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ORIGINAL ARTICLE

# Anti-proliferative and anti-invasive effects of ferulic acid in TT medullary thyroid cancer cells interacting with *URG4/URGCP*

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**Abstract** Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA), a common dietary plant phenolic compound, is abundant in fruits and vegetables. The aim of present study is to investigate the effects of FA on cell cycle, apoptosis, invasion, migration, and colony formation in the TT medullary thyroid cancer cell line. The effect of FA on cell viability was determined by using CellTiter-Glo assay. $IC_{50}$ dose in the TT cells was detected as 150 μM. *URG4/URGCP* (upregulated gene-4/upregulator of cell proliferation) is a novel gene in full-length mRNA of 3.607 kb located on 7p13. It was determined that FA caused a decrease in the expression of novel gene *URG4/URGCP*, *CCND1*, *CDK4*, *CDK6*, *BCL2*, *MMP2*, and *MMP9*, a significant increase in the expression of *p53*, *PARP*, *PUMA*, *NOXA*, *BAX*, *BID*, *CASP3*, *CASP9*, and *TIMP1* genes in TT human thyroid cancer cell line by using real-time PCR. It was found that FA in TT cells suppressed invasion, migration, and colony formation by using matrigel invasion chamber, wound healing, and colony formation assay, respectively. In conclusion, it is thought that FA indicates anticarcinogenesis activity by affecting cell cycle arrest, apoptosis, invasion, migration, and colony formation on TT cells.

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**Keywords** Ferulic acid · Thyroid cancer · TT cells · *URG4/URGCP*

**Abbreviations**

| | |
|---|---|
| CCND1 | CyclinD1 |
| CTG | CellTiter-Glo |
| PARP | Poly(ADP-ribose) polymerase |
| *URG4/URGCP* | Upregulated gene-4/upregulator of cell proliferation |
| ECM | Extracellular matrix |
| MMP | Matrix metalloproteinase |
| MTC | Medullary thyroid cancer |

## Introduction

Thyroid cancer is the most common endocrine malignancy. Its incidence is higher in females than in males and has been increasing since the mid-1990s in the USA [1]. While thyroid cancer is usually seen in papillary and follicular types, the third most common thyroid cancer is medullary thyroid cancer (MTC). MTC is a neuroendocrine malignancy that is derived from parafollicular C cells of the thyroid gland [2, 3]. MTC is also responsible for 5–8 % of all thyroid cancer cases, and 5- and 10-year survival of MTC is 80 and 70 %, respectively [4, 5]. In patients with MTC, cervical lymph node metastases and distant metastases can be seen [6]. Degradation of the extracellular matrix (ECM) is an important process in cancer growth, invasion, and metastasis [7]. The members of matrix metalloproteinase (MMP) family are important proteinases due to the degradation of extracellular matrix in metastatic process [8]. The available chemotherapy and radiotherapy treatment methods are not sufficiently effective in metastatic

MTC. New therapies have developed with molecular studies in cancer, and less toxic therapies have been investigated.

In order to find new therapeutic drug for treatment of cancer, studies on cell cycle genes, such as cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, and genes that have a role in apoptosis and response to DNA damage, and genes that have important role in invasion and metastasis are increasing day by day. Phenolic compounds, which are available in fruits and vegetables, generally show anticarcinogenic activity by affecting cell cycle and apoptotic signaling pathways [9]. Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA), a common dietary plant phenolic compound, is abundant in fruits and vegetables such as blueberry, cranberry, pear, cherry (sweet), apple, orange, grapefruit, cherry juice, apple juice, lemon, peach, potato, lettuce, and spinach [10]. It was reported in previous studies that FA had cytotoxic effect in many cell lines such as human cervical cancer (HeLa), human bladder cancer, and human glioblastoma cancer (U87MG) cells [11–13]. Moreover, in a previous study, it was shown that FA had radioprotective effect on human lymphocytes [14].

