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Research Article | Araştırma Makalesi

DETERMINATION OF THE EFFECT OF RUTIN ON EPITHELIAL TO MESENCHYMAL TRANSITION IN PROSTATE CANCER CELLS

EPİTELYALDEN MEZENKİMALE GEÇİŞ ÜZERİNE RUTİNİN ETKİLERİNİN PROSTAT KANSERİ HÜCRELERİNDE BELİRLENMESİ

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ABSTRACT

Objective: The difficulties experienced in the treatment of prostate cancer and the excess of side effects due to chemotherapy have brought the search for alternative treatment strategies. In recent studies, it is known that Rutin (RUT) has an anti-cancer effect on cancer cells. Our study aimed to determine the effects of RUT on epithelial to mesenchymal transition (EMT) in prostate cancer cells, for the first time.

Methods: The anticancer effect of RUT in prostate cancer cells (PC-3) was examined by WST-1, Annexin V ELISA, DAPI and Acridine Orange staining, and the anti-cancer and anti-metastatic properties of RUT were evaluated with the Scratch Assay test. The expression level of *Bax, Bcl-2, Snail, Twist, Vimentin* and *E-cadherin* genes was evaluated by RT-PCR.

Results: The cells were treated with RUT (500, 750, 1000 and 1500 µM) for 24 and 48 hours. The viability rates decreased with increasing RUT concentration depending on dose and time ($p<0.01$). Free Annexin V protein decreased, and apoptotic cells increased depending on the increasing RUT concentration ($p<0.01$). There was a reduction in the migrating cells, especially at 750 and 1000 µM RUT concentrations. There was a significant increase (14.22-fold) in *E-cadherin* mRNA level after 1500 µM RUT treatment ($p<0.01$). Additionally, the mRNA level of *Snail, Twist* and *Vimentin* decreased at higher RUT concentrations ($p<0.01$).

Conclusion: The existence of RUT's potential to inhibit epithelial to mesenchymal transition and promote apoptosis has been demonstrated. It is also recommended to explore the effects of RUT on EMT *in vivo.*

Keywords: Prostate cancer, rutin, epithelial to mesenchymal transition, apoptosis

ÖZ

Amaç: Prostat kanseri tedavisinde yaşanan zorluklar, kemoterapi nedeniyle görülen yan etkilerin fazlalığı alternatif tedavi stratejileri arayışını gündeme getirmiştir. Son yıllarda yapılan araştırmalarda Rutin (RUT)'in kanser hücreleri üzerinde anti-kanser etki gösterdiği bilinmektedir. Bu nedenle çalışmamızda RUT'un prostat kanseri hücrelerinde epitelyalden mezenkimale geçiş (EMT) üzerindeki etkilerinin ilk defa belirlenmesi amaçlanmıştır.

Yöntem: Prostat kanseri hücrelerinde (PC-3) RUT'un antikanser aktivitesi WST-1, Annexin V ELISA, DAPI boyama ve Akridin Oranj (AO) boyama ile belirlenerek, RUT'un anti-kanser ve anti-metastatik özellikleri Scratch (Çizik) Assay testi ile değerlendirildi. *Bax, Bcl-2, Snail, Twist*, *Vimentin* ve *E-kaderin* genlerinin mRNA ifade düzeyi RT-PCR analizi ile belirlendi.

