medium (DMEM/F12 / 20% FBS / 0.2% penicillin-streptomycin) at 37°C/5% CO_2. The culture medium was replaced every day until the stem cells reach confluency. Cells from the second passage (P2) were used during experiments. AdMSCs were characterized by immunofluorescence staining of CD29, CD31, CD54 and CD90 molecules. Briefly, for immunostaining, AdMSCs grown on a culture dish were washed in phosphate buffered saline (Biochrom AG, Germany) and fixed for five minutes in methanol at -10°C. After fixation, cells were incubated for an hour with primary antibodies (in a dilution of 1:100) for CD29, CD31, CD54 and CD90 molecules and 45 minutes with a secondary antibody (donkey anti-goat Ig-TR). The cells were mounted with mounting medium and visualized under the fluorescence microscope (Olympus, Japan). Isolated ADMSCs was also characterized by osteogenic, chondrogenic, and adipogenic differentiation. For the chondrogenic, osteogenic and adipogenic differentiation potential of the cells, it was carried out by applying special-containing media at different time intervals. Osteogenic differentiation was confirmed by positive alizarin red staining of mineralized matrix after 21 days of culture. Chondrogenic differentiation was confirmed by alcian blue staining of sulfated proteoglycan-rich matrix after 14 days of culture. Adipogenic differentiation was confirmed by oil red O staining of lipid droplets after 14 days of culture.

Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cytotoxicity (23). It is a standardized calorimetric method that assesses the ability of viable cells to form MTT formazan by the mitochondrial enzyme suc-

cinate dehydrogenase. After the cells treated with royal jelly samples cells, well-plates were cultured at 37°C and 5% CO_2. For assessment of cellular viability, at the end of the incubation periods (24, 48, and 72 hours), the culture medium was replaced with 200 µL medium containing 10% MTT and plates were incubated for 4 hours at 37°C. The solution was removed and replaced with isopropanol alcohol and the absorbance was measured on a spectrophotometer microplate reader (µQuantTM, BioTek® Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm. Each experiment was performed in a 96-well plate and designed to have six replicates at three different time points.

Statistical analysis

Statistical significance was determined with the GraphPad Prism 5.00 program. The viability of cells in different royal jelly doses and regions were compared using repeated measures variance analysis. One-way analysis of variance (ANOVA) with Tukey post hoc test was used to compare viability in each concentration. Bonferroni post-test was used to compare values at same time interval. Data were expressed as mean ± standard derivation (SD) and it was considered significant when the p-value was less than 0.05.

Results

Royal jelly samples are numbered according to the regions where they are collected. Accordingly, it is numbered as follows. RJ1: Balıkesir, RJ2: Bursa 1, RJ3: Çanakkale, RJ4: Yalova, RJ5: Bursa 2. Results of palynological analysis of samples were represented at the Table 1.

***Castanea sativa* plants have overrepresented pollen content and should be 70-90% for dominance.

Isolated AdMSCs were used in this study. For this purpose, cells were thawed and cultured under standard condition. When confluency was observed immunofluorescence, staining was performed to identify AdMSCs. The staining showed that these cells were positive for surface markers CD29, CD54 and CD90, and negative for CD31.

Cell viability was assessed using MTT assay, and the percentage of cell viability calculated according to the following equation: The percentage of cell viability: OD of treated cells/ OD of control cells×100.

MTT assay results of cells were obtained after treatment with different doses of different royal jelly's in three consecutive days. Different RJ groups and all dilutions of groups didn't have any cytotoxic effects on AdMSCs after 24h incubation (Figure 1).

Table 1. Palynological analysis results of royal jelly samples

RJ No	Dominant pollen (>45%)*	Secondary pollen (16-44%)*	Minor Pollen (3-15%)*	Trace Pollen (<%3)*	Region
RJ1	Brassicaceae (58.33%)	*Castanea sativa* (20.83%)	Fabaceae (8.33%) *Salix* spp. (8.33%) Rosaceae (4.16%)	-	Balıkesir
RJ2	-	*Castanea sativa* (44.44%) Apiaceae (18.51%)	Fabaceae (14.81%) Brassicaceae (11.11%) Asteraceae (3.70%) *Scabiosa* spp. (3.70%) *Echium* spp. (3.70%)	-	Bursa 1
RJ3	*Castanea** sativa* (75%)	Fabaceae (25%)	-	-	Çanakkale
RJ4	*Castanea** sativa* (100%)	-	-	-	Yalova
RJ5	Brassicaceae (50%)	*Castanea sativa* (41.66%)	Chenopodiaceae (8.33%)	-	Bursa 2

* ≥45% Dominant (D), (16-44%) Seconder (S), (3-15%) Minor (M), (<3%) Trace (T).

