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Research Article

Royal Jelly Decreases MMP-9 Expression and Induces Apoptosis in Human 5637 Bladder Cancer Cells

Narges Fazili[1,2], Zahra Soheila Soheili[1*], Saeid Malekzadeh-Shafaroudi[2], Shahram Samiei[3], Shamila D. Alipoor[1], Nasrin Moshtaghi[2], Abouzar Bagheri[4]

[1] *Department of Molecular Medicine, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran*
[2] *Department of Biotechnology and Plat Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
[3] *Blood Transfusion Research Center High Institute for Research and Education in Transfusion Medicine, Tehran, Iran*
[4] *Department of Clinical Biochemistry and Genetics, Mazandaran University of Medical science, Iran*

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Abstract

Royal jelly (RJ) as a traditional medicinal agent has a variety of pharmacological benefits. In the present study, the effects of the royal jelly were investigated on the urinary bladder cancer cell line 5637 (HTB-9). MTT assay was performed to determine the percent of cell viability at different concentrations of the royal jelly. Moreover, *in vitro* wound-healing assay was applied to investigate the effects of RJ on cell migration. The activities and gene expression levels of matrix metalloproteinase 2 and 9 were assessed by zymography and Real time PCR, respectively. It was confirmed that royal jelly (RJ) at the concentration of 0.7 mg/ml exerted a significant inhibitory effect on HTB 5637 cells and reduced cell viability to 72% in comparison to the control cultures (P-value<0.009) during the first 72h of treatment. Furthermore, royal jelly Significantly decreased the bladder cancerous cell migration capacity, and induced a significant decrease in the transcriptional level of the MMP9 after 72h (50% of the controls; P-value<0.049). However, R.J.S did not impose any effect on the expression level and activity of matrix metalloproteinase 2. The results indicated the potential of RJ as a promising natural anti-proliferative and anti-metastatic drug.

***Keywords*:** Bladder Cancer, Royal jelly, MMP-9, MMP2

Introduction

Bladder cancer (BC) is one of the most widely spread malignancies of the genitourinary tract all over the world. Despite of considerable achievements in its treatment, the global control of the disease remains problematic (Saginala et al., 2020) .

Currently, chemotherapy of bladder tumors is considered as an efficient therapeutic method, but most of the chemotherapy regimens are associated with acute systemic toxicity and cause the pain, irritation, myelosuppression, and neuropathy (Saini et al., 2011; Volpe et al., 2013). The establishment of new innovative approaches of cancer treatment still remains a challenge. In this regard, natural products with anticancer properties are in the focus of studies (Cieckiewicz et al., 2012).

Royal jelly (RJ), as a natural product, is well known for its beneficial pharmacological effects, and has received particular attention in the treatment of different cancers (Didar et al., 2019; Izuta et al., 2009; Miyata and Sakai, 2018; Ramanathan et al., 2018; Zhang et al., 2017). RJ is the food of queen bees and larvae for the first three days, but after that, just queen larvae are fed with it (Shi et al., 2011). RJ consists of a family of proteins called major RJ protein (MRJP), which constitute 80-90% of total RJ protein (Zhang et al., 2012). Chemically RJ includes water (50-60%), protein (18%), carbohydrates (15%), lipids (3-6%), mineral salt (5%), and vitamins; together with a large number of bioactive substances such as 10-hydroxy-2-decenoic acid (Viuda-Martos et al., 2008).

It has been shown that RJ has anti-allergic, anti-inflammatory, and antioxidant activities (Ramanathan et al., 2018). In addition it has relieving effects of damaged tissues with high levels of oxidative stress and inflammation (Didar et al., 2019). RJ also can stimulate antibody production, immune cell proliferation, and immunomodulatory mechanisms in inflammatory conditions (Karadeniz et al., 2011).

* Corresponding author's e-mail address: soheili@nigeb.ac.ir, zssoheili@gmail.com

Tumor cells are characterized by an enhanced rate of cell proliferation and loss of apoptosis. Mixing the whole RJ with AKr leukemia cells, Ehrlich carcinoma, TA3 mammary carcinoma, and 6C3HED lymphosarcoma, suppresses the tumor growth (Bincoletto et al., 2005).