Bulgular: PC-3 hücrelerine, farklı konstrasyonlarda (500, 750, 1000 ve 1500 µM) ve farklı zamanlarda (24 ve 48 saat) RUT uygulandı. Canlılık oranlarının doza ve zamana bağlı olarak artan RUT konsantrasyonuna bağlı olarak azaldığı gözlendi ($p<0.01$). Ayrıca artan RUT konsantrasyonuna bağlı olarak serbest Annexin V proteinin azaldığı ve apoptotik hücrelerin arttığı belirlendi ($p<0.01$). Özellikle 750 ve 1000 µM RUT konsantrasyonlarında göç eden hücre sayısında azalma olduğu ortaya kondu. RT-PCR analizi sonucu elde edilen verilerde 1500 µM RUT muamelesi sonrası *E-Kaderin* mRNA seviyesinde 14.22-kat olarak belirgin bir anlamlı artış olduğu belirlendi ($p<0.01$). Diğer yandan EMT sürecinde yer aldığı bilinen *Snail, Twist* ve *Vimentin* mRNA ifade düzeyinin artan RUT konsantrasyonuna bağlı olarak azaldığı ortaya konuldu ($p<0.01$).

Sonuç: RUT'un epitelden mezenkimal geçişi engelleme ve apoptozu teşvik edici potansiyelinin varlığı ortaya konulmuştur. Ayrıca RUT'un EMT üzerine olan etkilerinin daha ileri moleküler analizler ve *in vivo* olarak araştırılması önerilmektedir.

Anahtar Kelimeler: Prostat kanseri, rutin, epitelyalden

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Introduction

Further research into the various signaling pathways involved in the pathogenesis of cancer is necessary since it is a very complex and multifaceted disease defined by the unrestricted proliferation of aberrant cells that have the capacity to assault and spread throughout the body.[1] Prostate cancer is one of the most commonly diagnosed cancers with high fatality rates, particularly among men.[2] According to the American Cancer Society, men have a 1 in 9 risks of being diagnosed with cancer during their lifetime. An estimated 1 in 41 men dies from prostate cancer.[2] Although the incidence of prostate cancer is related to age, the rate of diagnosis increases with age.[2] For the treatment of advanced prostate cancer and increased patient compliance, novel therapies also are required.

The biggest family of polyphenolic chemicals originating from plants, flavonoids have a variety of biological effects in a wide range of mammalian cell systems.[3] In recent years, the performance of flavonoids to prevent and treat prostate cancer has become the focus of attention due to the inverse relationship between a diet enriched with flavonoids and prostate cancer incidence.[4] One of the most well-known flavonoids, rutin (RUT), is best known for its anti-inflammatory and antioxidant capabilities.[5] RUT is a plant-derived flavonoid found in various vegetables, fruits and medicinal plants.[1] RUT, a phytochemical with anti-carcinogenic properties, is one of the powerful antioxidants with biochemical effects, particularly in cancer treatment.[6] In the literature, many studies have shown that RUT inhibits cancer cell proliferation through cell cycle arrest and apoptosis in some cancer types.[3,6,7] It has been shown that RUT inhibits the *in vitro* proliferation of cancer cell.[7]

The epithelial to mesenchymal transition (EMT) program is defined as a condition that causes tumor metastasis, in which epithelial cells slowly transform into mesenchymal cells, where they lose epithelial functions.[8] The earliest occurrence in EMT can be expressed as the loss of cell polarity and occurs due to the loss of epithelial proteins (E-cadherin and Vimentin).[8] By regulating the expression of the cadherin family of proteins (Snail and Twist) modulate cell adhesion.[9] Snail and Twist stand out as EMT-inducing transcription factors and have an important role in EMT.[10] The results of many studies have shown a relationship between EMT markers and poor prognosis in carcinoma types, including but not limited to prostate, breast, liver, lung, pancreatic and bladder carcinomas.[10]

The pharmacokinetic restrictions and unfavorable side effects of chemotherapeutic medicines in the treatment of metastatic prostate cancer have recently drawn attention to the development of novel anticancer agents.[11] The pleiotropic tasks of EMT related to metastasis and therapy resistance create great therapeutic opportunities to increase the effectiveness of chemotherapy, targeted therapy or immunotherapy, and to prevent EMT.[11] Therefore our work was aimed to investigate the effects of RUT on EMT in prostate cancer cells.