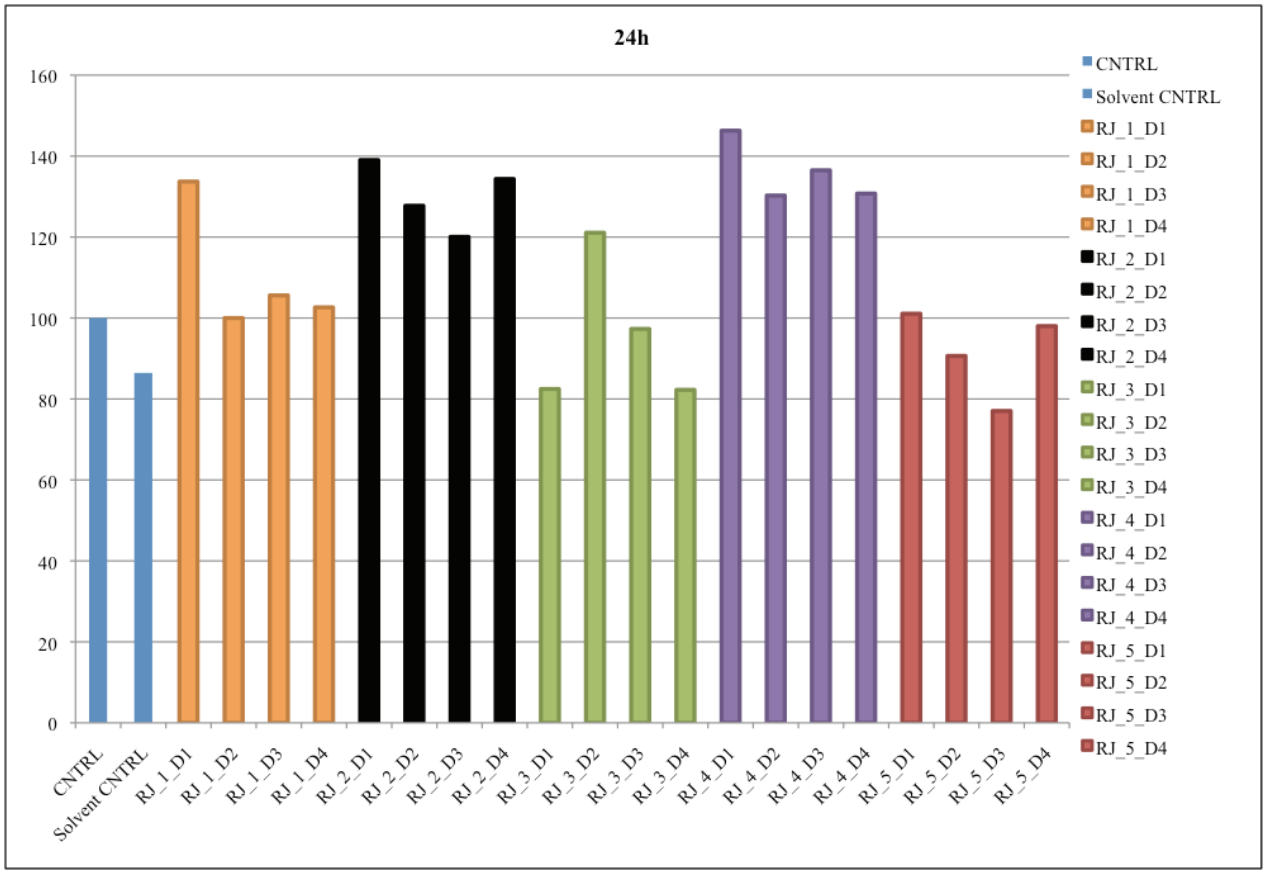

Figure 1. MTT assay of royal jelly's (RJ1, RJ2, RJ3, RJ4, and RJ5) on AdMSCs after 24h. (CNTRL: control, SOLVENT CNTRL: Solvent control, RJ: Royal Jelly, D: Dilution)

