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Anticancer, and antioxidant activities of royal jelly on HT-29 colon cancer cells and melissopalynological analysis

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Abstract: Royal jelly (RJ) is reflecting the flora around the apiaries as a result of the foraging activity of honeybees and therefore, pollen analysis could be used to verify its botanical/geographical origin. Colon cancer treatment by means of chemotherapeutics agents reflects a substantial problem for the organism due to high toxicity to other viable cells of the body. To explore a chemopreventive approach to enhancing colon cancer treatment efficacy, the antioxidant and antiproliferative effects of RJ in HT-29 colon cancer cells were assessed. Cell proliferation was performed by WST-1 assay, index of apoptosis was calculated by TUNEL Assay, antioxidant activities were examined through analysis of reactive oxygen species (ROS), lipid peroxidation (LPO), total antioxidant status (TAS) levels and superoxide dismutase (SOD) and catalase activities. Expression of caspase-3 protein levels were analysed via immunohistochemical staining, mRNA and protein levels of apoptotic and antiapoptotic genes were studied by qRT-PCR and western blotting experiments, respectively. RJ treatment led to increase in ROS and LPO levels, decrease in SOD and catalase activity, TAS level, increase in apoptotic index and caspase-3 activation, upregulation of levels of apoptotic genes and proteins of intrinsic and extrinsic pathway (Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, -10) and downregulation of antiapoptotic Bcl-2 both at transcriptional and translational levels. Although there are several studies on the physicochemical properties and composition of royal jelly, melissopalynological studies are limited. In this study, we also investigated the pollen content of RJ and the use of pollen analysis as a geographical origin determination.

Keywords: Colon cancer, Turkish royal jelly, caspase-3, antioxidant, pollen analysis

1. Introduction

Royal jelly (RJ) is a yellowish milky creamy bee product that is secreted from the mandibular and hypopharyngeal glands of the worker honeybee and is the special nutrient of the larva of the queen honeybee (*Apis mellifera*) (Pavel et al., 2011). Proteins represent the major content of RJ (27%–41%). Among these, over 80% are soluble proteins (MRJPs). These proteins are assumed to be the main biomolecules responsible for the specific physiological roles of RJ in the development of queen honeybee. RJ proteins have been shown to induce cell proliferation in previous studies (Filipič et al., 2015; Ramanathan et al., 2018: Dobritzsch et al., 2019). Trans-10-hydroxy-2-decenoicacid (10-H2DA), 10-hydroxydecanoic acid (10-HDAA), and sebacic acid (SEA) are abundantly found fatty acids in the content of RJ. They, together with proteins, contribute to several types of biological activities of RJ that include anticancer, antimicrobial, immunomodulatory, antioxidative, and antihypertensive effects (Coutinho et al., 2018; Park et al., 2019; Ahmad et al., 2020; Park et al., 2020; Guo et al., 2021). RJ also comprises amino acids, organic acids (Lercker et al., 1981), vitamins, minerals, flavonoids, phenolic acids and carotenoids (Viuda-Martos et al., 2008; Bengü et al., 2020). Among several types of phytochemicals existing in RJ, the phenolic and flavonoids such as acacetin, apigenin, chrysin, galangin, kaempferol, pinocembrin, hesperidin and quercetin contribute to its antioxidant activity (Viuda-Martos et al., 2008, Pasupuleti et al., 2017, Kocot et al., 2018). Flavonoids also possess anticancer activities (Liu et al., 2010; Zhang et al., 2018; Hazafa et al., 2019) owing to their high antioxidant capacity along with their capability to affect several

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signalling pathways some of which include, inhibition of cell proliferation, induction of apoptosis, and cell cycle arrest as reviewed in (Premratanachai and Chanchao, 2014; Pasupuleti et al., 2017).

Colon cancer is reported as the third most common kind of cancer after lung and breast cancer and the fourth leading reason of cancer-related death around the world (Rejhova et al., 2018). Colon cancer treatment by means of chemotherapeutics agents reflects a substantial problem for the organism due to high toxicity to other viable cells of the body. Lately, there are several research motivating the use of bee products for the prevention and treatment of cancer disease (Badolato et al., 2017; Farooqi et al. 2019). However, antioxidant and anticancer effects of RJ on HT-29 colon cancer cells have not been studied in detail at a cellular level previously. Therefore, the aim of this study was to investigate antioxidant and anticancer effects of RJ and the mechanisms underlying these effects on HT-29 colon cancer cells.

The royal jelly is a secretion of hypopharyngeal and mandibular glands of honeybees and not a directly plant-originated product, but it contains pollen grains as a result of partial digestion of pollen and honey (Witherell, 1978). The honey bees consume pollen stored in the honeycombs (bee bread) to produce royal jelly (Anderson et al., 2014). So, the pollen grains are transferred to the royal jelly from the honey stomach and also it could be contamination from body parts of the bees (Piana et al., 2006; Dimou et. al., 2007). Royal jelly is reflecting the flora around the apiaries as a result of the foraging activity of honeybees (Piana et al., 2006) and therefore, pollen analysis could be used to verify its botanical/geographical origin.