Methods

Cell Culture Conditions

RUT (Tokyo Chemical Industry) was purchased commercially PC-3 cell line was used as the prostate cancer cell line and is available in Department of Biology. PC-3 cells were cultured in RPMI 1640 medium containing 10% FBS (Gibco) and penicillin/streptomycin at 37°C and 5% CO_2. HUVEC cells were used only for viability analysis. HUVEC cells were cultured in DMEM medium containing 10% FBS and penicillin/streptomycin at 37°C and 5% CO_2.

Cell Viability Analysis

The WST-1 assay utilize tetrazolium salts to measure metabolic activity in active cells. According to the assay methodology, cellular mitochondrial dehydrogenases convert the tetrazolium salt to formazan. For this reason WST-1 analysis was performed to determine the cytotoxic effect of RUT in PC-3 cells. For this purpose, cells were first seeded in 96-well cell culture plates at $2x10^4$ cells/well. They were treated with different concentrations of RUT (500, 750, 1000 and 1500 µM) for 24 and 48 hours. After, WST-1 dye addition to the cell culture medium incubated for 1-4 hours at 37°C, and measurements were made in a microplate reader (Chromate) at 450 nm wavelength. Control cell viability not treated with RUT was accepted as 100%, and viability rates of experimental cells were expressed as "%".

ELISA Analysis

Annexin V ELISA analysis was made to determine the apoptotic effect of RUT on PC-3 cells. For this purpose, cells were first cultured in six-well plates at $1x10^5$ cells/well. They were treated with different concentrations of RUT (500, 750, 1000 and 1500 µM) for 24 hours. Following incubation, the Human Annexin V ELISA kit (ab119503) was used in accordance with the manufacturer's instructions.

Acridine Orange (AO) and DAPI (4',6-diamidino-2-phenylindole) Staining

AO and DAPI staining was used to evaluate the effect of RUT on cell and nuclear morphology in PC-3 cells. For this purpose, cells were first cultured in six-well plates at $2x10^5$ cells/well. They were treated with different concentrations of RUT (500, 750, 1000 and 1500 µM) for 24 hours. The cells were fixed with a 4% paraformaldehyde (PFA). Then, the cells were washed and treated with AO dye (100 mg/mL) and DAPI, and the images were taken under a fluorescent microscope (Olympus).

Scratch Assay

The scratch assay was performed to explore the anti-migrating capacity of RUT. For this purpose, the cells were first cultured in six-well plates at $2x10^5$ cells/well and the "+" shape was given to the wells with a 1000 ml pipette tip. They were treated with RUT (500, 750, 1000 and 1500 µM) for 24 hours. After incubation, cells were fixed with a 4% paraformaldehyde (PFA) solution for 30 minutes. Afterward, the images were taken under an inverted microscope (Nikon).

RT-PCR Analysis

To determine the effect of RUT on the mRNA levels of *Bax, Bcl-2, Snail, TWIST1, Vimentin* and *E-cadherin* in PC-3 cells, RT-PCR analysis was performed. For this purpose, cells were seeded in T_{25} cell culture flasks at $1x10^6$ cells/well. They were incubated with different concentrations of RUT (500, 750, 1000 and 1500 µM) for 24 hours. After incubation, RNA isolation was performed using Xtrazol (Biofroxx) according to the appropriate kit protocol. The obtained RNA levels were measured at 260 nm in a spectrophotometer (Thermo Fisher Scientific) and after controlling the quality and concentration, 5 µg of RNA was converted into cDNA using appropriate kits containing Reverse Transcriptase. The obtained cDNA is diluted with nuclease-free distilled water and after the primers suitable for *Bax, Bcl-2, Snail, TWIST1, Vimentin* and *E-cadherin* genes are obtained from the company, the appropriate reaction mixture was prepared. As a final step, the reaction was carried out by adjusting the RT-PCR conditions and the number of cycles (Bio-Rad, CFX Connect).