After 48h, both D3 and D4 of RJ3 produced higher percentage of reduction compared to control even though no significant difference was found between all groups ($P > 0.05$) (Table 2 and Figure 2). Unlike the RJ3, in all other groups and dilutions was no significant decreasing percentage of reduction. MTT results also indicated that after 72h incubation, RJ2 and RJ4 dilutions (D1: 100 mg, D2: 200 mg, D3: 500 mg, D4: 1000 mg) significantly different from control group ($P<0.05$) (Table 2 and Figure 3). However, there were no differences between dilutions. On the other hand, the highest dilutions of RJ3 cause more cell death with IC50 potential after 72h incubation (Table 2). After 72 hours, toxic effect was observed in all groups except RJ5. Statistically, the highest concentration of RJ5 has been shown to have growth-stimulating activity on AdMSCs compared to control ($P< 0.05$) after 72h (Table 2). The results of statistical analysis indicated that there is not any significant difference with the solvent control (DMSO group) and the control group.

Discussion

Honeybees collect pollen from flowers to meet their protein needs (17-19). The pollen, they collect can be detected in bee products. One of the easiest and most reliable ways to detect this is palynological examination (16). Because of that, in this study palynological analysis were done at the Turkish royal jelly samples. According to these results, we determined, Brassicaceae pollen type dominantly at RJ1 and RJ5 whereas *Castanea sativa* pollen type were found dominantly at RJ3 and RJ4. Beside this, we couldn't find any dominant pollen type at RJ2 (Table 1). Palynological analysis in royal jelly samples show the phyto-

Table 2. MTT viability assay for royal jelly samples

Viability	RJ1				RJ2				RJ3				RJ4				RJ5			
Mean± SD	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
24h	0.415± 0.13	0.310± 0.06	0.327± 0.05	0.318± 0.04	0.431± 0.07	0.396± 0.07	0.372± 0.04	0.417± 0.07	0.256± 0.02	0.375± 0.11	0.302± 0.09	0.255± 0.07	0.454± 0.08	0.404± 0.03	0.423± 0.07	0.405± 0.06	0.313± 0.05	0.281± 0.02	0.238± 0.01	0.303± 0.03
48h	0.262± 0.06	0.233± 0.06	0.260± 0.04	0.196± 0.02	0.223± 0.05	0.174± 0.03	0.214± 0.09	0.180± 0.03	0.285± 0.05	0.202± 0.03	0.160± 0.02	0.163± 0.02	0.345± 0.05	0.281± 0.04	0.279± 0.07	0.312± 0.03	0.207± 0.01	0.163± 0.01	0.123± 0.01	0.154± 0.02
72h	0.129± 0.01	0.099± 0.01	0.125± 0.01	0.120± 0.01	0.117± 0.01	0.114± 0.01	0.161± 0.01	0.116± 0.02	0.143± 0.01	0.119± 0.01	0.097± 0.01	0.086± 0.01	0.166± 0.02	0.126± 0.01	0.146± 0.01	0.122± 0.01	0.294± 0.02	0.304± 0.05	0.298± 0.04	0.348± 0.01
*P**													0.0014				0.0014			

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; D1: 100 mg, D2: 200 mg, D3: 500 mg, D4: 1000 mg; SD: Standard deviation;
* Bonferroni post-test