Although there are several studies on the physicochemical properties and composition of royal jelly, melissopalynological studies are limited (d'Albore and Bernardini, 1978; Nagai et al., 2001; Barth, 2005; Piana et al., 2006; Dimou et. al., 2007; Dimou et. al., 2013). In the present study, we also examined the pollen spectrum of Bingöl royal jelly and investigated the possibility of determining the geographical origin of royal jelly.

2. Materials and methods

2.1. Materials

Colon cancer cells (HT-29; ATCC® HTB-38™) were generously received from Uludağ University. Human umbilical vein endothelial cells (HUVECs; ATCC® CRL-1730™) were provided from Plant, Drug, and Scientific Research Center (Eskişehir, Turkey). Royal Jelly was purchased from local beekeepers registered with Bingöl beekeepers association. RJ was produced during spring 2019 by local beekeepers in Bingöl and stored at -20 °C. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) and malondialdehyde bis were acquired from Merck (Germany). Dulbecco's Modified Eagle Medium and Fetal Bovine Serum were bought from GIBCO (Gibco, USA).

2.2. Cell culture

HT-29 and HUVECs were grown at 37 °C in a moistened 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM) with the addition of 10% FBS, 1% penicillin-streptomycin in 25 cm^2 cell culture flasks for 24 h. Following day, the cells were treated with different concentrations of RJ between 0.15 and 10 mg/mL for 24 h. The RJ was dissolved in DMEM. The control cells were grown in the same medium without addition of RJ.

2.3. Cell cytotoxicity assay

Cytotoxicities of Turkish RJ were analysed by Water Soluble Tetrazolium-1 (WST-1) cell viability assay kit as explained in our previous work (Ayna, 2020). HT-29 cells were treated as explained in cell culture. Subsequent to that, 3 µL of WST-1 compound was added to each well. Afterward, the colon cancer and HUVEC cell suspensions were incubated for 2 h and the absorbance was measured at 450 nm wavelength (reference: 630 nm).

2.4. Detection of intracellular ROS level

Cellular ROS creation was evaluated by use of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay kit purchased from Abcam, MA, USA, as previously defined (Aykutoglu et al., 2020). The cells were treated as given in cell culture. Following treatment and growing the cells, 1×10^6 HT-29 cells were collected and incubated for 1 h at 37 °C in the presence of 2 µM DCFH-DA and fluorescence was measured.

2.5. Examination of LPO level

In order to evaluate malondialdehyde (MDA) level in RJ exposed HT-29 cells, a minor adjustment of the previously published protocol was utilised (Özbolat and Ayna, 2021). HT-29 were grown as described in cell culture. HT-29 were collected and centrifugation was performed for 5 min at 30,000 rpm. Later, the cell suspensions were treated with LPO assay mixture containing 70% w/v trichloroacetic acid and 1 mL of 0.8% w/v thiobarbituric acid and was incubated for 0.5 h at 95 °C. Subsequent to that, the solution was cooled on ice bath for five min and then, the cell mixtures were centrifuged at 15,000 rpm for 10 min. The absorbance measurement of the supernatant was taken at 532 nm.

2.6. Measurement of CAT and SOD activity and total antioxidant status (TAS)

The cells were treated as stated in cell culture. The assays were performed following the manufacturer's instructions provided with the catalase assay kit (Elabscience), human TAS and SOD assay ELISA kit (SunRed).

2.7. TUNEL assay

The assay was performed following the procedure supplied with the ApopTag kit (Milipore). The HT-29 were seeded

in a 6-well plate covering approximately 5 × 10^5 cells/well. The cells were seeded as described in cell culture. Later, the HT-29 were observed under an inverted light microscope. At least three pictures were obtained for each treated group and Apoptotic Index (AI) was calculated as described previously (Darendelioglu, 2020).

2.8. Caspase-3 staining

Immunohistochemical staining experiments were carried out by use of UltraVision LP Large Volume Detection System (Santa Cruz Biotechnology). HT-29 were grown as given in experimental groups. 1% paraformaldehyde was used to fix the cells, washed with phosphate-buffered saline (PBS) and permeabilized with ethanol:acetic acid (2:1 v/v). After that, the HT-29 were stained as explained in the procedure supplied by the producer and they were imaged with an inverted light microscope.

2.9. QRT-PCR analysis

The mRNA expression level of the apoptotic *Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, -10* genes and antiapoptotic *Bcl-2* genes were studied by quantitative real-time expression assay kit (Jena Bioscience) in Rotor-Gene Q (Qiagen). mRNA was isolated by Jena Bioscience Isolation Kit following the procedures provided by the supplier. cDNA synthesised was performed according to kit protocols (Jena Bioscience). The expression levels of proapoptotic, apoptotic and antiapoptotic genes and *β-actin* as a housekeeping gene were analysed as given previously (Ayna, 2021). mRNA levels of apoptosis connected genes were normalised using *β-actin* .