Statistical Analysis

"SPSS 22.0" statistical program was used to evaluation and $p<0.05$ was accepted as statistically significant. Differences between cell viability percentages were evaluated by one-way analysis of variance (Post-Hoc Tukey). The differences in mRNA expression levels, which vary depending on the dose and time, were compared statistically with the web-based analysis software (https://www.qiagen.com/tr/shop/genes-and-pathways/). All analyzes were carried out in Kocaeli University, Department of Biology, Biotechnology laboratory.

Results

The Effects of RUT on Cell Viability

WST-1 analysis was used to determine the cytotoxic effect of RUT on PC-3 and HUVEC cell lines (Figure 1). Our findings demonstrate that the proliferation rates of cells treated with different concentrations of RUT (62.5, 125, 250, 500, 1000 and 1500 µM) for 24 and 48 hours decreased significantly depending on the RUT concentration and time in PC-3 cells (Figure 1A, $p<0.05$). However, it was determined that RUT at 500 and 750 µM concentrations had a toxic effect on HUVEC cells for 48 h (Figure 1B). Based on the data obtained from the viability analysis results, RUT concentrations of 500, 750, 1000 and 1500 µM were determined to be used for 24 hours, respectively, in further analysis.

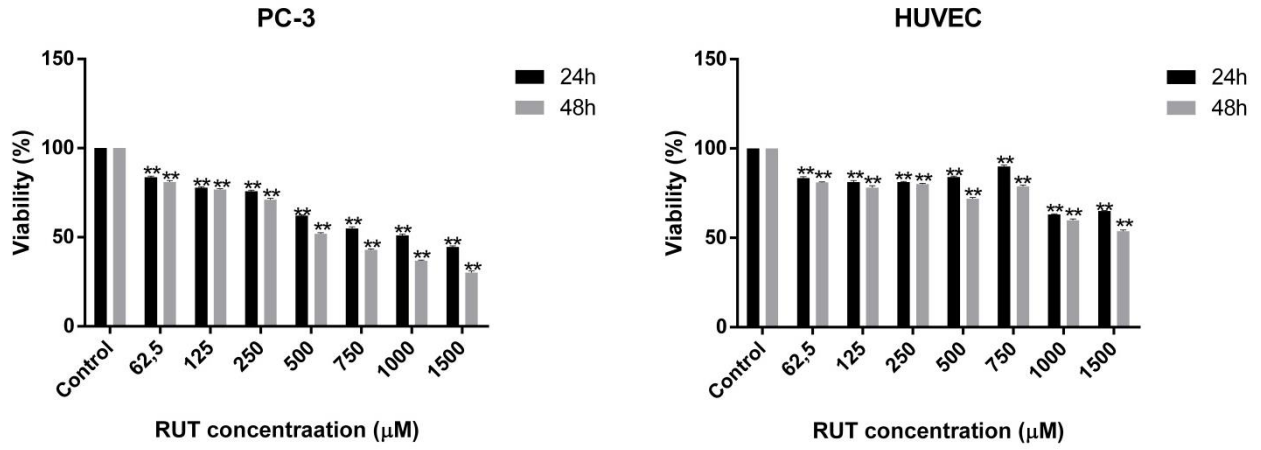

Figure 1. The cytotoxic effect of RUT in PC-3 and HUVEC cells. The viability rate was determined after treated with different concentrations of RUT (62.5, 125, 250, 500, 750, 1000 and 1500 µM) and statistically analyzed for 24 and 48 h (*$p<0.05$, **$p<0.01$).

The Effects of RUT on free Annexin V Protein Level

It was observed that there was a significant decrease in free Annexin V protein level ($p<0.01$, Figure 2). It has been determined that 500, 750, 1000 and 1500 µM RUT treatment decreased free Annexin V protein levels (7.12, 5.85, 4.82, 3.87 and 3.39 ng/mL, respectively) in PC-3 cells ($p<0.05$). Therefore, it was determined that RUT induced apoptosis depending on the increasing concentrations.

