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ARAŞTIRMA MAKALESİ **RESEARCH PAPER**

Investigation of Apoptosis-mediated Cytotoxic Effects of Royal Jelly on HL-60 Cells

Hasan ULUSAL[1]* Mehmet Akif BOZDAYI[2] Hatice Kübra YIGIT DUMRUL[3]
Melek Sena TARAKÇIOĞLU[4] Abdullah TAŞKIN[5]

[1]*Department of Medical Biochemistry, Faculty of Medicine, Gaziantep Islam Science and Technology University, Gaziantep, Türkiye*
[2]*Department of Medical Biochemistry, Mehmet Akif Inan Education and Research Hospital, Sanliurfa, Türkiye*
[3]*Department of Physiology, Faculty of Medicine, Gaziantep University, Gaziantep, Türkiye*
[4]*Department of Medical Biochemistry, Faculty of Medicine, Gaziantep University, Gaziantep, Türkiye*
[5]*Department of Nutrition and Dietetics, Faculty of Health Sciences, Harran University, Sanliurfa, Türkiye*

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*: https://orcid.org/0000-0003-3890-2088
: https://orcid.org/0000-0003-0459-0629
: https://orcid.org/0000-0002-6780-229X
: https://orcid.org/0000-0001-5649-3965
: https://orcid.org/0000-0001-8642-1567

***Corresponding author's:**
Hasan ULUSAL
Department of Medical Biochemistry, Faculty of Medicine, Gaziantep Islam Science and Technology University, Gaziantep, Türkiye
✉: hasan_ulusal@hotmail.com

Abstract: In recent years, the use of nontoxic natural products that can be effective on cancer cells as new agents has attracted the attention of scientists in order to reduce the negative side effects of existing cancer drugs and their toxicity to normal cells. Some in vivo and in vitro studies have shown that royal jelly (RJ) inhibits cell proliferation and induces apoptosis. In this research, we aimed to investigate the effects of RJ on proliferative and apoptotic processes in the human acute promyelocytic leukemia cell line (HL-60). The HL60 cell line was treated with different concentrations of RJ for 24, 48, and 72 hours. The half maximum inhibitory concentration (IC_{50}) of RJ was determined using 3-(4.5 dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide test (MTT) and the proliferation activity of HL-60 cells was evaluated. Flow cytometry analysis was performed to measure apoptosis in HL-60 cells. IC_{50} values for RJ were calculated as 13.98, 6.45, and 2.06 mg/mL for 24, 48, and 72 hours, respectively. Flow cytometry results also showed that RJ had apoptotic effects at the concentrations found. The results showed that RJ treatment significantly induced apoptosis and reduced the proliferation of HL-60 cells. This study shows that RJ can be a complementary treatment against HL-60 acute myeloid leukemia cells due to its anticancer and antiproliferative effects.

Keywords: Anti-cancer, apoptosis, hl-60, mtt, royal jelly.

Arı Sütünün HL-60 Hücreleri Üzerindeki Apoptoz Aracılı Sitotoksik Etkilerinin Araştırılması

***Sorumlu yazar:**
Hasan ULUSAL
Gaziantep İslam Bilim ve Teknoloji Üniversitesi, Tıp Fakültesi, Tıbbi Biyokimya Anabilim Dalı, Gaziantep, Türkiye
✉: hasan_ulusal@hotmail.com

Öz: Son yıllarda mevcut kanser ilaçlarının olumsuz yan etkilerini ve normal hücrelere olan toksisiteyi zararlarını azaltmak için kanser hücrelerinde etkili olabilecek nontoksik doğal ürünlerin yeni ajan olarak kullanılması bilim insanlarının dikkatini çekmektedir. Bazı in vitro ve in vivo çalışmalar, arı sütünün hücre proliferasyonunu inhibe ettiğini, apoptozu indüklediğini göstermiştir. Bu çalışmada, arı sütünün insan akut miyeloid lösemi hücre hattında proliferatif ve apoptotik süreçlerdeki etkilerini araştırmayı amaçladık. HL60 hücre hattı, 24, 48 ve 72 saat boyunca farklı konsantrasyonlarda arı sütü ile muamele edildi. 3-(4,5 dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür testi (MTT) kullanarak arı sütünün yarı maksimum inhibitör konsantrasyonu (IC_{50}) belirlendi ve HL-60 hücrelerinin proliferasyon aktivitesi değerlendirildi. HL-60 hücrelerindeki apoptozu ölçmek için flow sitometresi analizi gerçekleştirildi. Yapılan hesaplamalarda arı sütü için IC_{50} değerleri 24, 48 ve 72 saat için sırasıyla 13,98, 6,45 ve 2,06 mg/mL bulundu. Flow sitometri sonuçlarıde arı sütünün bulunan konsantrasyonlarda apoptotik etkilere sahip olduğunu gösterdi. Sonuçlar, arı sütü tedavisinin HL-60 hücrelerinin proliferasyonunu önemli ölçüde azalttığını ve apoptozunu indüklediğini gösterdi. Bu çalışma, arı sütünün antikanser ve antiproliferatif etkilerinden dolayı HL-60 akut miyeloid lösemi hücrelerine karşı tedavide tamamlayıcı olarak kullanılabileceğini göstermektedir.