2.10. Western blot analysis

The effects of RJ treatments on protein expressions of β-Actin, Bax, cyt-c, p53, cas-3, -6, -8, -9, Bcl-2 and NFκB in HT-29 cells were studied by Western blotting analysis. Protein concentrations were determined by Coomassie Blue G250. The same amounts of protein samples were prepared in Laemmli buffer and run onto 15% SDS-PAGE. Afterward, the proteins were transferred to nitrocellulose membranes. The membranes were rinsed twice in TBS-0.05% Tween-20 (TBS-T) for 5 min and blocked for an hour prior to using the primary antibody in 5% Bovine Serum Albumin. β-Actin, Bax, cyt-c, p53, cas-3, -6, -8, -9, Bcl-2, and NFκB were used as primary antibodies. The membranes were incubated in the existence of these antibodies at 4 °C during the overnight. The blots were then washed in TBS-T for 5 min and incubated in the presence of antimouse secondary antibody, at 37 °C for 90 min. The density of protein bands quantified in X-ray films purchased from ECL (Advansta, CA) was settled to detect specific binding and was densitometrically examined with a photo analysis system (GelDoc, Bio-Rad, USA).

2.11. Pollen analysis

A simplified version of the d'Albore and Bernardini's (1978) methodology by Dimou et al. (2007) was carried out on the melissopalynological analysis of royal jelly. Royal jelly (0.5 g) dissolved in 10 mL KOH 2.5% (w/w) and the sample was centrifuged 10 min at 3000 rpm. The supernatant was decanted, the precipitate was resuspended in 10 mL distilled water and was centrifuged again. Then, the precipitate was smeared with 1 mm^3 basic fuchsin glycerine gelatine and placed on a slide. Glycerine gelatine was liquefied and mixed on a heating plate and a coverslip was covered on it. The slide was left upside down and became ready for microscopic examination after six h. At least 300 pollen grains were identified by using light microscopy (×40 and ×100 magnification) and relative frequency as a percentage of the total number of pollen was calculated for each pollen taxa. Dimou's frequency classes were used to classify pollen taxa: "I": found in (<1%), "II": very few (1%-3%), "III": few (3%-15%), "IV": frequent (>15%) (Dimou et al., 2007). In order to identify the pollen content of the royal jelly sample Bingöl University reference pollen collection created by the project "2017K124000-BÜBAP-PİKOM-Bitki.2018.002" and literature data were used (Sorkun, 2008; Polat et al., 2020; Polat et al. 2021).

2.12. Statistics

All experiments were repeated at least three times. Statistical analysis and comparable data groups were assessed using GraphPad Prism 5 by one-way ANOVA Newman-Keuls post-hoc Test; $p < 0.05$ was considered as significant.

3. Results

3.1. Cell proliferation assay

Growth inhibition of human colon cancer cells by different concentrations of RJ was examined on human colon cancer HT-29. The viability of the colon cancer cells was significantly and concentration-dependently reduced by RJ (Figure 1). We also investigated the effect of RJ on normal HUVECs viability, and the results showed that there was no effect on the proliferation of the cells under the concentrations tested (data not shown).

3.2. Detection of intracellular ROS and antioxidant parameters

The effects of RJ treatment on ROS generation in HT-29 cells were evaluated. The results revealed that RJ at a concentration of 0.3 mg/mL significantly increased ROS level in HT-29 cells while 0.5 mg/mL of RJ had a negligible effect on ROS levels (Figure 2A). To assess whether RJ contributed to the apoptosis of HT-29, an LPO assay was performed at 0.3 and 0.5 mg/mL of RJ to detect MDA levels. As shown in Figure 2B, the level of MDA in HT-29 cells remarkably increased in the presence of 0.3 mg/mL RJ while 0.5 mg/mL RJ had a negligible effect on MDA levels in HT-29 cells. The antioxidant effects of RJ in colon

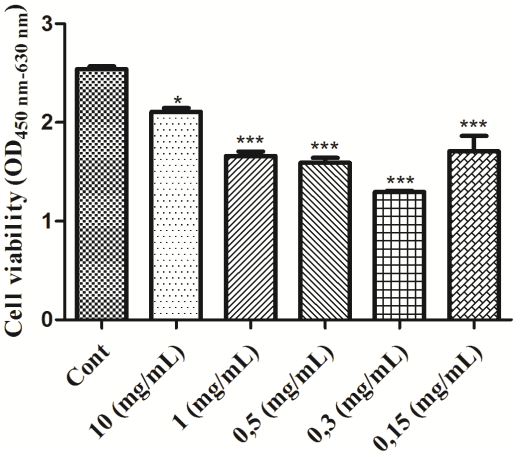

Figure 1. Effects of different concentrations of Turkish RJ on HT-29 cell proliferation. The cell proliferation was assessed by WST-1 assay. Experimental data are presented as mean ±SEM (replicate treatments ≥ 3). ***p < 0.001 points out statistically significant differences between cont and other groups; ns: not significant

cancer cells were also extensively examined within this study. The results showed that RJ treatment (0.3 mg/mL) significantly lowered the activities of SOD (Figure 2C) and CAT (Figure 2D) activity, TAS levels (Figure 2E) indicating that RJ could support health in humans by stimulating endogenous factors and protecting membranes from oxygen radical-mediated damage.