The Effects of RUT on Cell and Nucleus Morphology

Morphological changes caused by different concentrations of RUT in PC-3 cells and nuclei were visualized by AO and DAPI staining (Figure 3). In PC-3 cells, it was observed that apoptotic morphological changes increased in cells and nuclei depending on the increasing concentration of RUT. Chromatin condensation, cell and/or cytoplasmic shrinkage were observed in PC-3 cells. In addition, nuclear blebbing was determined especially in PC-3 cells that were treated with 1000 and 1500 µM RUT. Compared to the control

group, PC-3 cells treated with RUT showed disruption of cell membrane integrity, nuclear blebbing and cytoplasmic shrinkage. It was determined that apoptotic cell and nucleus changes were observed the most in PC-3 cells, especially in 1000 and 1500 µM RUT in PC-3 cells.

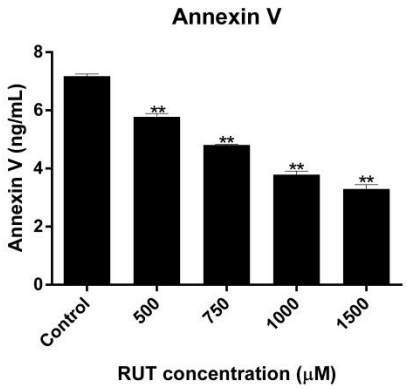

Figure 2. The effect of RUT on the level of free Annexin V protein in PC-3 cells. The level of free Annexin V protein was determined after treated with different concentrations of RUT (500, 750, 1000, and 1500 µM) and statistically analyzed for 24 h (**p<0.01).

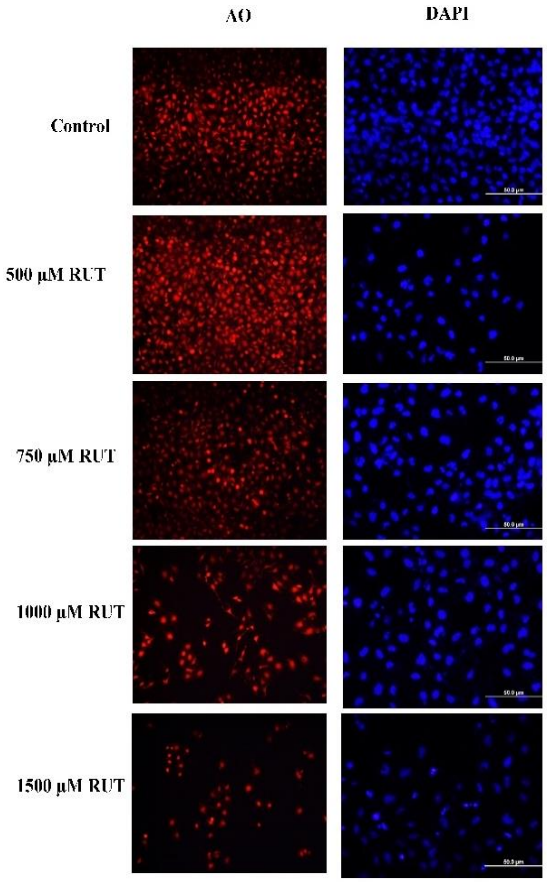

Figure 3. The effect of RUT on cell and nucleus morphology in PC-3 cells. The cells treated with RUT (500, 750, 1000 and 1500 µM), general morphology by AO staining and nuclear morphology by DAPI staining were visualized.

The Effects of RUT on Cell Migration

The effect of RUT on the migration capacity of cells was visualized by performing a scratch assay (Figure 4). PC-3 cells treated with RUT showed that the rate of migration of cells was decreased especially at 750 and 1000 µM RUT. These results reveal that RUT has anti-migratory properties as well as apoptotic properties in PC-3 cells.