Digestion of a type IV collagen, an essential component of the basement membrane, provides the initial step of cell invasion, and matrix metalloproteinases (MMPs) play the crucial role in this process. MMPs belong to the zinc-dependent family of endo-peptidases. They can induce tumor cell proliferation by recruiting growth factors (Sugiyama et al., 2012). Among the matrix metallic proteinases, the gelatinases MMP-2 (72KDa) and MMP-9 (92KDa) have key roles, and promote the degradation of physical barriers of the cell invasion (Gialeli et al., 2011; Kessenbrock et al., 2010). Overexpression of MMP-2 and MMP-9 in different types of human cancers such as breast, colon, prostate, bladder, and ovarian cancers have been illustrated (Aparna and Brundha, 2020; Ricci et al., 2015)

In this study, we tried to investigate the effects of RJ on 5637 cells' migration, gene expression pattern of metalloproteinases, and enzymatic activities of MMP-2 and MMP-9. These cells are considered as a highly metastatic and differentiated type of the transitional cell carcinoma (TCC) (Ricci et al., 2015).

Materials and Methods

Preparation of soluble extract of royal jelly

Fresh RJ was collected from the Kazeroon, Iran. The RJ was obtained from four days old larvae of queen honeybees, and was prepared as follows: Briefly, neutral RJ was suspended in PBS at the concentration of 0.01g/ml. The suspension was then centrifuged at 15000*g* for 15min at 4°C and separated into three layers; the clear top layer was pooled and sterilized using 0.22µm filters (Millipore, Munich, Germany) and stored at -80°C freezer until use.

Cell culture and cell viability assay

The 5637 cells were obtained from the ATCC (Manassas, VA, USA), and cultured in the Roswell Park Memorial Institute medium (RPMI 1640) (Gibco; Carlsbad, CA, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (Gibco), 25 mM HEPES (Gibco), 100units/ml penicillin (Sigma, Munich, Germany), and 100ug/ml streptomycin (Sigma) in the presence of 5% CO_2 at 37°C. For the experimental procedure, cells were seeded at a primary density of 10^3 cells/cm^2 in 96-well plates (Costar, Munich, Germany), and exposed to 0.05 to 0.7 mg/ml of RJS or PBS as control. Cell proliferation assay was performed 72h post treatment according to the study performed by Nakayaet et al. (Nakaya et al., 2007) .

Cellular viability was measured using the colorimetric MTT assay based on their metabolic activities. Briefly, the medium was refreshed, and 20µl MTT solution (5 mg/ml in PBS) was added per well. The cells were then incubated for 4h. Formazan crystals were dissolved in dimethyl sulfoxide (100µl/well) (Sigma). The absorbance intensities were measured at 490 nm with a reference wavelength of 620 nm (Titertek Multiskan ELISA reader, Labsystems Multiskan, Roden, Netherlands).

In vitro wound-healing assay

Cells were seeded at a primary density of 10^4 cells/cm^2 in a 12-well plate (Costar, Munich, Germany) overnight, and were treated with RJS. A plastic blue micropipette tip was used to introduce a scratch in a straight line. Cells were exposed to 0.7 mg/ml RJS or RPMI for another 24h (RJ/RJ and RJ/RPMI groups respectively). Cell migration towards the wound closure was monitored by a phase-contrast microscope (OLYMPUS, Japan). Images were captured 24-hours later using an inverted light microscope to observe cellular migration and to count the migrated cells.

Zymography

Concentrated conditioned media from 5637 cells culture were collected, centrifuged and concentrated using Whatman centrifuge tube filters 6, 12, 24, 48, and 72h post RJ-treatment, and were run on 10% polyacrylamide gel containing 20mg/ml gelatin. Following the electrophoresis, a renaturing buffer (2.5% Triton X-100 in distilled water) was used for washing the gel. Then, the gel was incubated in the zymography buffer (0.15M NaCl, 10mM $CaCl_2$, 0.02% NaN3 in 50mM Tris–HCl, pH 7.5) for 72h at 37°C. In the next step, the gel was incubated in the Coomassie brilliant blue (0.05% Coomassie blue R-250, 25% ethanol, and 10% acetic acid in distilled water) for 1h, and finally, it was de-stained (20% isopropanol and 10% acetic acid in distilled water). Bands densities were measured by the by image J software. (Rasband, 2011).

Quantitative Real-time PCR

Total RNA extraction and cDNA synthesis were performed using an RNA extraction kit (Qiagen,

Table 1. Primers that have been used in the quantitative RT-PCR experiments.