3.3. TUNEL assay and Immunohistochemical staining for caspase-3

The effect of RJ on triggering apoptotic pathway in HT-29 cells was also assessed by TUNEL assay. The results revealed that RJ stimulated apoptosis by resulting in the formation of DNA strand breaks and a rise in AI (Figure 3). To further understand whether RJ-induced cell death was observed as a result of apoptosis, activation of caspase-3, a key executioner of the apoptotic pathway, was explored. Caspase-3 was considerably activated by the exposure to RJ (Figure 4).

3.4. QRT-PCR analysis

Finally, we investigated whether the expression of proapoptotic and antiapoptotic genes in HT-29 cells would be affected by RJ. The expression levels of the apoptotic *Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, -10* genes and antiapoptotic *Bcl-2* gene were measured by quantitative PCR (Figure 5). Treatment with RJ enhanced the levels of *Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, -10* while decreased the level of antiapoptotic *Bcl-2* as compared with control cells (Figure 5).

3.5. Western blot analysis

To further understand bio-molecular mechanisms of the antiapoptotic impacts of RJ on HT-29 colon cancer cells (Figure 6A and B), the protein expressions of Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, and antiapoptotic Bcl-2 were examined. The level of the apoptotic proteins remarkably increased and Bcl-2 level considerably decreased after RJ

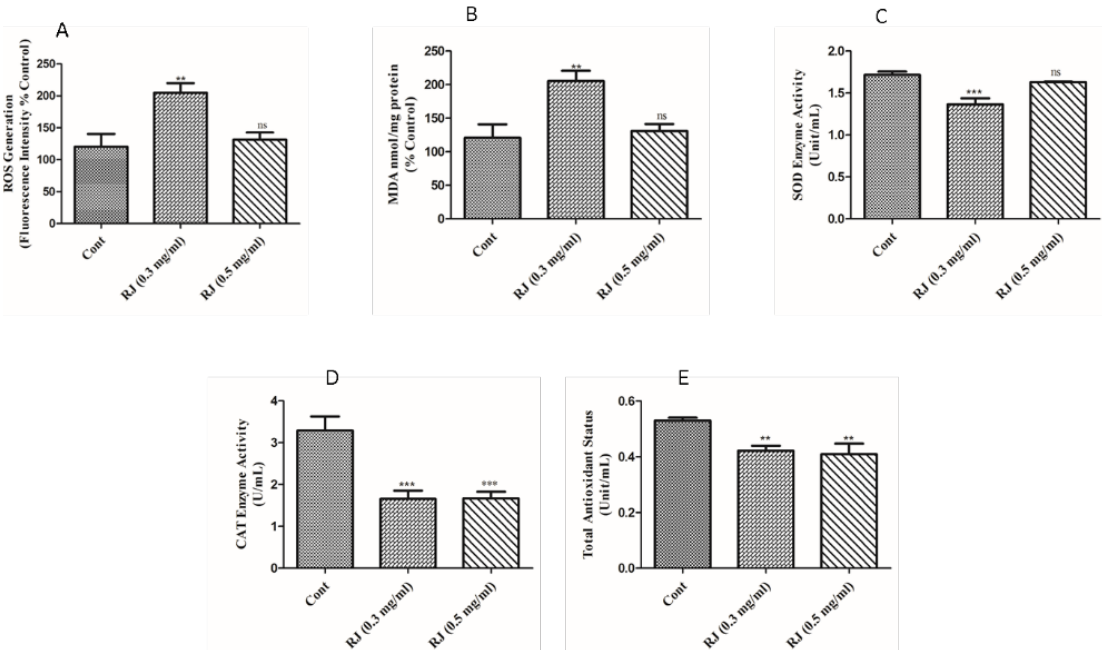

Figure 2 The effect of RJ treatment on ROS generation (A), MDA levels (B), SOD and CAT activities (C, D), and TAS (E) levels in HT-29 cells. Experimental data are given as mean ± SEM (replicate treatments ≥ 3). ***p < 0.001, **p < 0.01, *p < 0.05 point out statistically significant differences between cont and other groups.

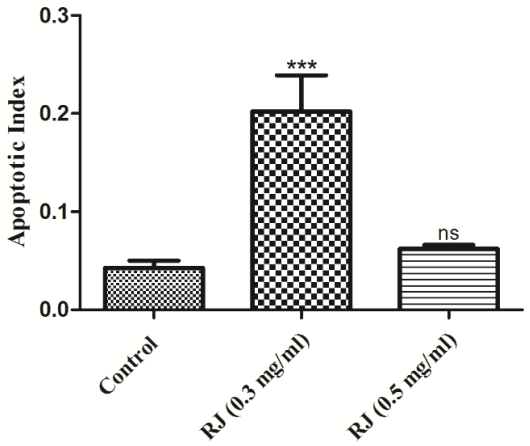

Figure 3 The effect of Turkish RJ treatment as compared to cont on apoptosis in HT-29. Apoptotic index was calculated according to the TUNEL assay instruction. Experimental data are given as mean ± SEM (replicate treatments ≥ 3). ***$p < 0.001$ points out statistically significant differences between control and other groups; ns: not significant.

treatment. These results further suggest that RJ could have an apoptotic role in HT-29 cells.