Anahtar kelimeler: Arı sütü, hl-60, anti-kanser, apoptoz, mtt.

INTRODUCTION

Cancer has been defined as one of the most important health problems in the world for nearly a century. A cancerous tumor refers to a colony of cells that proliferate more frequently and uncontrollably than normal tissue and have invasive and metastatic properties. The World Health Organization estimated that total cancer-related deaths in 2018 were 9.6 million, and new cases were approximately 18.1 million (Upreti et al., 2022). According to the World Health Organization's 2050 report, 27 million cancer cases and 17.1 million deaths are expected annually (Kumar & Jaitak, 2019). The most obvious characteristics of cancer cells are their resistance to cell death signals and their immortality. Programmed cell death consists of three main categories: necrosis programmed cell death, autophagic cell death and apoptosis. These three death pathways together can determine the future of cancer cells, but due to their separate importance, studies on cancer treatment focus on the triggering pathways of apoptosis (Upreti et al., 2022; Xiang et al., 2019). In adults, prostate cancer, ovarian, cervix, breast, lung, liver, colorectal, stomach, and leukemia are the most common neoplasms; leukemia has the highest incidence among children and adolescents (Michlewska et al., 2019). Leukemia is a type of cancer that results from the uncontrolled growth of immature blood cells derived from abnormal hematopoietic stem cells in the bone marrow and accounts for about 3% of all new cancer cases. Currently available cancer treatments include chemotherapy, surgery, radiotherapy, immunotherapy, chemical-based drugs, etc. (Upreti et al., 2022; Xiang et al., 2019). Chemotherapy is considered to be the main treatment strategy method for leukemia patients, but these treatments show high toxicity and relatively low efficacy in patients and can also put patients under excessive physical and mental stress (Hsiao et al., 2021; Ji et al., 2014). In addition, it has been observed that even with the best available treatment method used in acute myeloid leukemia, the 5-year survival rate of patients is low, and the response to existing chemotherapeutics has been found to be reduced (Salama et al., 2022; Suleiman et al., 2020). Therefore, the search for alternative treatments in this field is of primary importance. Although many studies have been conducted in recent years for alternative treatments, the use of agents obtained from natural sources has attracted interest in cancer treatment due to their low toxicity to healthy cells and tissues, their effectiveness against cancer cells and their low cost (Suleiman et al., 2020; Upreti et al., 2022; Xiang et al., 2019). In general, natural products are advantageous because they are easy to obtain and relatively safe.

In recent years, the great increase in interest in the food industry and natural products has revealed the importance of royal jelly (RJ) and its unique pharmacological and therapeutic properties (Miyata & Sakai, 2018). RJ is a white and viscous substance excreted from the mandibular and hypopharyngeal glands of the worker honeybee Apis mellifera. It is known as a “super food” that only the queen bee consumes throughout her life. RJ is also a special nutrient source for 2-3 days for honeybee larvae. This super food is considered to be the main reason for the longer lifespan and fertility of the queen bee compared to other bees (Pasupuleti et al., 2017). RJ is considered a functional food because it has a number of pharmacological activities (hypotensive, vasodilatory, anti-hypercholesterolemic, antimicrobial, antitumoral, anti-inflammatory, and antioxidant). The various pharmacological properties of RJ are due to its unique and rich composition consisting of polyphenols, flavonoids, minerals, lipids, vitamins, carbohydrates, proteins, as well as various biologically active substances (Bagameri et al., 2023; Kunugi & Mohammed Ali, 2019; Viuda-Martos et al., 2008). In vivo and in vitro studies have shown that RJ inhibits cell proliferation and induces apoptosis (Albalawi et al., 2022; Bagameri et al., 2023; Jovanović et al., 2022; Kul Köprülü et al., 2022; Salama et al., 2022).