Upregulator of cell proliferation 4 (*URG4/URGCP*) is a gene in full-length mRNA of 3.607 kb located on 7p13. It was firstly found that *URG4/URGCP* contributed to hepatocarcinogenesis by promoting growth and survival in tissue culture and tumor development in nude mice [15]. In another study, it was shown that URG4 downregulation via small interfering RNA (siRNA) might cause cell cycle arrest by downregulating cyclin D1 in SGC7901 and MKN28 gastric cancer cells [16]. Moreover, in previous studies, it was shown that *URG4/URGCP* was upregulated in gastric cancer [16], osteosarcoma [17], and leukemia [18]. Consequently, *URG4/URGCP* is a novel gene which plays an important role in cell cycle regulation and may be molecular target in cancer treatments.

In this study, the effect of FA in human TT medullary thyroid cancer cell line on cell cycle signal transmission, gene expression in apoptosis pathways, invasion, colony formation capacity, and wound healing was investigated in order to understand molecular mechanism and therapeutic activity.

## Materials and methods

### Cell culture

TT human thyroid cancer cell line (obtained from ATCC, USA) was used in this study. TT cells were grown in RPMI-1640 medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μg/ml), and 10 % (*v*/*v*) heat-inactivated fetal calf serum at 37 °C in a saturated humidity atmosphere containing 95 % air and 5 % $CO_2$. TT cells were treated with 50 μM, 75 μM, 100 μM, 150 μM, 200 μM, 300 μM, 400 μM, 500 μM, 750 μM, and 1 mM ferulic acid by solving in medium for 72 h, considering a time- and dose-dependent manner.

### Cytotoxicity assay

Cytotoxicity assays and determination of $IC_{50}$ dose of FA in TT cells were performed by using trypan blue dye exclusion test and CellTiter-Glo (CTG) assay as indicated in the manufacturers' instruction.

### CellTiter-Glo® luminescent cell viability assay

Cells were seeded in 96-well tissue culture plates and incubated for 24 h without reagent. After addition of reagents, cells were incubated for 24, 48, and 72 h and cell viability was assessed by using CTG mixture as recommended by supplier. ATP-based luminometric measurement from the metabolically active cells in the culture was determined by this method. ATP was quantified spectrophotometrically at 560 nm using luminometry. Viability was calculated using the background-corrected absorbance as follows:

$$\text{Viability}\ (\%) = \text{A of experiment well} \Big/ \text{A of control well} \times 100$$

### RNA isolation and real-time PCR

Total RNA was isolated from the cells exposed to $IC_{50}$ doses of ferulic acid with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by using Transcriptor First-Strand cDNA Synthesis Kit (Roche, Germany). *URG4/URGCP*, *CCND1*, *CDK4*, *CDK6*, *p53*, *PARP*, *PUMA*, *NOXA*, *BAX*, *BCL2*, *BID*, *CASP3*, *CASP9*, *MMP2*, *MMP9*, and *TIMP1* gene expression was performed on real-time RT-PCR according to the SYBR Green qPCR Master Mix (Thermo Scientific, USA) protocol. RT-PCR assay was performed using gene-specific primers. The expression results were proportioned to the *GAPDH* gene (housekeeping gene) expressions to calculate relative expression rations. Primer sequences are given in Table 1.

### Cell migration and invasion assay

Invasion activities of control and dose group cells were determined according to the BioCoat Matrigel Invasion Chamber guide (BD Biosciences). The cells with serum-free RPMI-1640 medium were seeded at a density of $2\times10^5$ cells/well onto the upper chambers of Matrigel-coated filter inserts and serum-containing RPMI-1640 medium (500 μl) was added to