Gene Accession Number	Name	Primers (Qiagen)
P04406	GAPDH	Q101192646
P14780	MMP-9	Q100040040
P08253	MMP-2	QT00088396

Hilden, Germany) and cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. SYBR Green PCR Master Mix (Invitrogen) was used to perform quantitative RT-PCR experiments with the following program: an initial denaturation (95°C for 15min); 40 cycles of amplification, including (95°C for 15s, 52–60°C for 17s, and 72°C for 25s). The gene expression levels were normalized to the expression level of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

Statistical Analyses

Two-tailed Student's t-test (Microsoft Excel 2010) was used to evaluate the statistical significance of the data, and *P*-values less than 0.05 were considered statistically significant. All data were expressed as mean ± standard error (SE).

Results

The Effects of RJS on 5637 cells viability

Based on the result obtained by MTT assay, a significant reduction in the viability of 5637 cells was detected (72%) following the treatment by RJS at the concentration of 0.7mg/ml (Figure 1). This RJ concentration (0.7mg/ml) was used in subsequent experiments.

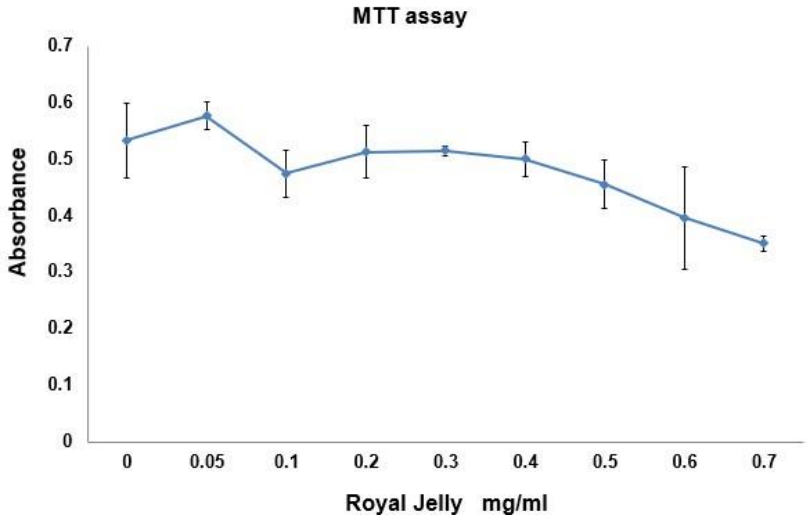

Figure 1. The Effect of RJS on 5637 cells viability, MTT assay. Dose-dependent effect of the RJ against the 5637 cells viability is shown following 72h exposure to 0.05-0.7mg/ml of RJS according the study performed by Nakayaet et al (Nakaya et al., 2007). The maximum effect was observed in 0.7mg/ml concentration.

The effects of RJS on 5637 cells migration and wound healing capabilities

Upon RJS treatment, 5637 cells' migration was significantly inhibited in comparison to control samples (Figure 2). The cells in both treatment groups (RJ/RJ and RJ/RPMI) were treated with RJS, 24h before scratching. In RJ/RJ group; RJS treatment was continued for another 24h following the introduction of the wound. The rate of cell migration decreased significantly (0.25- and 0.5-fold decrease in RJ/RJ and RJ/RPMI groups, respectively) (P-value <0.049). Based on the results, RJS treatment reduced cell migration irrespective of the superiority or precedence of RJ application (Figure 2).