Our aim in this study was to examine the apoptotic and cytotoxic effects of RJ, which has a rich content, on HL-60 cells, an acute promyelocytic cell line. For this purpose, we applied RJ to HL-60 cancer cells and examined its cytotoxic effects with MTT test and calculated IC_{50} value. In addition, Annexin V test was applied with flow cytometry method to examine the apoptotic effects of RJ on HL-60 cells. With these analyses, we aimed to reveal the effect of RJ on proliferative and apoptotic processes.

MATERIAL AND METHOD

Reagent and Chemicals: Dimethyl sulfoxide (DMSO) (Molecular biology grade) was purchased from Sigma Aldrich. RPMI-1640 medium (with L-glutamine), phosphate buffer saline (PBS) (Without Calcium, magnesium), Penicillin-Streptomycin 100X solution, and fetal bovine serum (FBS) (US Origin) were purchased from HyClone. 3-(4,5 dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromür (MTT) (Ultrapure) was purchased from VWR. FITC Annexin V Apoptosis Detection Kit with PI was purchased from Biolegend.

Cell line and cell culture: Human acute myeloid leukemia (HL-60) cell line was obtained from ATTC (Manassas, VA, USA). HL-60 cells were thawed in a water bath set at 37°C and PBS was added and centrifuged at 1200xg for 5 min to remove DMSO. Then, the cells were washed. HL-60 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, %1 penicillin/streptomycin at 95% humidity, 37°C, and 5% CO_2 in an incubator. When the cells covered approximately 80% of the flask, the cells were taken out with a pipette and centrifuged at 1200xg for 6 min to remove the medium. The

pellet was diluted with 6 mL of medium and viable cell counts were performed using trypan blue on a Neubauer slide under an inverted microscope. The counted cells were used for proliferation analysis and apoptosis measurement.

Cell proliferation analysis: This assay is a colorimetric assay that measures the reduction of MTT reagent by mitochondrial succinate dehydrogenase. The anti-proliferative activity of RJ at different concentrations (100 mg/mL, 10 mg/mL, 1 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 0.001 mg/mL) on HL-60 cells was evaluated using the MTT method. HL-60 cells were seeded in 24-well plates at approximately $1x10^5$ cells/well. RJ prepared at different concentrations was added to the cells in 3 replicates and incubations were applied for 24, 48, 72 hours. At the end of each incubation, 100 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37 °C, and then the medium was removed and 1000 µL of dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals on an orbital shaker. The absorbance was measured at 570 nm in a plate microplate reader (H1 Synergy, Biotek, USA). All analyses were performed in triplicate for each RJ dose. Cell viability was calculated as the ratio of the absorbance of treated cells divided by the absorbance of untreated control cells (the same amount of solvent was applied to the cells), and IC_{50} values for each incubation time were calculated with the help of a program (https://www.aatbio.com/tools/ic50-calculator).

A*poptosis measurement by flow cytometry:* Royal jelly-induced apoptosis in the HL-60 cell line was measured by flow cytometry using Annexin V-FITC. HL-60 cell suspension was seeded into 24-well cell plates as 1 ml at $1x10^5$ cells/well. Based on the MTT results, the apoptotic effects of the concentrations of RJ corresponding to the IC_{50} value in the 24, 48 and 72 h assays were evaluated in the flow cytometry device. The plates containing the HL-60 cell suspension were taken from the incubator and the cells were aspirated from the wells. Then, the cells were transferred to a centrifuge tube and centrifuged at 1200xg for 5 minutes. After centrifugation, the supernatant was discarded, 3 mL of cold PBS was added to the pellet and centrifuged again. This process was repeated 2 times. After centrifugation, the pellet was homogenized in 200 µL of PBS. 100 µL of this homogenate was taken and transferred to a flow tube. 5 µL of Annexin and 5 µL of propidium iodide (PI) included in the kit were added to each flow tube containing cells and vortexed for 1-2 seconds. This suspension was incubated for 15 minutes in the dark at room temperature and then 350 µL of binding buffer was added. The flow tube was gently shaken to obtain a homogeneous mixture and cell samples were analyzed on a flow cytometer (BD FacsLyric, USA).