**Table 1** Primer sequences of the genes used in this study

| Name | Primer sequence |
|---|---|
| *GAPDH* | F: TTCTATAAATTGAGCCCGCAGCC |
| | R: CCGTTGACTCCGACCTTCAC |
| *URG4/URGCP* | F: CGGGAGATGGGACAGTTTTA |
| | R: CATGGTGTTGAGGAGTGTGGG |
| *CCND1* | F:AGCTCCTGTGCTGCGAAGTGGAAAC |
| | R:AGTGTTCAATGAAATCGTGCGGGGT |
| *CDK4* | F: ATGTTGTCCGGCTGATGGA |
| | R: CACCAGCGTTACCTTGATCTCCC |
| *CDK6* | F: AGACCCAAGAAGCAGTGTGG |
| | R: AAGGAGCAAGAGCATTCAGC |
| *P53* | F: ATCTACAAGCAGTCACAGCACAT |
| | R: GTGGTACAGTCAGAGCCAACC |
| *PARP* | F:ACACCCCTTGCACGTACTTC |
| | R:GATGGGTTCTCTGAGCTTCG |
| *PUMA* | F: GACCTCAACGCACAGTACGAG |
| | R:AGGAGTCCCATGATGAGATTGT |
| *NOXA* | F:ACCAAGCCGGATTTGCGATT |
| | R:ACTTGCACTTGTTCCTCGTGG |
| *BAX* | F: AGAGGATGATTGCCGCCGT |
| | R: CAACCACCCTGGTCTTGGATC |
| *BCL2* | F: TTGGCCCCCGTTGCTT |
| | R:CGGTTATCGTACCCCGTTCTC |
| *BID* | F: CCTACCCTAGAGACATGGAGAAG |
| | R: TTTCTGGCTAAGCTCCTCACG |
| *CASP3* | F: GCAGCAAACCTCAGGGAAAC |
| | R: TGTCGGCATACTGTTTCAGCA |
| *CASP9* | F: GGCTGTCTACGGCACAGATGGA |
| | R: CTGGCTCGGGGTTACTGCCAG |
| *MMP2* | F:TCTCCTGACATTGACCTTGGC |
| | R:CAAGGTGCTGGCTGAGTAGATC |
| *MMP9* | F:CCTTGTGCTCTTCCCTGGAG |
| | R:GGCCCCAGAGATTTCGACTC |
| *TIMP1* | F:ACCATGGCCCCCTTTGAGCCCCTG |
| | R:TCAGGCTATCTGGGACCGCAGGGA |

the lower chambers. Then, the cells were incubated at 37 °C for 24 h. After of incubation, filter inserts were removed from the wells. The cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with crystal violet. The cells that invaded the lower surface of the filter were counted under a light microscope. Each experiment was repeated three times. Percentage of invasion was calculated using control and matrigel membrane cell count as follows:

$$\text{Invasion}\ (\%) = \frac{\text{The number of cells in matrigel matrix basement membrane}}{\text{The number of cells in control membrane}} \times 100$$

### Colony assay

For colony formation analysis, the cells were digested with trypsin, counted using trypan blue dye exclusion test, and seeded in six-well plates at a density of $10^3$ cells per well. The medium was changed every 3 days for 10 days until visible colonies formed. Colonies were fixed methanol for 10 min and stained with crystal violet.

### Wound healing assay

The control and dose group cells were plated at $10^6$ cells per well of 60×15 mm style cell culture dishes and grown overnight at 37 °C with 5 % $CO_2$. The 80 % confluent control group and dose group cells were treated with 150 μM ferulic acid after a straight line scratch was made on a confluent monolayer of cells using a sterile 200-μl plastic pipette tip. To remove debris and smooth the edge of the scratch, the cells were washed with 2 ml serum-free RPMI-1640. Images of the TT cell proliferation were taken at 0, 16, and 24 h after the scratch. The scratch assay was performed in triplicate.

### Statistical analysis

The analysis of the findings has been made with the ΔΔCT method and quantitated with a computer program. The comparison of the groups has been performed with "Volcano Plot" analysis, from "RT$^2$ProfilesTMPCR Array Data Analysis," which is assessed statistically using the "Student's *t* test." Moreover, parametric and nonparametric analysis of doses and controls have been evaluated with the SPSS 17.0 statistical analysis program ($p < 0.05$ is significant statistically).