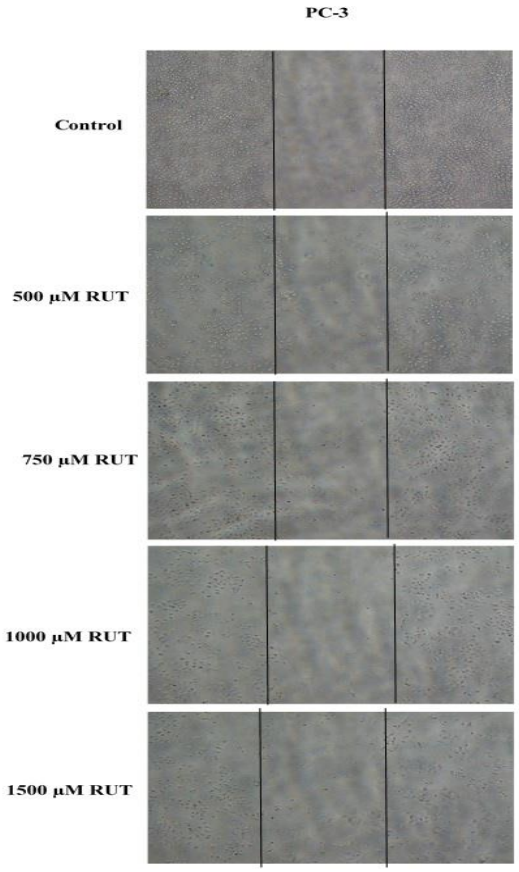

Figure 4. The effect of RUT on migration capacity in PC-3 cells. The cells treated with RUT (500, 750, 1000 and 1500 µM) and visualized.

The Effects of RUT on Gene Expression Levels

The effect of RUT on mRNA levels of *Bax, Bcl-2, E-cadherin, Snail, Twist* and *Vimentin* in PC-3 cells was determined by RT-PCR analysis (Figure 5). It was determined that EMT-inducing factors *Snail, Twist* and *Vimentin,* and *Bax* gene expression with pro-apoptotic effect decreased due to increasing RUT concentration, and there was a significant increase in mRNA levels of *Bcl-2* and *E-Cadherin* gene expression, which have anti-apoptotic activity. (p<0.01).

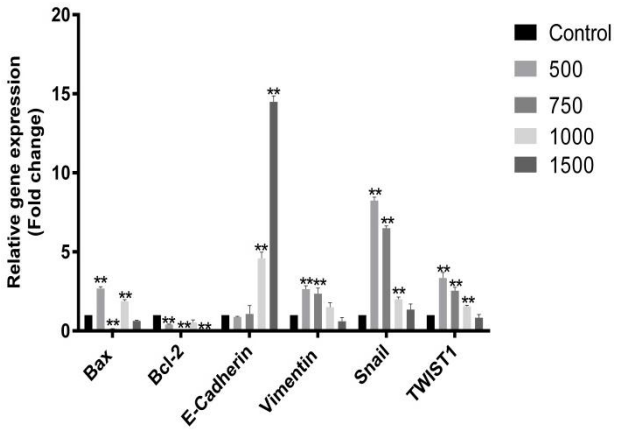

Figure 5. The effect of RUT on mRNA levels of *Bax, Bcl-2, Snail, TWIST1, Vimentin* and *E-cadherin* in PC-3 cells was determined by RT-PCR analysis and statistically analyzed (**p<0.01).

The increase in *Bax* expression and the decrease in *Bcl-2* expression, which supported the increase in apoptosis with the increase in RUT concentration, supported the Annexin V ELISA analysis and AO staining images, and the decrease in the expression of *Snail, Twist* and *Vimentin.* Together with the gene-level data we obtained, the existence of RUT's potential to inhibit mesenchymal transition from the epithelium and promote apoptosis has been demonstrated.