Statistical Analysis: SPSS 25.0 program was used for statistical analysis of the data. Shapiro-Wilk test was used to evaluate whether the means of the variables showed normal distribution. One-Way ANOVA test was used to compare the means of variables that showed normal distribution between more than two groups. Bonferroni post-hoc test was used to compare the groups with each other, and Dunnet post-hoc test was used to compare the control group with other groups. T-Test was used for parametric evaluation of 2 independent groups for flow cytometry data. $p<0.05$ was considered statistically significant.

RESULTS

According to MTT analyses, IC_{50} values of RJ concentrations at 24, 48 and 72 hours were calculated as 13.98, 6.45 and 2.06 mg/mL, respectively, indicating that RJ inhibited the proliferation of HL-60 cells in a dose and time dependent manner.

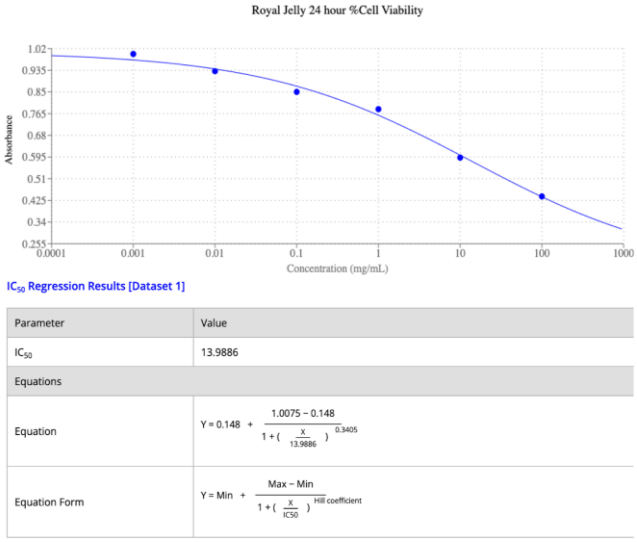

Figure 1. Calculation of IC_{50} value for RJ after 24 hours of application.

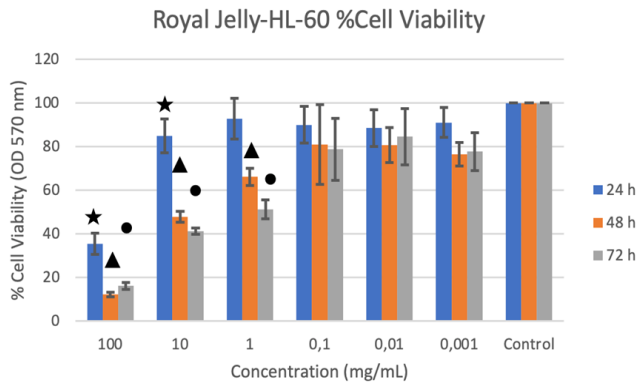

Figure 2. Effects of different concentrations of RJ on HL-60 cell line. Experimental data are represented as mean ±SEM (replicate treatments:4). $p<0.05$ was considered statistically significant. ★; significant differences between control and other groups for 24 h, ▲; significant differences between control and other groups for 48 h and ●; significant differences between control and other groups for 72 h.

Dose-dependent viability graph of RJ application for different incubations is given in Figure 2. In the comparison of RJ at different doses with the control group, it was found that there was a significant difference after 10

mg/mL for 24 hours, and there were significant differences after 1 mg/mL in 48 and 72 hour applications.