## Results

### Cytotoxic activity—CellTiter-Glo assay

TT cell death upon treatment with FA was assessed by the CellTiter-Glo assay. Time- and dose-dependent decrease patterns were found in the viability of TT cells. For this purpose, the expression changes of genes are evaluated by treating different concentration of FA with at the 24th, 48th, and 72nd hours in TT cells. In our study, $IC_{50}$ doses (inhibitory concentration where 50 % of the cells die) in the TT cells were detected as 150 μM at 72nd hour by CTG assay (Fig. 1).

### Real-time PCR

After total RNA was isolated from control and ferulic acid-treated cells, the cDNA synthesis have been performed by using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany). The expression analysis of *URG4/*

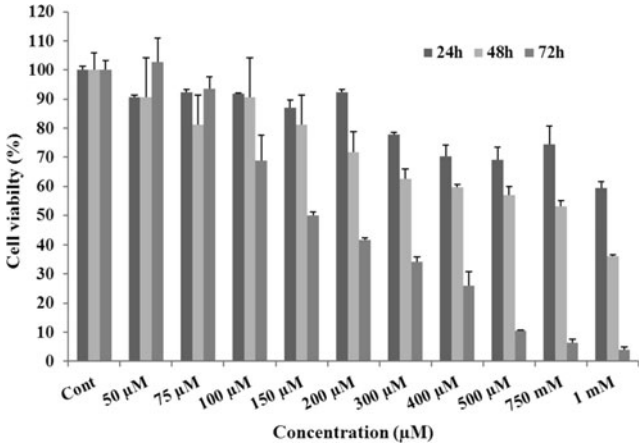


**Fig. 1** Effect of FA on the viability of TT cells. The cells were treated with FA and at different concentrations and time intervals, and their proliferation was assessed by CTG assay. Data are the average results of three independent experiments. *$IC_{50}$ dose of ferulic acid in TT thyroid cancer cells was detected 150 μM at 72nd hour

*URGCP*, *CCND1*, *CDK4*, *CDK6*, *p53*, *PARP*, *PUMA*, *NOXA*, *BAX*, *BCL2*, *BID*, *CASP3*, *CASP9*, *MMP2*, *MMP9*, and *TIMP1* were studied on real-time RT-PCR according to the SYBR Green qPCR Master Mix (Thermo Scientific) protocol. Real-time PCR analysis showed that *URG4/URGCP*, *CCND1*, *CDK4*, *CDK6*, *BCL2*, *MMP2*, and *MMP9* expression was reduced in dose group cells, compared with the control group cells. *p53*, *PARP*, *PUMA*, *NOXA*, *BAX*, *BID*, *CASP3*, *CASP9*, and *TIMP1* expression was increased in dose group cells, compared with the control group cells (Table 2; $p<0.05$).

**Table 2** The mRNA expressions change of genes relative to *GAPDH* mRNA expression were studied on real-time PCR

| | Gene name | Fold change ($p<0.05$) |
|---|---|---|
| 1 | *URG4/URGCP* | −8.0743 |
| 2 | *CCND1* | −2.5915 |
| 3 | *CDK4* | −2.1959 |
| 4 | *CDK6* | −3.4759 |
| 5 | *p53* | 11.5497 |
| 6 | *PARP* | 3.7724 |
| 7 | *PUMA* | 4.8130 |
| 8 | *NOXA* | 2.9093 |
| 9 | *BAX* | 2.8586 |
| 10 | *BCL2* | −14.357 |
| 11 | *BID* | 2.9675 |
| 12 | *CASP3* | 2.9639 |
| 13 | *CASP9* | 2.8091 |
| 14 | *MMP2* | −18.6117 |
| 15 | *MMP9* | −2.2969 |
| 16 | *TIMP1* | 2.4225 |

### Migration and invasion assay

By matrigel invasion chamber assay, the cell invasion was significantly inhibited in the dose-treated group, compared with the control group. Invasive cells are shown in Fig. 2a. Data of invasion % of the two groups were shown as follows: control group 52±3.32 % and dose group 28±1.6 % (Fig. 2b).