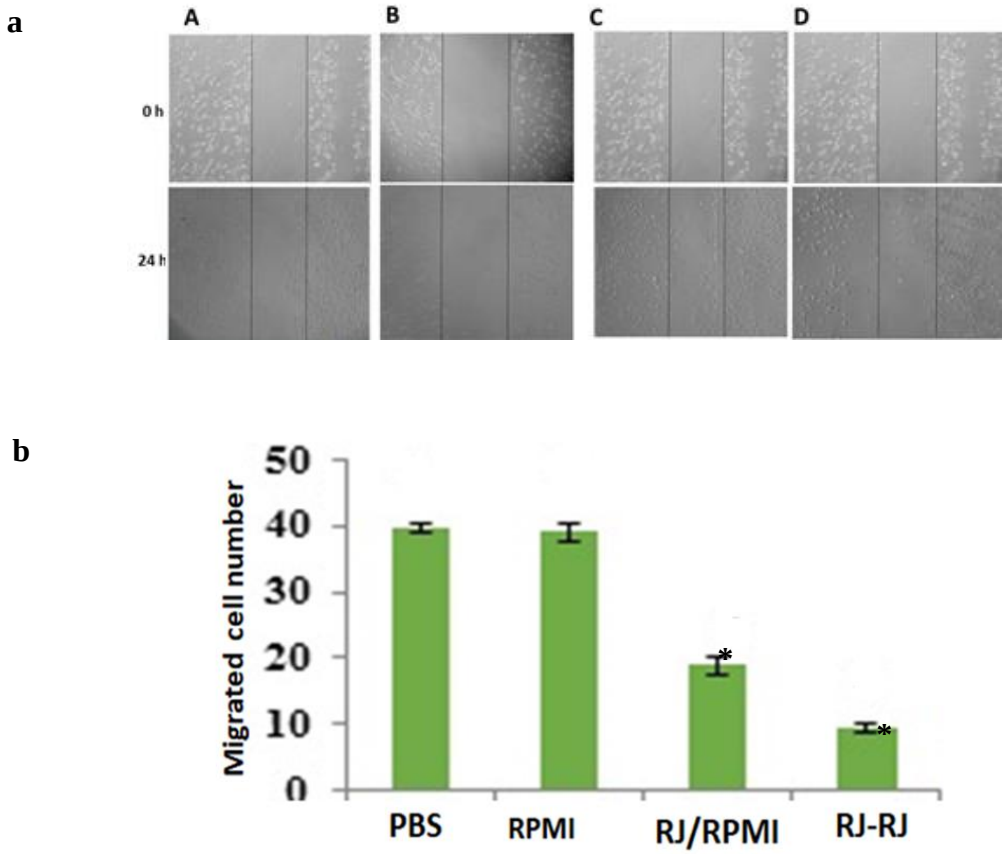

Figure 2. 5637 cells' migration is negatively affected by the RJS treatment. a: Results from the scratch assay: (A) untreated cells, (B) PBS-treated cells, (C) RJ-RJ treated cells (RJS concentration 0.7mg/ml; continued RJS treatment 24h after scratching), and (D) RJ-RPMI treated cells (treated with RJS only before scratching). The first photographs were taken at the zero-time point and the second ones were taken 24h later. Representative images are shown for each group (magnification ×100). b: Quantitative results of migration assay. The cells that had been migrated from the edge of scratched area were counted following the imaging by the inverted microscope. PBS: PBS treated cells, RPMI: untreated cells; RJ-RPMI: treated with RJS only before scratching. RJ-RJ: continued RJS treatment 24h after scratching.

The effects of RJS on MMP gelatinolytic activity

Zymography was used to assess the MMP gelatinolytic activity upon the treatment of 5637 cells with 0.7mg/ml of RJS (Figure 3a). No significant changes were detected in MMP-2 activity levels 6, 12, 24, 48, and 72h post-treatments (Figure 3b). Due to the long exposure time, MMP-9 was digested, and its activity was not detectable.

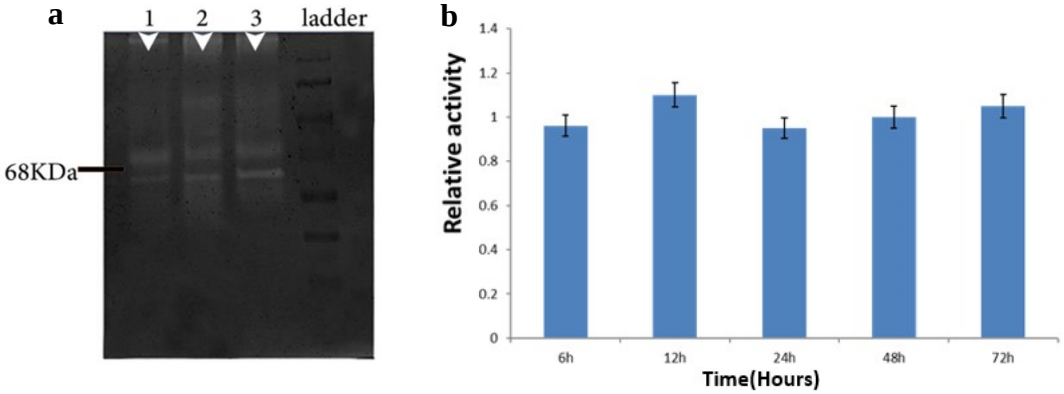

Figure 3. Zymography of conditioned media from 5637 cells. (a) Representative image of MMP-2 activity, gelatin zymography, 12h post-treatment: 1) PBS, 2) untreated, and 3) RJS treated cells. (b) Quantitative results from the zymography experiments on samples collected from 6h to 72h post treatment (p value<0.05). The relative activity in each band was estimated by image J software. (Rasband, 2011).