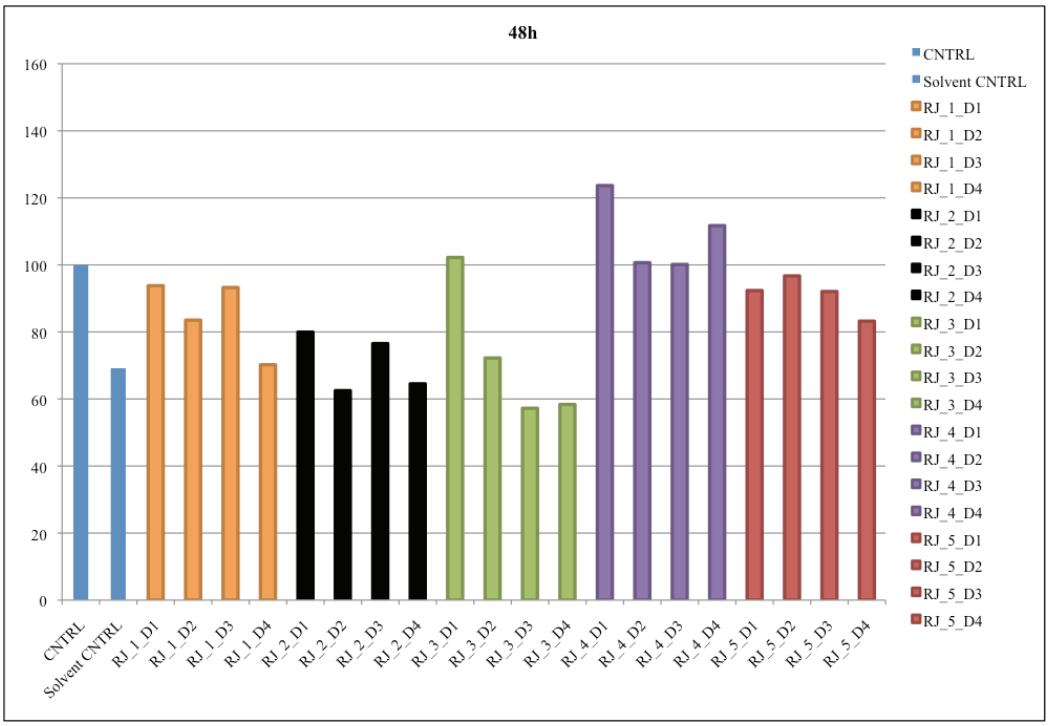

Figure 2. MTT assay of royal jelly's (RJ1, RJ2, RJ3, RJ4, and RJ5) on AdMSCs after 48h. (CNTRL: control, SOLVENT CNTRL: Solvent control, RJ: Royal Jelly, D: Dilution)