### Colony formation assay

Colony formation analysis performs by using colony formation assay. It is observed that colony formation decreased in the 150 μM FA-treated group, compared with the control group (Fig. 3a). Data of colony numbers of the two groups were shown as follows: control group (502.3±18.4) and FA group (205.4±12.16) (Fig. 3b).

### Wound healing assay

Effects of FA on cell migration were detected by wound healing assay. According to results, FA reduced cell migration in TT cells, compared with the control group. Figure 4 shows the images at 0, 16, and 24 h.

## Discussion

Cancer therapy is difficult due to the resistance that cancer cells develop to chemotherapeutic drug and its toxicity, despite developing treatment methods. A number of phytochemicals can affect genes that have important role in cell cycle, apoptosis, DNA damage, invasion, and metastasis [19]. FA, also one of the common phytochemical phenolic acids, has properties such as anti-inflammatory, antiviral immunoprotective, and antioxidant [20–22]. Pharmacological properties of FA such as antimicrobial, anti-inflammatory, antioxidant, antimutagenic, and anticarcinogenic have been investigated; however, limited information is available in the literature on this subject. In the present study, it was aimed to investigate the molecular mechanism and effects of FA in human TT medullary thyroid cancer cell line. Karthikeyan et al. reported that FA enhanced the effects of radiation by decreasing cell viability, survival, antioxidant status, and colony formation, and increasing intracellular ROS levels, lipid peroxidation markers, and oxidative DNA damage in HeLa and ME-80 human cervical carcinoma cells [23]. On the other hand, Prasad et al. showed that pretreatment with FA protected the human blood lymphocytes, normal cells, by preventing the decreases in the radiation-induced glutathione, superoxide dismutase, catalase, and glutathione peroxidase activities [14]. In the previous study, it was shown that FA significantly suppressed the proliferation of cells by blocking the cell cycle in G0/G1 phase in ECV304 human umbilical vein endothelial

