



## The Effects of Royal Jelly Samples Collected from Sivas Province on the Proliferation of Endothelial Cells

Safa Uğur Koçköprü<sup>1</sup>, Sevgi Durna Daştan<sup>2,3,\*</sup>

<sup>1</sup>Department of Biology, Institute of Science, Sivas Cumhuriyet University, Sivas, Türkiye

<sup>2</sup>Department of Biology, Faculty of Science, Sivas Cumhuriyet University, Sivas, Türkiye

<sup>3</sup>Beekeeping Development Application and Research Center, Sivas Cumhuriyet University, Sivas, Türkiye

\*Corresponding author

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### ABSTRACT

Honey, bee venom, royal jelly, pollen, propolis and other bee products are natural products that are used to protect and maintain human health, to create a strong and dynamic health condition, and used as an ethnopharmacological medicine in the therapy of some illness. The use of different bee products to strengthen the immune system is still very common today. In this study, it was aimed to determine the in vitro effects of royal jelly samples collected from Sivas province in HUVEC cell culture conditions by determining the healing endothelial damage. In this context, royal jelly samples were obtained from the province of Sivas, where beekeeping is carried out intensively and successfully, in the 2022 harvest period. The effect of royal jelly extracts on the cell proliferation of endothelial cell culture was investigated. Repair of endothelial cell damage, cell growth and development and migration values were measured using the HUVEC cell line, and the data were evaluated with SPSS, GraphPad, ImageJ programs. Although there were significantly important increases in cell growth, development and cell viability values in HUVEC cells treated with different doses of royal jelly at the end of 24 and 48 hours, no statistically significant difference was found in terms of cell migration ratio compared to the control group.

[safaugurcle23@gmail.com](mailto:safaugurcle23@gmail.com)

<https://orcid.org/0000-0002-9127-7990>

[sevgidurna@gmail.com](mailto:sevgidurna@gmail.com)

<https://orcid.org/0000-0003-4946-5602>



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### Introduction

Sivas province has high potential in terms of beekeeping, because of its natural plant diversity and land structure. However, scientific studies on bee products of Sivas province are very limited. Recently, importance has been given to traditional and complementary medicine practices on a world scale. People benefit from bee products in order to protect their general health and to treat some diseases (Ekici and Gölgeli, 2021; Rice, 2019; Fratellone et al., 2016; Premratanachai and Chanchao, 2014). Apitherapy is derived from the Greek words "therapy", which literally means healing or healer, and "apia" meaning bee (Kekeçoğlu et al., 2021). Royal jelly is one of the products frequently used in apitherapy. Royal jelly is a special substance secreted by the hypopharyngeal glands in the head of 5-15 days old worker bees to feed the queen and larvae (Sorucu, 2019; Viuda-Martos et al., 2008). It is fluid in gel form, creamy-white in color. It has a sour taste and a strong phenolic odor. Its density is 1.1 g/cm<sup>3</sup> and it is soluble in water (Parlakpınar and Polat, 2021; Silici, 2019). The majority of royal jelly is water, and

it contains various minerals (Fe, Na, Ca, K, Zn, Mg, Mn and Cu), polyphenols, amino acids, hormones, vitamins (A, C, D, K, E and B complex), enzymes, nucleotides (Sipahi et al., 2021; Ahmad et al., 2020). While the queen bee is fed with royal jelly throughout her life, the worker bees are fed only with royal jelly for a certain period of their lives. This diet determines the lifespan and reproductive capacity of bees. Royal jelly; it is stated that it is a functional food with its properties that increase endurance, relieve mental tension, relax, increase appetite and energize, as well as prevent and heal various diseases (Premratanachai and Chanchao, 2014; Ramadana and Al-Ghamdi, 2012). It has also been shown to have neurotrophic and neuroprotective effects, directly affect the differentiation of brain cells, and have a stimulating effect on the central nervous system. Proteins in the composition of royal jelly have antioxidant properties, therefore, positive results have been reported in studies aimed at delaying aging, protecting the liver and reducing the negative effects of radiation (Apan et al., 2021; Akyol,