3.6 Melissopalynological analysis

As a result of melissopalynological analysis, pollen of 38 plant taxa belonging to 18 different families were determined (Table 1). *Paliurus spina-christi, Trifolium* sp., *Centaurea* sp., Rosaceae, Apiaceae, and *Astragalus* sp. were the taxa observed with highest frequency in pollen spectrum of royal jelly.

4. Discussion

Bee products are being intensively used in traditional medicine for ages. The raw constituents, water or ethanol extracts, and isolated biologically active molecules from these products have been shown to have some interesting bio-activities that include antimicrobial (Fontana et al., 2004; Bengü et al., 2020), antiinflammatory (Yang et al., 2018) and anticancer (Miyata and Sakai, 2018) properties. The bee products have also been commonly utilised for treating several immune correlated syndromes in addition to the treatment of some tumours (Al-Kushi et al., 2018). Bee products have been reported to induce *in vitro* cellular apoptosis in numerous cancerous cell lines including prostate, lung, and liver cancers. These biologically active natural products may, hence, verify to be beneficial as part of innovative besieged therapies for different cancer types some of which include breast and colon cancers (Premratanachai and Chanchao, 2014).

Royal jelly (RJ) is a yellowish milky creamy bee product that is secreted from the mandibular and hypopharyngeal glands of the worker honeybee and is the special nutrient of the larva of the queen honeybee (*Apis mellifera*) (Takahama and Shimazu, 2006). It has been reported that RJ has a potential antitumor activity in mice (Kimura, 2008). Studies confirmed anticancer activity of RJ as it induced apoptotic and antiproliferative pathways in tumour cells (Kocot et al., 2018). Colon cancer disease is one of the primary reasons for death on the Earth. In colon or other kinds of cancer, biochemical approaches are considered as a therapeutic strategy. However, antioxidant-rich bee products have also been used as biotherapeutics to decrease reappearance and adverse effects in patients with colon cancer (Turan et al., 2015). A detailed literature search revealed that there was no data regarding the anticancer activity of RJ on HT-29 colon cancer cells. The aim of this study was to analyse the effect of different concentrations of RJ on apoptotic genes in extrinsic and intrinsic pathways of HT-29 colon cancer cells.

The study of antiproliferative and cytotoxic properties of Turkish RJ against HT-29 cells demonstrated that it can prevent the growth of these cells in a concentration-dependent way, with a selectivity towards cancerous

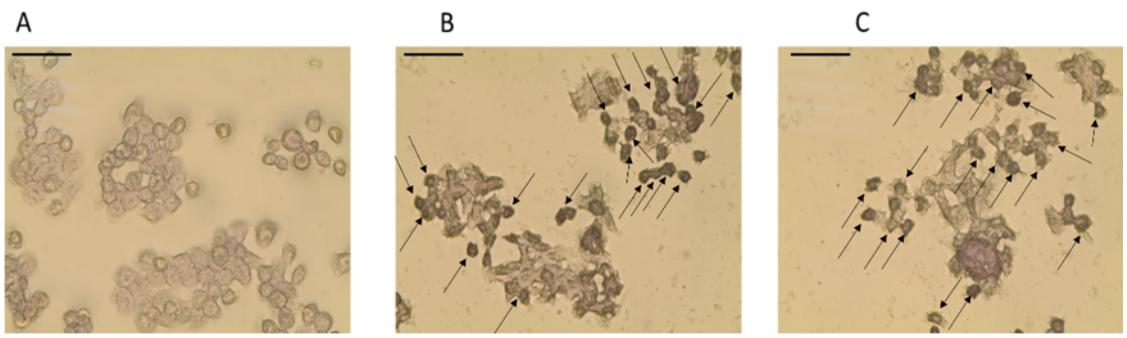

Figure 4 The effect of Turkish RJ treatment as compared to cont on apoptosis related active caspase-3 in HT-29 cells. The cells were photographed under the inverted light microscope. Caspase-3 expressing cells are characterized by their dark brown color. A: Control, B: 0.3 mg/mL RJ, C:0.5 mg/mL RJ. The arrows show the caspase-3 expressing HT-29 cells. Bar scale: 100 μm