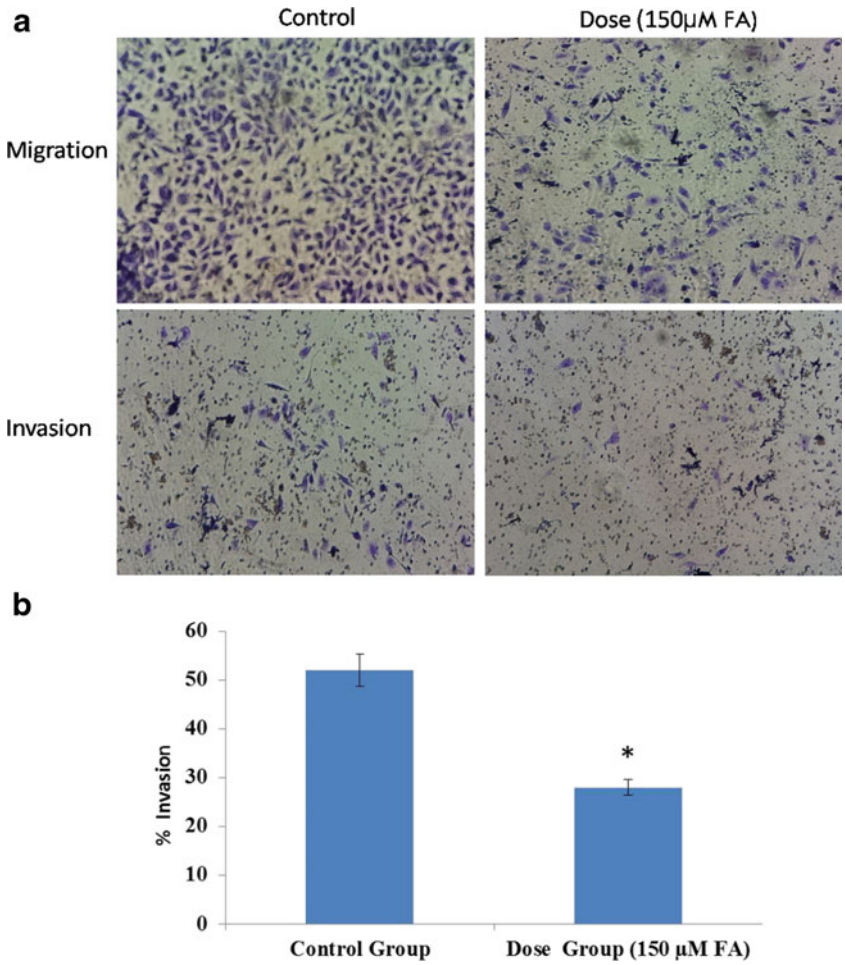


**Fig. 2** **a** Migration and invasion assay of TT cells. Cells that passed through the membrane were counted in ten representative areas. **b** Summary graph for invasion were also shown respectively. Data was presented as mean±SD, $n=3$, $^*p<0.05$

cell line. It was also reported that the inhibition of cell proliferation was associated with the decrease of CCND1 expression and phosphorylation of retinoblastoma protein, and the increase of p21 expression [24]. Tan et al. reported that FA was found at the highest level from selected polyphenolic compounds in brewers' rice by using ultra performance liquid chromatography. Moreover, they showed that $IC_{50}$ dose of brewers' rice extract was 21.88±12.43 μg/ml in HT-29 human colorectal cancer cell lines and it decreased cell proliferation via the induction of apoptosis [25].

In the present study, cytotoxic effects of FA in TT cells were detected in a time- and dose-dependent manner by CellTiter-Glo assay and $IC_{50}$ dose of FA in TT cells was found as 150 μM at 72nd hour. Moreover, in the present study, the anticancer mechanism and anti-invasive effect of FA were demonstrated by analyzing the expression of novel gene *URG4/URGCP*, *CCND1*, *CDK4*, *CDK6*, *p53*, *PARP*, *PUMA*, *NOXA*, *BAX*, *BCL2*, *BID*, *CASP3*, *CASP9*, *MMP-2*, *MMP-9*, and *TIMP-1* in TT cells.

In our previous study, we showed that FA suppressed the invasion and inhibited cell proliferation via genes that have important role in cell cycle, apoptosis, and DNA damage in PC-3 and LNCaP human prostate cancer cell lines [26]. Çıtışlı et al. showed the temozolamide and *URG4/URGCP* relationship in SH-SY5Y human neuroblastoma cells. They reported that temozolamide inhibited cell proliferation by inducing cell cycle arrest via *CCND1*, *CCND2*, *CDK4*, and *URG4/URGCP* gene expression changes and stimulating apoptosis in SH-SY5Y neuroblastoma cells [27]. This study tried to determine how FA affects novel gene *URG4* in TT human thyroid cancer cell line. According to this study, the mRNA expression of *URG4/URGCP*, *CCND1*, *CDK4*, and *CDK6* were significantly downregulated in FA-treated cells compared with control cells ($p<0.05$). Satiroglu-Tufan et al. reported that proliferation of HepG2 hepatoblastoma cells was suppressed when *URG4/URGCP* was downregulated through RNA interference-mediated silencing. Moreover, it was also shown that overexpression of *URG4/URGCP* increased *CCND1* mRNA expression, whereas downregulation of *URG4/URGCP* decreased *CCND1* mRNA expression [28]. Therefore, it may be suggested that FA caused cell cycle arrest by modulating gene expressions of *URG4/URGCP*, *CCND1*, *CDK4*, and *CDK6* in TT human thyroid cancer cell line. Anticancer effect of FA has been investigated; however, until now, there has been no investigation on *URG4/URGCP* expression in TT cells and also FA. Moreover, limited studies related to *URG4/URGCP* are available in the literature.