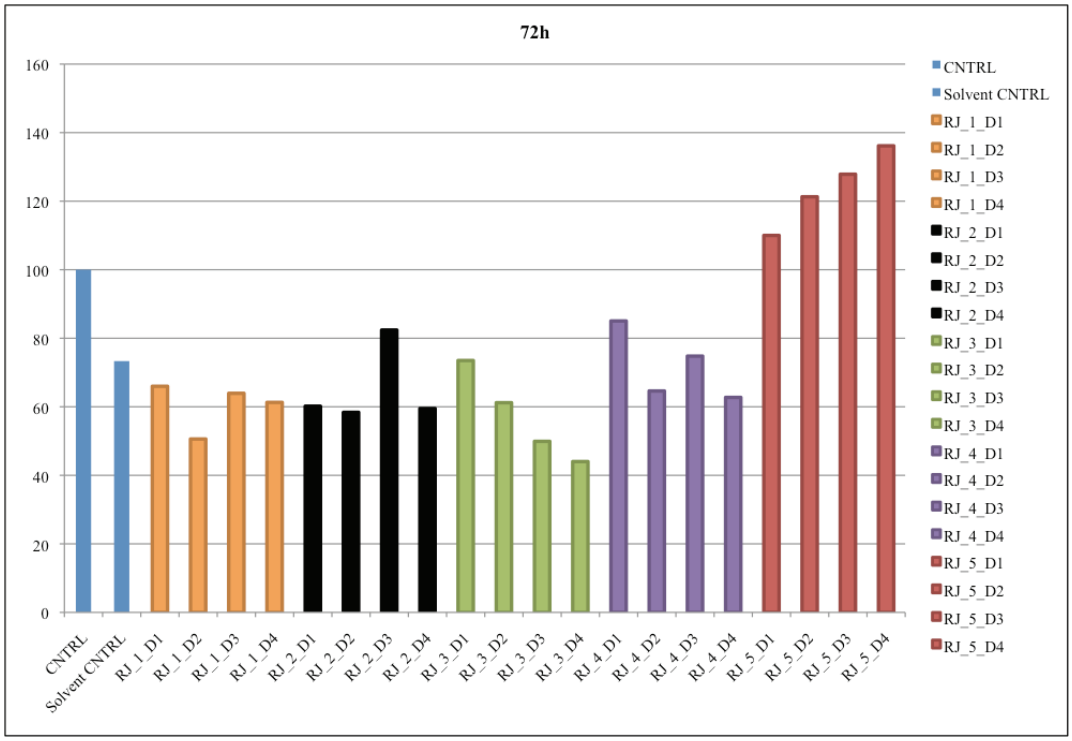

Figure 3. MTT assay of royal jelly's (RJ1, RJ2, RJ3, RJ4, and RJ5) on AdMSCs for 72h. (CNTRL: control, SOLVENT CNTRL: Solvent control, RJ: Royal Jelly, D: Dilution)

geographical origin of the product so it is important for the geographical marking and authenticity (15, 20). Even this importance, there aren't too much study about royal jelly palynology (20). Piana et al., (15) and Luz et al., (20) found *Castanea* pollen type the highest percentage at the samples. In our study we also found *Castanea* pollen type dominantly at the two royal jelly samples. Beside *Castanea sativa* we found Brassicaceae, Fabaceae, *Salix* spp., Rosaceae, Apiaceae, Asteraceae, *Scabiosa* spp., *Echium* spp. and Chenopodiaceae family types. These pollen taxa results were compatible the region's botanical origin, where the royal jelly samples collected (24).

Royal jelly consists of a large number of bioactive substances such as 10-HDA (25-31), minerals (27), adenosine (32), and antibacterial protein (6), a stimulating factor which induce development of genital organs in mouse model (33) and 350 kDa protein (34) that stimulates the proliferation of human monocytes.

Here, in this study RJ's that consistent different composition of chemical substances was analysis for their proliferation potential on AdMSCs. The MTT test used for this purpose is one of the most sensitive, quantitative and reliable colorimetric analyses that measure the viability, proliferation and activation of cells. The test is based on the production of dark blue/purple formazan, the water insoluble product, by reducing the cellular mitochondrial dehydrogenase enzyme in living cells by the water-soluble yellow substrate 3-(4,5-dimethylthiazol-2yl) -2,5-diphenyl tetrazolium bromide. The amount of formazan produced is directly proportional to the number of cells (35). In this study, analysis of different concentrations of different regions royal jelly's on cell viability revealed that the viability of cells was not related to the concentrations of royal jelly's, except RJ5. Dose dependent response was observed only after RJ5 treatment on 3th day where the viability of cells increases. Cells treated with the lowest concentration of RJ5 produced lower percentage of viability (Figure 3). Highest concentration of royal jelly might serve a more suitable pH for the cells to grow and proliferate compared to lower concentrations of the royal jelly. Hence, this data supports the results of earlier studies where royal jelly proteins were reported to stimulate the proliferation of U-937 monocytes (34) and hepatocyte DNA synthesis and albumin production (36). This is achieved by the activation of several important intracellular signalling factors that play a role in stimulating hepatocyte DNA synthesis. The 57-kDa protein in royal jelly also plays a role in the protection of cells from apoptosis (37). In another studies researches claimed that royal jelly promotes cell growth through its content (growth factors or hormones) (4, 38). Narita et al., (39) reported that RJ stimulates the proliferation of mouse osteoblast-like MC3T3-E1 cells. They also indicated that oral administration of RJ to mice caused an increase in bone ash content and an up-regulation of type I procollagen gene expression.

All results indicated that further studies are still needed. The results of cell viability test highlight the potential differences of royal jelly samples on stem cell growth stimulation. Further work will now be needed to test which signalling pathways are activated by RJ's content.

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Correspondence:
Aslı Özkök,
Hacettepe University, Bee and Bee Products Application and Research Center (HARUM), Beytepe, Ankara, Turkey,
Tel: +90(312) 2977161, Fax: +90(312) 2992028,
E-mail: asozkok@gmail.com, aozkok@hacettepe.edu.tr