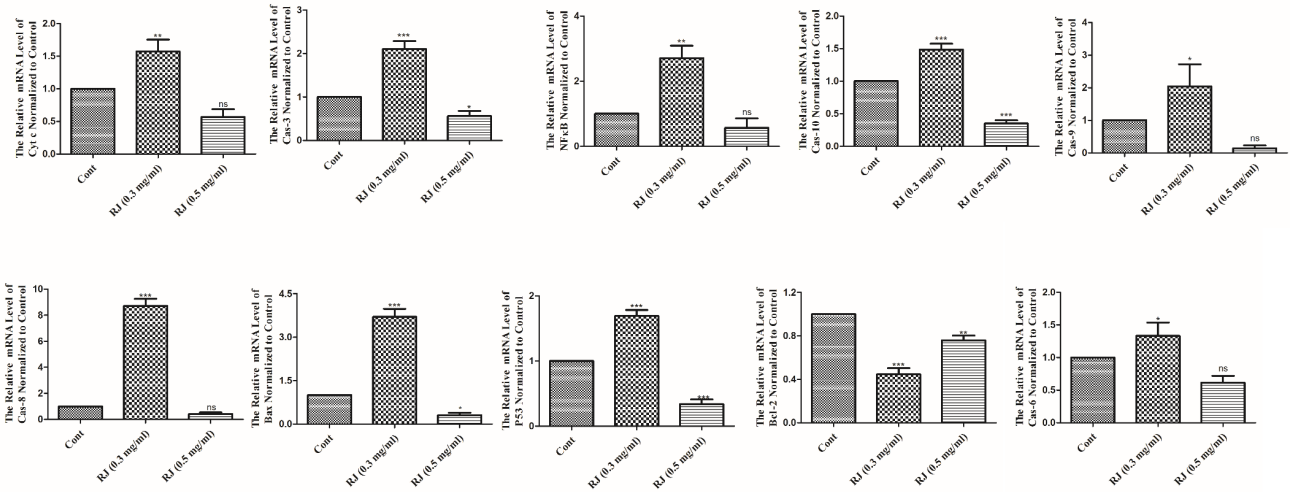

Figure 5 The effect of Turkish RJ on level of apoptosis related gene expressions in HT-29. Cells were treated as stated in experimental groups. mRNA levels of proapoptotic Bax, antiapoptotic Bcl-2 and apoptotic cyt c (C), cas-3, -6, -8, -10, p53 and NFKb were measured by using QRT-PCR analysis. Experimental values are expressed as mean ± SEM (Replicate treatments ≥ 3). ***p < 0.001, **p < 0.01, *p < 0.05 indicate statistically significant differences between cont and other groups; ns: not significant.