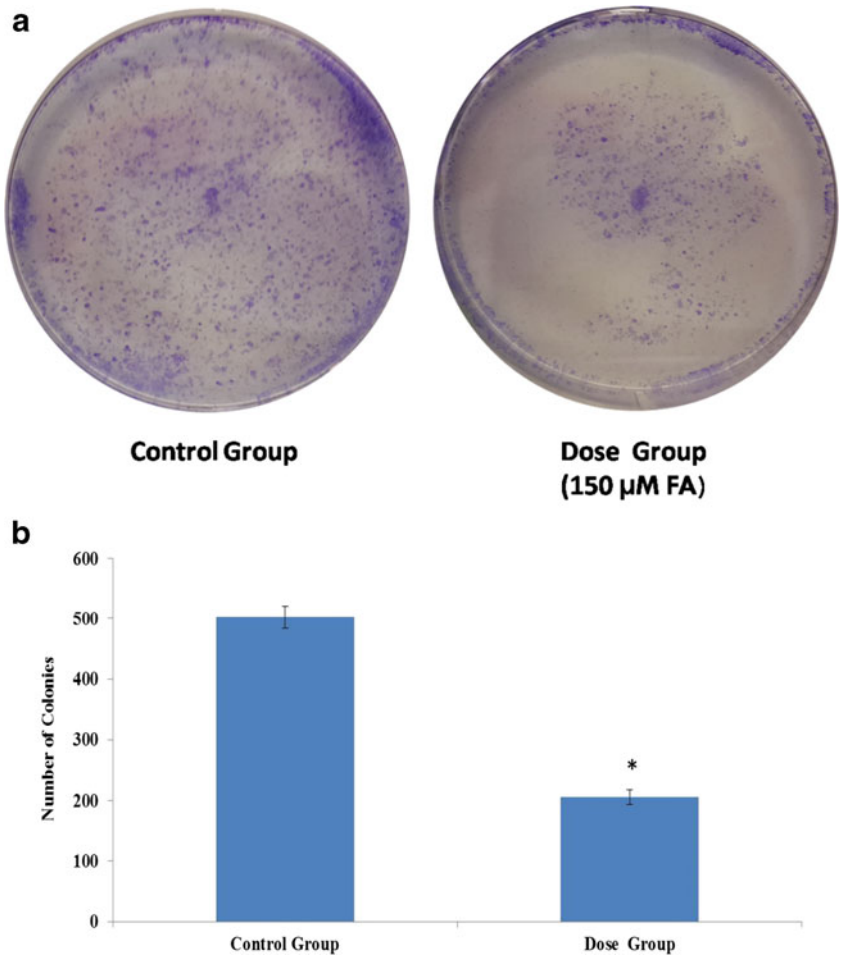


**Fig. 3** **a** FA decreases TT cells' colony formation; **a** colonies were stained with crystal violet. **b** The number of colonies was significantly decreased in cells treated with ferulic acid compared with the control cells. Data are expressed as mean±SD, $n=3$, $^{*}p<0.05$

In the previous a study, it was reported that the combination of FA and 2-deoxy-D-glucose induced apoptosis by modulating *TP53*, *CDKN1A (p21)*, *nuclear factor kB (NFkB)*, *BAX*, and *CASP3* in NCI-H460 lung cancer cells [29]. *PARP* and *p53* genes have an important role in response to DNA damage. The members of BCL2 family include proapoptotic and antiapoptotic proteins. Whereas PUMA, NOXA, BAX and BID are proapoptotic members, BCL2 is an antiapoptotic member. PUMA and NOXA might mediate the apoptosis by inducing via p53 [30]. *Caspases* associated with apoptosis is initiator caspases and the executioner caspases. Whereas CASP9 is one of the initiator caspases, CASP3 is one of the executioner caspase in apoptosis pathway [31]. In the present study, a significant increase in the expression of *p53*, *PARP*, *PUMA*, *NOXA*, *BAX*, *BID*, *CASP3*, and *CASP9* genes, and a decrease in the expression of *BCL2* were seen in the FA