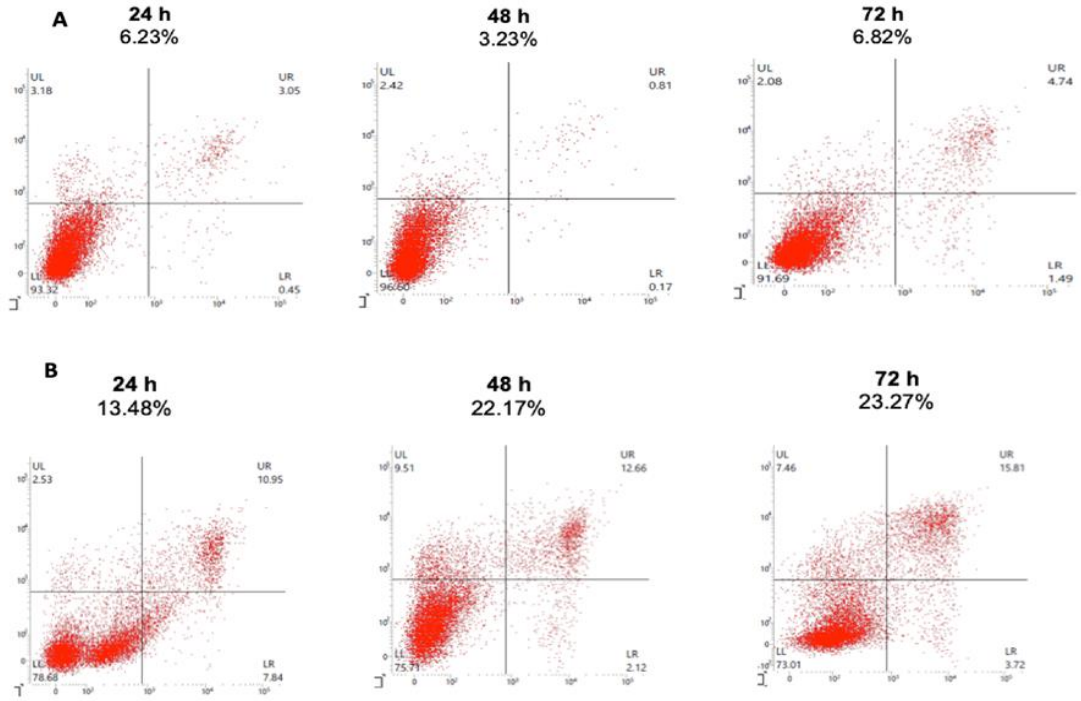

Figure 3. Apoptotic cell percentages in HL-60 cells determined by flow cytometry at 24, 48, 72 hours, (A) Apoptotic cell rate in cells containing medium without RJ (B) Apoptotic cell rate in cells applied with RJ.

At the end of 24, 48 and 72 hours of incubation; According to the Annexin V-FITC test results, the percentage of apoptotic cells in HL-60 cells with RJ was found to be 13.48%, 22.17% and 23.27%, respectively. In the control group without RJ, the apoptotic cell rate was 6.23%, 3.23% and 6.82%, respectively (Figure 3). When flow cytometry results were examined in 24, 48 and 72 hour incubations, a statistically significant difference was found between the royal jelly and control group ($p<0,05$) (Figure 4).The results showed that RJ treatment supported HL-60 cell apoptosis and encouraged HL-60 cells to the apoptotic pathway.

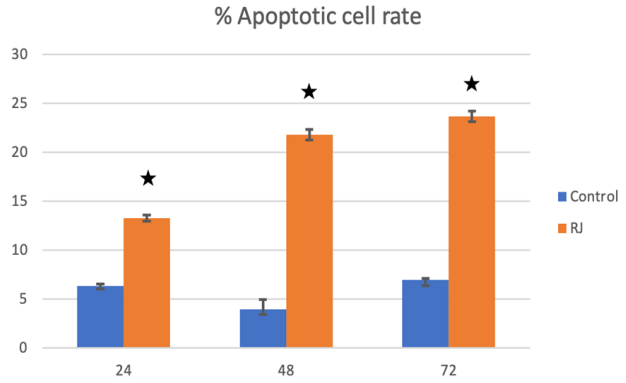

Figure 4. Flow cytometry results for different time. Experimental data are represented as mean ±SEM (replicate treatments:2). $p<0.05$ was considered statistically significant. ★; significant differences between control and RJ.

DISCUSSION AND CONCLUSION

Cancer, which has more than 30 different types, has recently been recognized as the leading cause of death worldwide (Albalawi et al., 2021). Today immunotherapy, targeted therapy, radiotherapy, and chemotherapy are the most common anticancer therapies used to treat patients with intermediate and advanced malignancies by prolonging survival and improving the quality of life to some extent. However, these treatments have been found to have many limitations and disadvantages. While the toxicities of the agents used in treatment significantly affect the quality of life of patients, some patients may discontinue treatment because they cannot tolerate these toxicities (Zhang et al., 2021). Therefore, the search for new natural methods with high efficacy and low toxicity in cancer treatment has gained more importance in recent years (Hegde et al., 2023; Moskwa et al., 2023; Sánchez-Martín et al., 2023). Natural products have long been one of the most valuable sources of medicines due to their high availability, broad efficacy, and low toxicity (Albalawi et al., 2021; Gullett et al., 2010; Sznarkowska et al., 2017). Products with these properties include bee products such as bee venom, pollen, RJ, bee bread, propolis, and honey. Bee products have been used in traditional medicine for the treatment of various diseases and injuries for thousands of years since ancient times. Several studies have confirmed