The effect of RJS treatment on MMP-2 and MMP-9 gene expressions

Real time PCR revealed that RJ induced a significant increase in the transcriptional level ofMMP-9 after 6h (2.8-fold *p-value* <0.02 respectively). However, a decreasing trend started from 24h post-treatment and continued until 72h. The maximum reduction was observed 72 h post treatment (0.5-fold, *p-value* <0.049) (Figure 4).

No significant changes were detected in MMP-2 mRNA level upon RJS treatment at different time points (Figure 4).

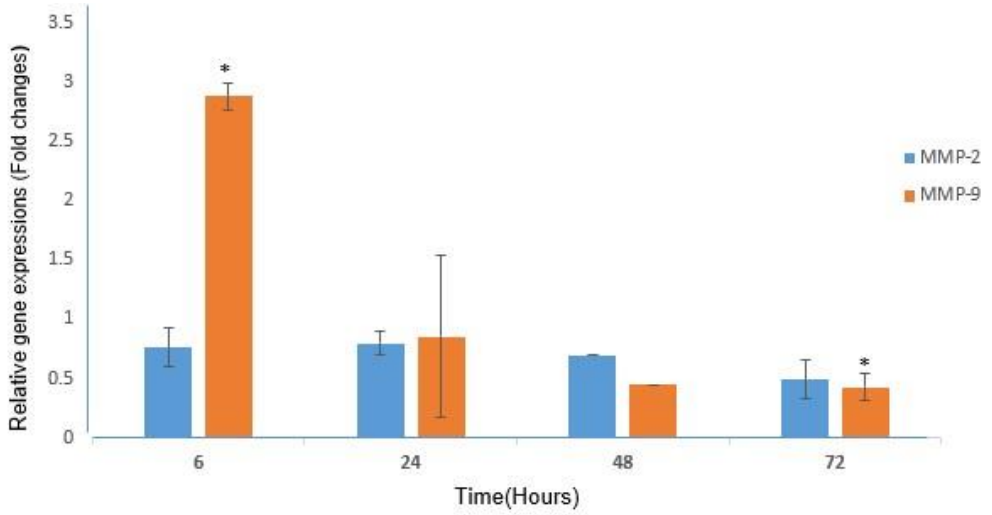

Figure 4. The Effect of RJS on MMP-2 and MMP-9 gene expressions. Relative expression of MMP-2 and MMP-9 in RJS treated samples were compared to PBS treated ones. The target genes expression was normalized to the GAPDH. Statistical significance was determined by Student's t-test (p<0.05).

Discussion

This study set out to determine the effect of RJ on the proliferation of the bladder cancer cell line 5637 and their invasiveness secretory indicators: MMP-2 and MMP-9. The anti-tumor activity of RJ has been suggested to be related to its fatty acid content, mainly $10H_2DA$, which is not a water-soluble compound. Here we investigated the inhibitory effects of the soluble supernatant of the RJ on the characteristics of the bladder cancer cells.

To determine the effects of RJS on the proliferation of 5637 cells, they were treated with different concentrations of the RJ supernatant (0.05 to 0.7mg/ml). Results from MTT assay showed that RJS decreases bladder cancer cell proliferation at the concentration of 0.7mg/ml. These anti-proliferative effects were dose-dependent. Furthermore, it was shown that 72h treatment of the cells with 0.7 mg/ml of RJS induces apoptosis. In a similar study, Erem et al (2006) reported that the addition of 0.02 to 0.5 mg/ml of the RJS to lymphocyte cultures, decline cell proliferation rates. However, the higher concentrations (more than 5mg/ml), conversely, increase cell proliferation capacities (Erem et al., 2006). Although, treatment of HeLa cells by RJ. P_{30} (a fraction of RJ protein extract) reduces the cells' proliferation on the first day of treatment (Salazar-Olivo and Paz-Gonzalez, 2005). Pervious experiments confirmed that of RJ decreases the number of human breast cancer cells (MCF-7), 72h. in our study based on scratch assay experiments, revealed that pretreatment of cells with 0.7mg/ml of RJS significantly reduces wound healing (p<0.01). On the contrary, another study reported that RJS at different concentrations (0.05, 0.001, and 0.00001mg/ml) could enhance cell migration in human dermal fibroblasts (Kim et al., 2010).