2015; Ramadana and Al-Ghamdi, 2012; Silici et al., 2011). Antibacterial, antioxidant, anti-tumor, anti-aging, immunomodulatory effects, success in respiratory system diseases such as allergic rhinitis, asthma, biological effects such as anti-ulcer, accelerated fracture healing, hair growth agent, aphrodisiac, spermatogenesis enhancing quality, cardioprotective and growth promoting effects have been described (Sorucu, 2019; Morita et al., 2012; Silici et al., 2011; Pavel et al., 2011; Çavuşođlu et al., 2009; Viuda-Martos et al., 2008). Generally, free amino acids, carbohydrates (fructose, glucose) and lipids are found in royal jelly (Parlakpınar and Polat, 2021; Silici, 2019). It is 10-hydroxydecanoic acid, an unsaturated fat molecule found only in royal jelly, that gives royal jelly its biological properties (10-HDA) (Sorucu, 2019). In some researches; It has been reported that the antibacterial and antioxidant properties of royal jelly are attributed to the 10-HDA substance in its composition (Apan et al., 2021; Nagai ad Inoue, 2004; Yatsunami and Echigo, 1985). 10-HDA is also considered the commercial quality of royal jelly. The ratio of this substance in royal jelly varies between 1.4-2% (Sorucu, 2019; Barker et al., 1959). In addition, 80-85% fatty acids, 4-10% phenols, 3-4% sterols and 0.4-0.8% phospholipids are the lipids found in royal jelly (Parlakpınar and Polat, 2021; Özdemir et al., 2021; Onbaşı et al., 2019; Silici, 2019). Royal jelly contains at least 17 amino acids, 8 of which are essential (methionine, valine, lysine, leucine, threonine, phenylalanine, isoleucine, and tryptophan) (Parlakpınar and Polat, 2021; Alvarez-Suarez, 2017). Royal jelly also contains phosphorus, sulfur, aluminum, and traces of nickel, chromium, tin, tungsten, antimony, titanium and bismuth (Parlakpınar and Polat, 2021; Silici, 2019; Sorucu, 2019). The pH value of royal jelly is 3,6. Likewise, royal jelly contains 1 mg/g of acetylcholine, which is important for nerve transmission, production and release of glandular secretions, and triggers the release of adrenaline. The main flavonoids (galangin, quercetin, camferol, and fisetin), flavanones (naringin, pinosembrin, and hesperidin) and flavones (chrysin, apigenin, luteolin, acacetin) are found in royal jelly (Parlakpınar and Polat, 2021; Silici, 2019). In this study, it was aimed to determine the effects of royal jelly samples collected from Sivas province on cell migration in in vitro conditions in cell culture medium and in this way, their effects on healing endothelial damage.

## Material and Method

### Preparation of Royal Jelly Samples

Royal jelly was obtained from beekeepers in the Zara district of Sivas province in the 2022 harvest period. Lyophilization process was carried out at Sivas Cumhuriyet University Advanced Technology and Research Center (CUTAM). The concentration of lyophilized royal jelly was adjusted to be 2 mg/mL, and samples were prepared at different dose concentrations between 1 µg/mL -1000 µg/mL concentrations by dilution.

### Cell Viability Assay

The human umbilical vein endothelial cell (HUVEC) line was used in in vitro cell culture studies. Cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and 2

mM L-glutamine. The HUVEC cultures were incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Briefly, cells were seeded into a 96-well plate at a density of 5 × 10<sup>3</sup> cells/well in the 200 µL medium. After incubation, cells were treated for 48 hours with royal jelly sample with different dose concentrations and cisplatin was used as a positive control. Cell viability was determined by MTT assays as described in Al-Janabi et al. (2020). 50 µl of MTT reagent (5 mg/mL diluted in culture medium without phenol red and PBS) was added and incubated for additional 4 hours. MTT was discarded and 200 µL of DMSO was added to dissolve the formazan crystals and incubated in an orbital shaker for 1 hour. The absorbance values of the MTT solution in the well were read with a microplate reader (ThermoScientific microplate reader) at a wavelength of 575 nm. GraphPad Prism software (version 8.0.1; GraphPad Software, Inc., La Jolla, CA, USA) was used to determine the IC<sub>50</sub> dose. The morphology of the cells after the applied with royal jelly samples' dose were assessed by inverted microscope (Topcu et al., 2022; Saygi and Cacan, 2021; Dastan et al., 2017; Daştan et al., 2016).

### Cell Migration Assay

We performed wound healing detection to determine the effect of royal jelly applied to HUVEC line on cell migration. Cells were added at a density of 5x10<sup>4</sup> cells per well of the culture-insert placed in a 6-well plate. Cells were incubated overnight in a 37°C incubator containing 5% CO<sub>2</sub> to adhere to the surface. Wound closure was photographed using Zen 3.1 (ZEN lite) programme on inverted microscope (Zeiss Axiovert A1) at 0, 24 and 48 hours, with wound formation time considered as 0 hour. Areas (%) covered by migrating cells were measured with the ImageJ 1.53t (USA) programme application (Menevse Cinar, 2022).