Discussion

RUT has been shown to decrease the cell viability of cancer cells.[6] RUT, has been shown to regulate several signaling pathways. More *in vitro* and *in vivo* researches are needed to assess the potential of RUT as an adjuvant and chemotherapeutic agent.[6] Therefore, in our study, the effect of RUT on the EMT program was evaluated for the first time. When we evaluated the obtained results, the suppressive effect of RUT on the EMT program was revealed for the first time.

It has been reported that up-regulation of Bcl-2 prevents cell death and creates disease pathogenesis with cytokine production. Bax protein initiates the process cascade via releasing cytochrome c out from mitochondria, which leads to apoptosis.[12] RUT causes growth inhibition in human glioblastoma cells by suppressing the expression of pro- and anti-apoptotic genes, induction of cell cycle arrest.[13] Similarly, Khan et al.[14] demonstrated that RUT causes apoptosis by arresting the cell cycle in cervical cancer cells. Additionally RUT induces apoptosis in colon cancer cells using mitochondria-mediated apoptotic pathways.[15] Satari et al.[6] found that after the combined treatment of anticancer drug (5-fluorouracil, 5-FU) and RUT at different concentrations to PC-3 cells, the application of 5-FU/ and RUT separately or using a combination, increased apoptosis in PC-3 cells. In addition, the combination of 5-FU/RUT has determined a high level of Bcl-2 protein suppression in PC-3 cells. In our study, *Bcl-2*, which has anti-apoptotic and pro-apoptotic activity, and *Bax* gene expression, which has the pro-apoptotic effect, decreased depending on increasing RUT concentration. The increase in *Bax* expression and decrease in *Bcl-2* expression, which supported the increase in apoptosis with the increase in RUT concentration, supported the Annexin V ELISA analysis and AO staining images.

EMT is linked to cancer growth, migration, and metastasis during prostate carcinogenesis, and mesenchymal markers and transcription factors are substantially elevated. Epithelial cell markers such as E-cadherin are downregulated. While there is a loss of cell-cell adhesion in the EMT process, mesenchymal markers such as vimentin and N-cadherin increase and allowing cells to migrate to secondary sites or organs. This process has been evaluated as a molecular target to inhibit EMT in prostate cancers.[16] RUT is also effective in colon adenocarcinoma cells by cell cycle arrest in G1 phase and regulation of microRNAs, messenger RNAs and transcription factors and exhibits anti-angiogenic properties *in vitro*.[17,18] Additionally Ben Sghaier et al.[19] demonstrated that RUT inhibits proliferation and decreases adhesion and migration of human lung and colon cancer cells. In our study, the increase in *E-cadherin* gene expression and the decrease in *Snail, Twist* and *Vimentin* expression after the highest concentration of RUT (1500 µM) treatment support the existence of its anti-migratory effect. Despite the promising anticancer effects of RUT in preclinical studies, barriers to its clinical transition have been observed. The low solubility, high metabolism, low gastrointestinal absorption, and low bioavailability of RUT limit its ability to reach appropriate concentrations in tumor tissues.[16] To increase the bioavailability and effectiveness of RUT as an anticancer agent, many strategies include the combination of RUT with other chemotherapeutic medications, RUT synthetic analogs, and RUT-nano-formulations are additional choices.

Conclusions

With the findings obtained at the end of the present study, an important development will be created in the preparation of biologically sourced, specific, biotechnologically innovative chemotherapeutics that can be used in the treatment of various cancer types. While increasing the benefit to the patient, undesirable side effects may decrease or disappear completely because the patient will be exposed to lower doses. We concluded that RUT has value as a new anti-metastatic agent and can be considered as promising multi-targeted nutraceutical agent that provides various health benefits in general.

Compliance with Ethical Standards

No ethics committee decision is required for the study.

Conflict of Interest

Authors declare that there is no conflict of interest regarding this work.

Author Contribution

Authors contributed equally to this work.

Financial Disclosure

None.

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