Our study has shown that RJS-treated 5637 cells induced no significant change in the MMP-2 gene expression. while, 6h following the treatment, the expression of MMP-9 was increased significantly. This is in accordance with the previous study reported the increased human dermal fibroblast cell migration 8h post-treatment. (Bergers et al., 2000).

In the present study, 24h post-treatment, the MMP-9 gene expression was down regulated, and within 72h it was declined to 0.5fold (P<0.049). The increasing level of MMP-9 (up to 6h) in RJS-treated cells could be attributed to the treatment duration. In accordance with our

data, a previous study revealed that the migration capacity of RJ-treated human dermal fibroblasts just increased up to 8h post-treatment and thereafter (up to 48h), it was remained equal to the migration capacity of control cells (Erem et al., 2006; Kim et al., 2010) in this study the whole RJ suspended in PBS was used. In another study, analyzing the protein content of the RJ demonstrated that defensin-1 peptide of the royal jelly induced an enhanced level of MMP-9 expression and wound healing in keratinocyte s, *in vitro*. In this study we apply RJ prepared in sterile deionized water at a concentration of 100 mg/ml as it was proposed previously (Bucekova et al., 2017). As a result, it may be concluded that RJ may exert distinct effects based on method of extraction.

According to the last updates, the effects of RJ extensively depend on the method of extraction and the treatment duration. In the current study, the upper soluble part of the RJS was utilized to determine its anti-proliferative and anti-migration potentials. It was reported that this soluble fraction of RJS contains major royal jelly protein 2 and 3 (MRJP2, MRJP3) (GUO et al., 2005; Nagai and Inoue, 2004).

Several mechanisms may be responsible for the observed decrease in the cell proliferation and migration upon the treatment. It is well known that tumor cells induce the production of inflammatory factors (Nagai and Inoue, 2004). The presence of inflammatory messenger polypeptides, such as tumor necrosis factor-alpha (TNF-α) and interleukins in the tumor microenvironment plays a critical role in the genomic rearrangement and angiogenesis (Zidi et al., 2010). TNF-α can induce 5637 cells' growth and MMP-9 gene expression, which is one of the major enzymes involve in cell migration and invasiveness. However, it has been suggested that it doesn't impose any effect on the MMP-2 expression levels (Lee et al., 2008). Moreover, it causes tumor cells to be tolerant against apoptosis (Huerta-Yepez et al., 2006). TNF-α induces pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8) (Zidi et al., 2010). Interestingly, a few studies indicated that RJS decreases some of the pro-inflammatory cytokines. Kohno (2003) showed that RJS inhibited TNF-α, IL-1, and IL-6 in the cultures of mouse peritoneal macrophages in a dose-dependent manner (Kohno et al., 2004). Also, the reduction of TNF-α in the human peripheral blood lymphocytes cultures were reported upon 72h incubation with RJS (Erem et al., 2006). It was reported that the water-soluble extract of RJ possesses the most potent immunomodulatory properties *in vitro*. It can decrease interleukin-2 (IL-2), and increase apoptosis in rat T-cells (Gasic et al., 2007). As mentioned before, MRJP3 is enriched in the water-soluble fraction of the RJ, and inhibits the production of some cytokines, such as IL-4, IL-2, and IFN-g, through the inhibition of the T cells proliferation (Okamoto et al., 2003).

Taken together, it can be concluded that RJS may indirectly affect MMP-9 expression levels by decreasing the TNF-α and IL-6 production. Reduced MMP-9 expression can reduce cell proliferation, and inhibit cell migration. It is also very interesting that decreasing the levels of TNF-α, diminishes cell tolerance against apoptosis

Collectively, these findings provide some further evidences that the soluble fraction of the RJS decreases 5637 cells' proliferation and migration. It reduces MMP-9 expression, which is commonly over-expressed in 60% of high-grade urothelial bladder tumors, and it is responsible for tumor invasiveness (Reis et al., 2012). Similar results are expected to be observed for other cell lines from the identical tissue. As well as, some differences which are inevitable.

Conclusion

Based on the results from the present study, RJS can promote apoptosis and decrease cell migration through the inhibition of cell proliferation and concurrent reduction of the MMP-9 gene expression in 5637 cells. RJ could be considered as a promising natural medication candidate. Its potential should be further investigated to be applied in combination with chemotherapeutics in advanced stages of bladder cancers.

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