### Statistical Analysis

GraphPad Prism software (version 8.0.1; GraphPad Software, Inc., La Jolla, CA, USA) was used to statistical data analysis. Descriptive and comparative analyzes were performed with IBM SPSS v23 software (IBM SPSS, Chicago, Illinois, USA). The statistical significance levels of the doses are given on the figures (\*P<0.05, \*\* P<0.01).

## Results and Discussion

### Cell Viability Results

HUVEC cell cultures, in which different doses of royal jelly were applied, were examined under the microscope after 24 and 48 hours of incubation, and the growth and development levels of the cells were determined (Figure 1). In the microscopic images, no significant differences were detected in the cell morphologies of the experimental group and control group, and it was observed that the growth and development amounts of the cells were close to each other (Figure 1). In addition, statistical analyzes were carried out using the absorbance values obtained as a result of the MTT application. The control and experimental groups were compared with the ANOVA test using absorbance values (Table 1). The difference in growth and development levels of HUVEC cells was examined according to the Anova test. Dunnet's multiple comparison

test was used to determine the level of difference between the groups in which the difference was detected (Table 2). In the comparison made according to the absorbance values obtained from the MTT test, significant differences were determined between the control and experimental groups in terms of cell viability rates (Table 1-2).

### Cell Migration Results

The cell migration test is based on the determination of the closure of the gap area opened in HUVEC cell cultures with the application of different doses of the royal jelly. With this test, the space created in the culture medium in which the HUVEC cell line is incubated is closed with the increase in the migration and proliferation rates of the cells, and the rate and amount of closure of the gap are measured. With the proliferation of cells, the cell-free space is invaded and closed by the cells. In this way, cell proliferation and migration rates are examined by microscopic imaging during 48 hour time. In this study, although it was determined that there were increases in cell growth and development, generally in cell migration with

the application of the different doses of royal jelly, it was defined that there was no statistically significant improvement. It showed the same level of cell proliferation as the control group. At the statistical level, no significant differences were found between the control and experimental groups (Figure 2-3). In microscopic evaluations with the application of high doses of royal jelly samples, it was noticed that the cells were more active and there were improvements in cell proliferation, but no significant differences were found between the control group and the experimental groups at the statistical level (Figure 2-3). In the images measured with the Image J program, it was determined that the area where the gap was created began to be closed by the cells very slowly, but the area where the gap was still in 48 hours could not be fully closed yet, and the gap area was clearly seen as an opening. Similar images were obtained in the control group and in all groups where different doses of royal jelly were applied (Figure 2-3). Using the Image J program, the amount of gap's area in the cell plate was measured in all groups and comparisons were made as percentage of area (Figure 4-5).

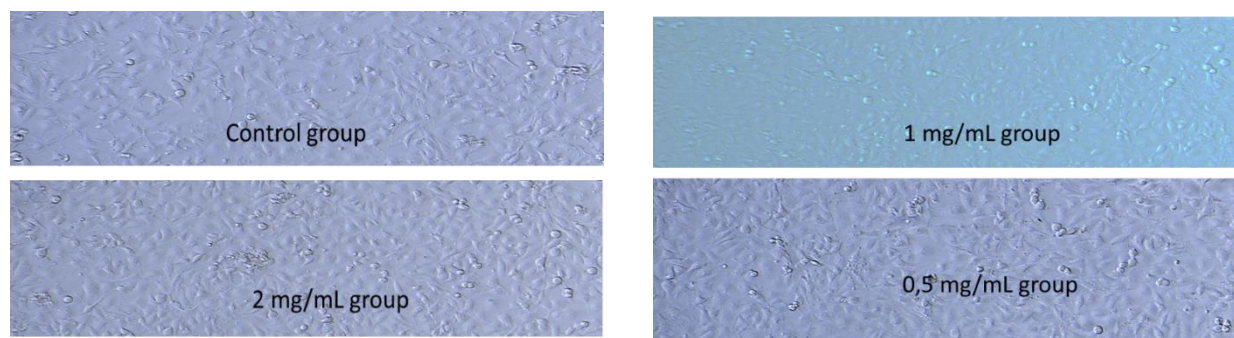


Figure 1. Microscopic view of HUVEC cells treated with different doses of royal jelly.

Table 1. Comparison of cell viability values in control and experimental groups according to Anova test.

Cell viability differencies from ANOVA statistic programme result						
	Differencies	Squares	DF	Mean of Squares	F	P
24 hour	Between Groups	0.256	8	0.032	25.343	0.01**
	Inside Group	0.028	22	0.001		
	Total	0.283	30			
48 hour	Between Groups	2.954	8	0.369	245.786	0.01**
	Inside Group	0.033	22	0.002		
	Total	2.987	30			

Table 2. Comparison of cell viability values in control and experimental groups according to Dunnet test.