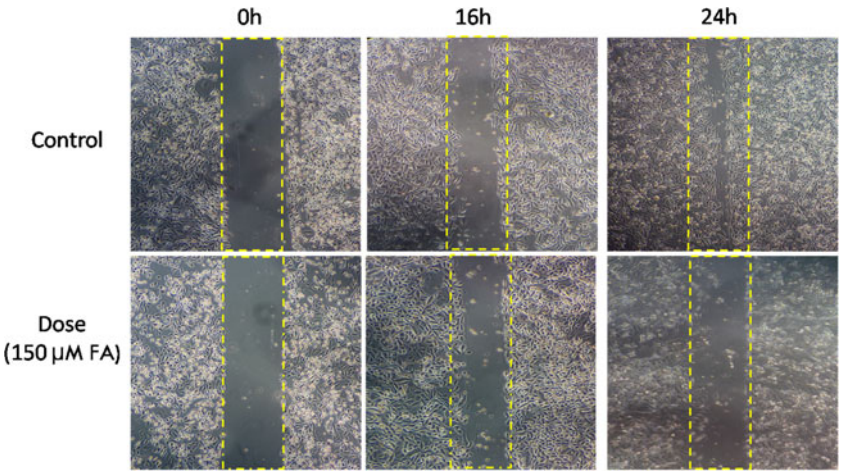


**Fig. 4** Wound healing assay results showed that ferulic acid reduced cell migration. Control and dose (150 μM FA) images at 0, 16, and 24 h were given

treatment group, compared with the control group in TT human thyroid cancer cell line. The increase in the expression of tumor suppressor and apoptotic genes and the decrease in expression of antiapoptotic proteins showed that FA had apoptotic activity in TT cells.

Metastasis is a significant process for tumor progression and tumor recurrence. Invasion and migration of cancer cells is important characteristic of malignant tumors for metastasis. The upregulation of MMPs in cancer has been correlated with tumor progression and metastasis [32]. In the previous study, it was shown by immunohistochemistry that the expression of VEGF-C and MMP2 proteins was upregulated in metastatic compared to nonmetastatic thyroid carcinoma [33]. Xing et al. reported that the overexpression of *URG4/URGCP* significantly increased the migration of human umbilical vein endothelial cells (HUVECs) and angiogenic capacity of hepatocellular carcinoma cells, and knockdown of *URG4/URGCP* decreased of angiogenic capacity of hepatocellular carcinoma cells [34]. Yuan et al. showed that FA could inhibit vascular smooth muscle cells migration in vascular endothelial growth factor (VEGF) induced conditions by inhibiting the *MMP-9* mRNA expression, and increasing the TIMP-2 protein expression [35]. In the previous study, it was observed that feruloyl-L-arabinose (FAA), active FA derivative, inhibited the migration and invasion in H1299 human lung cancer cells. It was suggested that FAA-mediated inhibition on migration and invasion of H1299 cells might be achieved by decreasing the MMP2 and MMP9 activities [36].

In the present study, a significant decrease in the expressions of *MMP-2* and *MMP-9* genes that have role in the degradation of the ECM, and an increase in the expression of *TIMP1*, inhibitor of metalloproteinases, were seen in the FA treatment group, compared with the control group in TT human thyroid cancer cell line. Moreover, it was also observed that FA significantly reduced cell invasion, cell migration, and colony formation dose compared with control cells in this study. FA may have also caused the decrease in cell invasion, cell migration, and colony formation by reducing the expression of *URG4/URGCP* gene.

In conclusion, genes that have important role in cell cycle, response to DNA damage, apoptosis, invasion, and migration have been seen as potential targets in order to find new therapeutic drug in the treatment of cancer. According to the results of this study, FA inhibits cell proliferation by inducing cell cycle arrest and apoptosis, and also decreases invasion, migration, and colony formation in TT medullary thyroid cancer cell line. Moreover, the interaction of FA and *URG4/URGCP* in TT medullary thyroid cancer cell line was firstly shown in this study. As a result, FA may be a novel candidate for the treatment of thyroid cancer. Therefore, it is necessary to conduct further studies with other cell lines and in vivo animal models to discover therapeutic effect and the molecular mechanism of FA on thyroid cancer.

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