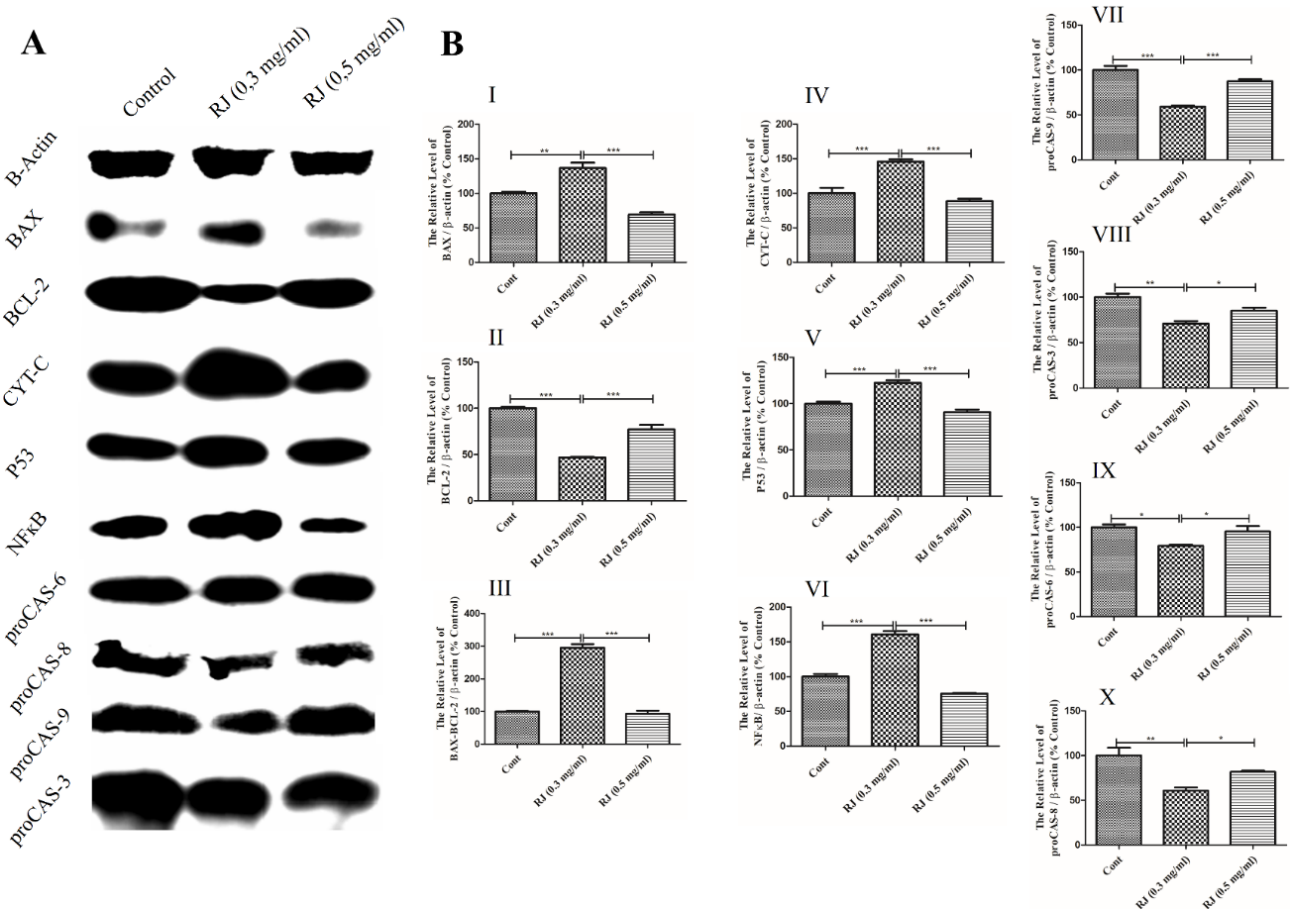

Figure 6 Antiapoptotic properties of Turkish RJ in HT-29 cells. Protein levels of proapototic Bax, antiapoptotic Bcl-2 and apoptotic cyt c (C), cas-3, -6, -8, -10, p53 and NFKb were measured by western blotting experiments. All data were expressed as mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05 indicate statistically significant differences between cont and other groups; ns: not significant.

Table 1. Pollen spectrum and frequency of royal jelly.

Family	Taxa	Pollen frequency (%)	Dimou's frequency class
Adoxaceae	*Sambucus* sp.	2.67	II
	Viburnum sp.	1.00	II
Amaranthaceae	Amaranthaceae	0.33	I
Apiaceae	Apiaceae	5.67	III
	Eryngium sp.	2.67	II
Asteraceae	*Ambrosia* sp.	0.33	I
	Centaurea sp.	7.33	III
	Cichorium sp.	2.67	II
	Gundelia sp.	1.00	II
	Xerathemum sp.	1.33	II
Boraginaceae	*Cynoglossum* sp.	0.67	I
	Echium sp.	3.00	III
	Myosotis sp.	0.67	I
Brassicaceae	Brassicaceae	1.33	II
Fabaceae	*Astragalus* sp.	5.67	III
	Fabaceae	4.00	III
	Lotus sp.	1.33	II
	Trifolium pratense L.	2.67	II
	Trifolium spp.	8.00	III
	Vicia sp.	1.00	II
Hypericacecae	*Hypericum* sp.	0.67	I
Lamiaceae	*Salvia* sp.	1.33	II
	Stachys sp.	2.00	II
	Teucrium sp.	1.33	II
Moraceae	*Morus* sp.	1.67	II
Plantaginaceae	*Plantago* sp.	2.67	II
Poaceae	Poaceae	1.33	II
	Zea mays L.	1.00	II
Polygonaceae	*Rumex* sp.	2.67	II
Ranunculaceae	*Ranunculus* sp.	4.00	III
Rhamnaceae	*Paliurus spina-christi* Mill.	8.00	III
Rosaceae	*Agrimonia* sp.	2.00	II
	Crataegus sp.	1.00	II
	Filipendula sp.	4.67	III
	Pyrus sp.	1.00	II
	Rosaceae	6.33	III
Salicaceae	*Salix* sp.	1.33	II
Scrophulariaceae	*Verbascum* sp.	367	III

cells as compared to the healthy HUVEC cells. In one of the studies, the effects of RJ on the growth of malignant fibrosarcoma cells in Balb/c mice were investigated. The results showed that the average size of tumours in mice receiving RJ was smaller than in the control group (Shirzad et al., 2013). In another study, the effect of RJ on leukaemia

and sarcoma cells in animal models was explored revealing no effect on leukaemia cells but has therapeutic effects on sarcoma cells (Taniguchi et al., 2003). Another antitumor evidence is that RJ has an immune-stimulating effect by preventing the myelosuppression induced by tumour evolution depending on the length of the treatment and of the RJ doses (Bincoletto et al., 2005).

Apoptosis is one of the main forms of cellular death which is linked with the characteristic morphological alterations that include condensation in chromatin and nucleus, DNA fragmentation, and apoptotic bodies formation. An increased LPO and ROS production were also shown to happen in apoptosis (Frión-Herrera et al., 2015). To understand whether the cellular processes of RJ treatment on cell survival can be related to specific induction patterns of ROS and LPO, we demonstrate that RJ resulted in a rise in ROS and LPO levels. Antioxidant protection machinery in the human body preserves the equilibrium between the neutralization and generation of ROS and contain SOD, CAT, and glutathione (Paulsen and Carroll, 2013). Nonetheless, disequilibrium between the generation of ROS and antioxidant protection machinery results in oxidative injury, which initiates cancer progression (Klaunig et al., 1998). ROS have also been associated with the mediation of the apoptotic pathway which is crucial for the eradication of damaged cells in cancer treatment (Jafari et al., 2013). In our study, the catalytic activities of CAT and SOD and level of TAS were considerably reduced in RJ-exposed HT-29 cells. The anticancer effects of RJ could be attributed to its antioxidant or prooxidant activity which, appears to be mainly dependent on the level of oxidative stress in cancer cells. If cancer cell survival is dependent on low levels of ROS and oxidative stress, the bee products act as prooxidant, thereby increasing ROS, LPO, and oxidative stress (Alamri, 2021). Decreased activity of SOD and catalase was probably observed due to increased superoxide radical, an important ROS production.