Time period	(I) The doses of royal jelly	(J) Control	Mean Difference (I-J)	Standart Error	P
24 hour	1000 µg/mL	Control	0.16654**	0.02450	0.001
	500 µg/mL	Control	0.20787**	0.02450	0.001
	250 µg/mL	Control	0.22420**	0.02450	0.001
	125 µg/mL	Control	0.22974**	0.02450	0.001
	62.5 µg/mL	Control	0.23080**	0.02450	0.001
	31.25 µg/mL	Control	0.23447**	0.02450	0.001
	15.63 µg/mL	Control	0.22000**	0.02450	0.001
48 hour	2000 µg/mL	Control	0.72480**	0.02675	0.001
	1000 µg/mL	Control	0.72377**	0.02675	0.001
	500 µg/mL	Control	0.74514**	0.02675	0.001
	250 µg/mL	Control	0.72907**	0.02675	0.01
	125 µg/mL	Control	0.73447**	0.02675	0.01
	62.5 µg/mL	Control	0.75237*	0.02675	0.01
	31.25 µg/mL	Control	0.74794*	0.02675	0.01
15.63 µg/mL	Control	0.74577*	0.02675	0.01	

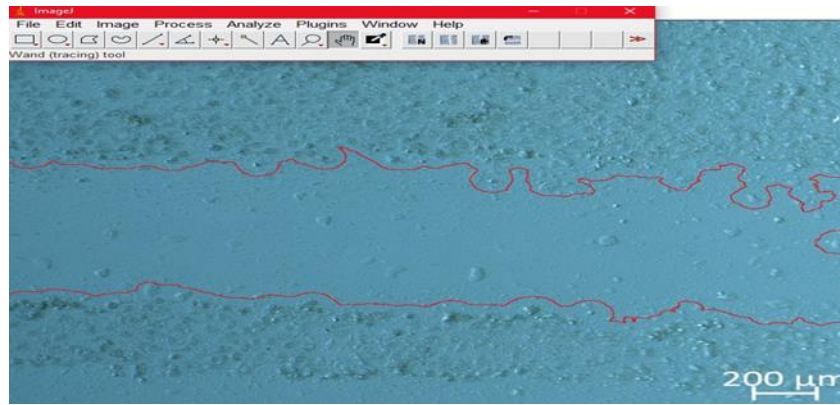


Figure 2. ImageJ program view and measurement of the gap area on HUVEC line.

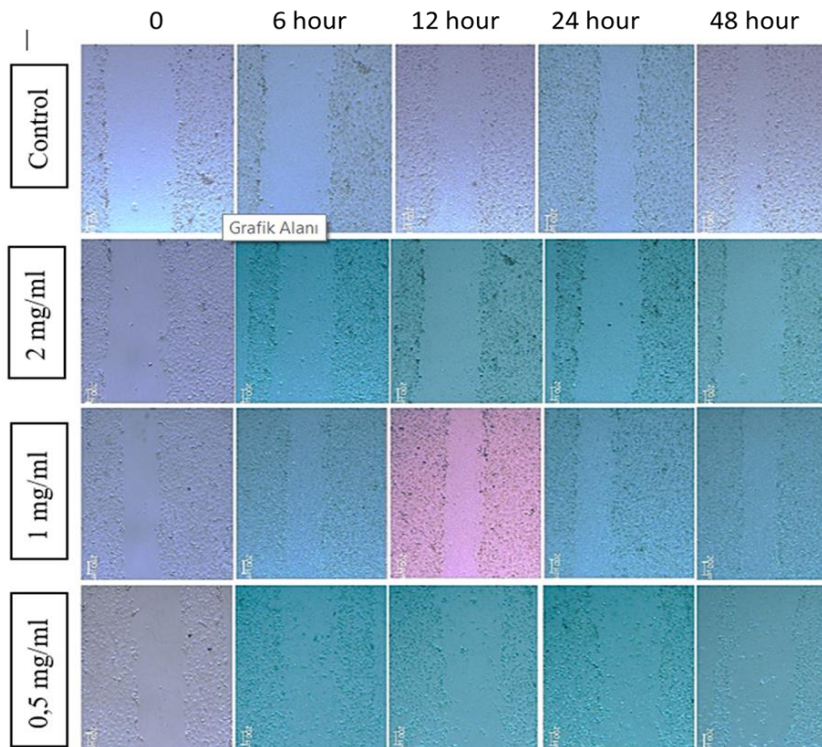


Figure 3. Microscopic imaging of migration and proliferation of cells at 0-48 hour time intervals in the cell-free space created in HUVEC culture placed in 6-well plates.