that bee products contain many active ingredients in their chemical composition and exhibit a wide range of biological activities, such as anticancer, antimutagenic, antioxidant, anti-inflammatory, antiviral, and antibacterial (Moskwa et al., 2023). In this research, we aimed to investigate the effects of RJ on proliferative and apoptotic processes in the HL-60 cell line.

Our experimental results showed that the IC_{50} values of RJ concentrations at 24, 48, and 72 h were 13.98, 6.45, and 2.06 mg/mL, respectively, according to MTT assays. These results showed that RJ inhibited the growth of HL-60 cells and the results were dose- and time-dependent.

Cell death, and more specifically apoptosis, is the priority of many anti-cancer therapies, from traditional chemotherapy and radiotherapy to newly developed targeted therapy and immunotherapy (Berthenet et al., 2020). The role of apoptosis in cancer has attracted great attention, with resistance to apoptosis being widely accepted as an acquired feature of cancer cells, providing them with survival advantages that promote tumor evolution and growth, as well as treatment failure. As a result, the effectiveness of cancer therapies is tightly dependent not only on the cellular damage they cause, but also on the ability to activate apoptosis in cells (Morana et al., 2022).

In our study, in order to detect cells undergoing apoptosis, Annexin V-FITC test analysis was performed with a single RJ concentration determined as a result of MTT analysis. At the end of 24, 48 and 72 hours of incubation; According to the Annexin V-FITC test results, the percentages of apoptotic cells in RJ HL-60 cells were found to be 13.48%, 22.17% and 23.27%, respectively. In the control group to which RJ was not added, the apoptotic cell rate was 6.23%, 3.23% and 6.82%, respectively. The fact that the apoptosis rate in control cells not treated with RJ was found to be lower than in cells treated with RJ clearly shows that RJ leads cells to apoptosis in the HL-60 cell line.

The data we obtained from cytotoxicity and apoptosis tests show that RJ is a natural substance that can affect cancer cells. When the studies in the literature are examined; a study in MCF-7 breast cancer cells found that RJ inhibited estradiol-induced cell proliferation (Nakaya et al., 2007). In WiDr colon cancer cells, treatment with RJ was observed to inhibit WiDr cells in a dose-dependent manner (Yang et al., 2018). In addition, RJ has been reported to have anti-proliferative activity in SH-SY5Y human neuroblastoma cells and in a mouse breast cancer model (Gismondi et al., 2017; Zhang et al., 2017). In metastatic renal cancer cells, RJ has also been shown to enhance the anti-cancer effects of molecularly targeted agents, while in human hepatocellular carcinoma cells (HepG2), RJ treatment has been shown to induce apoptosis (Miyata et al., 2020; Saad Al Shehri et al., 2023).

Previous studies from Turkey have explored the therapeutic potential of RJ on various cancer and neuroblastoma cell lines. For instance, its antiproliferative effects on HT-29 colon cancer cells have been demonstrated, highlighting its potential to inhibit cell growth and induce apoptosis. These findings suggest that RJ may exert significant cytotoxic effects on tumor cells by targeting key signaling pathways involved in cell survival and proliferation. RJ affected SH-SY5Y neuroblastoma cells in another study, indicating its anti-cancer properties in neuronal cell models. These findings contribute to a growing body of evidence that RJ could have diverse biological effects across different cell types, further supporting its potential role in cancer therapy (Ayna & Darendelioglu, 2022; Ayna et al., 2021; Caliskan et al., 2021).

As a result, cancer is one of the biggest health problems of our day. In addition to developing new drugs for the treatment of cancer, the use of natural products is among the frequently used methods. The RJ we used in this study stands out due to its rich nutritional content. Our findings have shown that RJ has an anti-proliferative effect on the HL-60 cell line and increases cell death by promoting apoptosis. These results provide clues that RJ can be used as a complementary supplement in cancer treatments. The effects of RJ on cancers will be more clearly revealed by investigating the combinations of RJ with existing chemotherapeutics and the effectiveness of RJ in cancer cell models.

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