Several indications of apoptosis used in this research provide a better understanding and more comprehensive picture of the influence of RJ on the viability of HT-29 cells. Initially, apoptosis, evaluated by the presence of double DNA strand breaks, was identified by the use of a TUNEL assay. The AI was evaluated by TUNEL assay and it was shown that RJ exposure remarkably increased AI in HT-29 cells.

Moreover, the expression of various proapoptotic and antiapoptotic genes and proteins in the intrinsic and extrinsic apoptotic pathways was found to be altered during apoptosis (Frión-Herrera et al., 2015). ROS produced by cellular activities have been shown to be able to function as cellular toxicants besides signalling compounds. ROS generations in Turkish RJ treated HT-29 cells might be linked with the induction of tumour suppressor protein p53. The levels of both p53 and ROS were shown to be considerably increased resulting to apoptosis in treated cells (Jaganathan and Mandal, 2010). It was demonstrated that p53 might enable apoptosis through activating target genes including proapoptotic proteins which adjust the activities of Bcl-2 family proteins (Macip et al., 2003). In a study, it was reported that honey triggered apoptosis of colon cancer cells was complemented by an increase in the level of Bax and by the decrease in the level of Bcl2 which are the downstream targets of p53 (Jaganathan and Mandal, 2010). The bee products and their content (chrysin) were also shown to increase the levels of caspase-3 in U937 leukaemia cells (Woo et al., 2004; Jaganathan and Mandal, 2010). In another study, 10-HDAwas shown to induce cell cycle arrest and apoptosis in A549 human lung cancer cells through ROS-mediated MAPK, STAT3, NF-κB, and TGF-β1 signaling pathways (Lin et al., 2020). In this study, treatment with RJ enhanced the levels of Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, -10 while decreased level of antiapoptotic Bcl-2 as compared with control cells both at transcriptional and translational levels in intrinsic and extrinsic apoptotic pathways. Proteins have been shown to be the major constituents of RJ content. RJ proteins were reported to trigger cell proliferation in some of the previous studies (Filipič et al., 2015; Ramanathan et al., 2018: Dobritzsch et al., 2019). Trans-10-hydroxy-2-decenoic acid (10-H2DA), 10-hydroxydecanoic acid (10-HDAA), and sebacic acid (SEA) are abundantly found fatty acids in the content of RJ. They, together with proteins, contribute to several types of biological activities of RJ that include anticancer, antimicrobial, immunomodulatory, antioxidative, and antihypertensive effects (Coutinho et al., 2018; Park et al., 2019; Ahmad et al., 2020; Park et al., 2020; Guo et al., 2021).

The value and demand of royal jelly are constantly increasing in today's world market thanks to its high nutritional and pharmaceutical value. Therefore, the production of royal jelly could provide an alternative income for beekeepers. However, as in other bee products, quality control is very essential in royal jelly. One of the most important quality control methods of royal jelly for preventing counterfeiting is the determination of the botanical origin (d'Albore and Bernardini, 1978; Piana et al., 2006; Dimou et al.,2007; Sabatini et al., 2009; Dimou et. al., 2013). In this study, we investigated the pollen content of royal jelly and the use of pollen analysis as a geographical origin determination.

It has been revealed that Bingöl royal jelly has a wide pollen spectrum. The pollen taxa detected in the royal jelly analysis is in accordance with the previous studies about the bee flora of Bingöl (Behçet and Yapar, 2019: Polat et al., 2020; Polat et al., 2021;). So that, the pollen content of royal

jelly is representative of the flora of the production area. The pollen spectrum of royal jelly differed from countries such as Mexico, Russia, Italy, China, and Brazil (d'Albore and Bernardini, 1978; Barth, 2005; Piana et al., 2006). On the other hand, several major pollen types determined in this study such as *Paliurus spina-christi*, *Trifolium* spp., *Centaurea* sp., *Ranunculus* sp., and *Verbascum* sp. have also been reported in royal jelly samples from Greece and Italy (d'Albore and Bernardini, 1978; Dimou et al.,2007; Dimou et. al., 2013). The geographical distance and floral variety are estimated to be effective on these differences/similarities. Thus, it is revealed that pollen analysis in royal jelly could be used as an important tool for geographical origin determination.

In this study, treating colon cancer cells with RJ resulted in a reduction in cell proliferation while no effect was observed on normal healthy cells. The RJ treatment at a concentration of 0.3 mg/mL resulted in an increase in ROS and LPO levels, decrease in SOD and CAT enzyme activity, TAS level, increase in apoptotic index and caspase-3 activation, upregulation of levels of apoptotic genes of intrinsic and extrinsic pathway (Bax, cyt-c, p53, NFκB, cas-3, -6, -8, -9, -10) and downregulation of antiapoptotic gene Bcl-2 both at transcriptional and translational levels.

Although this study highlighted that it shows anticancer and antioxidant activities, it has some limitations. In vivo studies of tested parameters and genes/proteins along with histopathological and additional biomarker assessment are needed to evaluate the effectiveness of the RJ on colon cancer treatment.

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Conflict of interest

No potential conflict of interest is reported by the authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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