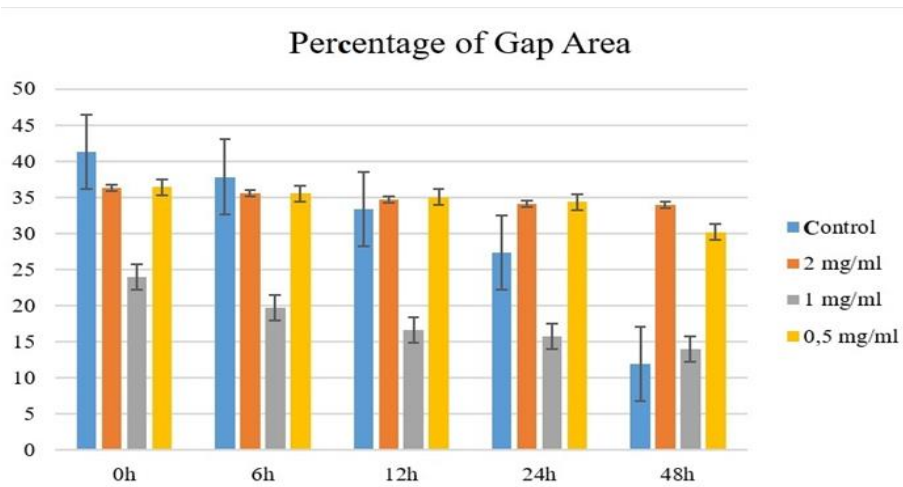


Figure 4. Percent gap area plot from the Image J program showing that the gap area created in HUVEC cell culture decreases with cell migration over time.

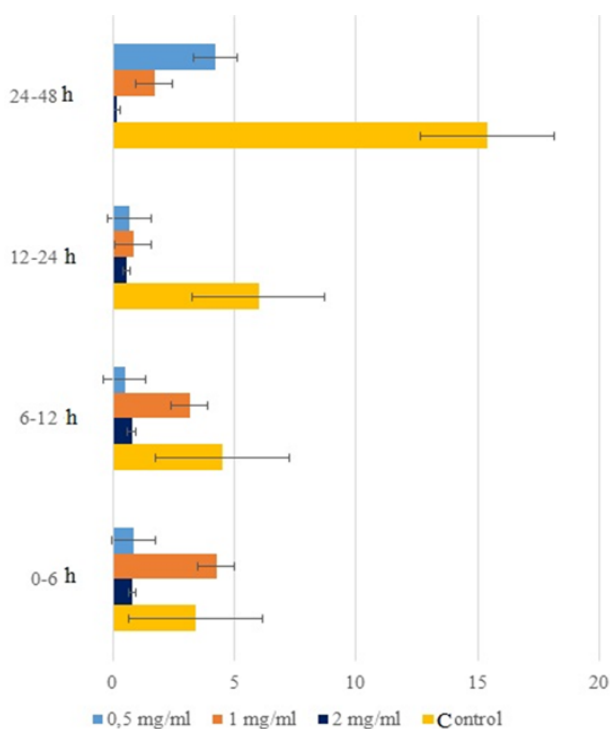


Figure 5. Percentage of cell migration in the gap area within the cell culture at periodic time intervals.

In the study conducted by Bayar (2020), it was determined that the cell migration accelerated from the 6th hour with the application of royal jelly, and the percentage of wound openings decreased in hyperglycemic cells in both normoxic and hypoxic environments (Bayar, 2020). After long-term exposure to hypoxia, it was determined that the migration potential of cells increased with the application of royal jelly in an environment containing high amounts of glucose. Within the scope of this study, in observing the potential effects on wound healing and cell migration with the application of royal jelly samples collected from Sivas province to HUVEC cells *in vitro*, it was observed that royal jelly slightly increased the wound healing potential, but there was no statistical difference between the control group and the experimental groups. . When we look at the groups that were treated with royal jelly, a small closure was observed in the wound distance for 48 hours, but there was no difference with the control group, the created wound cavity was similarly filled with cell migration rate and even the cavity was not closed enough in all groups for 48 hours.

## Conclusion

As a result, this study is a first in terms of evaluation of endothelial cell proliferation and wound healing potential of royal jelly samples from Sivas province and will attract the attention of the food, health and cosmetics sectors. In addition, the fact that it is a study to increase awareness in terms of health provides originality. With this study, it is possible to contribute to the universal knowledge base at a basic level and it is a preliminary study and it is thought that it will guide other studies to be done in the future.

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## Conflict of Interest

There is no conflict of interest between the authors.

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