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ORIGINAL ARTICLE

Suppression of Metastasis by *Citrus* Auraptene in a Mouse Model of Colorectal Cancer

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Abstract

Despite new approaches in adjuvant chemotherapy, colorectal cancer metastasis is still a major health concern worldwide. Herbal medications with anti-tumor effects are considered a suitable alternative for the treatment of malignancies. Auraptene, an abundant natural coumarin, exhibits anti-cancer activity against various types of cancer. However, the mechanisms by which auraptene impacts cancer invasion and metastasis remain unknown. The current study aimed to assess a possible inhibitory role of auraptene in colorectal cancer metastasis in a mouse model. The anti-proliferative and anti-migratory effects of auraptene were evaluated by 3–4,5-dimethylthiazol-2-yl-2,5 diphenyltetrazolium bromide (MTT) and cell migration assays, respectively. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) technique was used to determine the gene expression levels of matrix metalloproteinase (MMP)-2, MMP-9, E-cadherin, and vascular endothelial growth factor A (VEGF-A) and its cognate receptor (vascular endothelial growth factor receptor 1, VEGFR1) in tumor samples. Moreover, the western blot analysis was conducted to examine the expression of MMP-2 and MMP-9 proteins. According to the *in vitro* results, auraptene inhibited the proliferation and migration of CT26 cells. Furthermore, auraptene decreased the rate of metastasis by modulating the expression of MMP-2, MMP-9, E-cadherin, VEGF-A, and VEGFR1 genes. Accordingly, the expression levels of MMP-2 and MMP-9 proteins were significantly reduced. In addition, the pathological analysis revealed a marked degree of necrosis in tumor cells treated with auraptene. Based on our results, auraptene is likely to be a potent therapeutic agent for the treatment of metastatic colorectal cancer and may be a suitable candidate for adjuvant therapy due to its anti-metastatic effects.

Keywords Colorectal cancer · Matrix metalloproteinases · Angiogenesis · Metastasis

Introduction

Colorectal cancer is the second leading cause of cancer death and the third most commonly diagnosed cancer worldwide (Sung et al. 2021). Despite the rapid development of chemotherapeutic compounds over the past decade, metastasis is still a serious challenge in the treatment of colorectal cancer (CRC) (Valderrama-Treviño et al. 2017). 5-Fluorouracil (5-FU), which is the gold standard medication for the treatment of CRC, possesses numerous therapeutic effects; however, some obstacles, such as the high recurrence rate, have enhanced the need for alternative therapies (Fong et al. 1999). The rate of cell division is very high in cancer cells and these cells have the ability to escape from cell death (*i.e.*, apoptosis). Metastasis is more likely to occur when the size of the primary tumor grows. Therefore, inhibiting cancer cell proliferation

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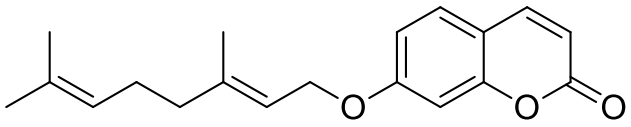
seems to be the critical first step in the progression of metastatic cancer (Weinberg and Weinberg 2013). Moreover, it is now established that a number of proteins are involved in the metastasis and invasion of cancer cells. Matrix metalloproteinases (MMPs) are a group of enzymes that digest the extracellular matrix and are responsible for the metastasis and invasion of cancer cells (Hooper et al. 2002). In addition to their proteolytic functions in physiological processes, MMP-2 and MMP-9 are also highly expressed in CRC, and some studies suggest that these two serum MMPs could be used as biomarkers of CRC invasion (Tutton et al. 2003; Said et al. 2014). It is worth noting that these two MMPs could be applied to predict the recurrence and invasion of CRC (Tutton et al. 2003; Araújo et al. 2015). E-cadherin is another key protein involved in cell-cell adhesion and tissue integrity (Deryugina and Quigley 2006). According to research, MMP-9 is closely related to E-cadherin since MMP-9 can cleave the ectodomain of E-cadherin, allowing cancer cells to migrate to other distant tissues/organs (Symowicz et al. 2007).

Angiogenesis or vascularization is one of the significant stages of cancer metastasis, and it has been shown that targeting this biological process could improve the treatment course of patients with CRC (Lopez et al. 2019). Vascular endothelial growth factor A (VEGF-A) and its cognate receptor (vascular endothelial growth factor receptor 1, VEGFR1) serve a crucial function in the survival of endothelial cells and the regulation of angiogenesis (Bhattacharya et al. 2017). Studies have indicated that VEGF-A is the most critical member of the VEGF family involved in the dissemination of tumor cells (George et al. 2001; Onogawa et al. 2004).

Natural or synthetic products that inhibit the function of these proteins with minimal side effects may be useful in limiting the metastatic spread of CRC. Auraptene (1,7-geranyloxycoumarin, **1**) is a monoterpene coumarin found in Rutaceae and Apiaceae families, especially in *Citrus* species, which is native to South and East Asia (Genovese and Epifano 2011; Kumar et al. 2012). In animal models of various cancers, including gastric, prostate, breast, and liver malignancies, auraptene appears to have numerous therapeutic effects (Derosa et al. 2016). With its antioxidant and chemopreventive properties, auraptene suppresses superoxide formation, reduces lipid peroxidation, and modulates glutathione *S*-transferase activity in mice (Tanaka et al. 1998). It was shown that the oral administration of auraptene prevented the metastasis of melanoma cells to the lungs and inhibited the growth of the metastatic tumor in mice (Tanaka et al. 2000). Hayashi et al. (2007) reported that auraptene reduced the risk of colon cancer in mice through the suppression of cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) and induced apoptosis in colon epithelial cells.



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The present study aimed to examine the inhibitory function of auraptene isolated from *Citrus* fruits in the proliferation and migration of CT26 cells as well as evaluate the anti-metastatic effects of this compound on the expression levels of MMP-2, MMP-9, E-cadherin, VEGF-A, and VEGFR1. To the best of our knowledge, this is the first report assessing the impacts of auraptene on metastasis in CT26 tumor-bearing mice.

Materials and Methods

Chemicals and Reagents

Auraptene was purchased from Tinab Shimi (purity of 96%; Mashhad, Iran; Lot number: T49500203). Dimethyl sulfoxide (DMSO), penicillin-streptomycin, and trypsin-EDTA were procured from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and RPMI 1640 medium were obtained from Gibco (Grand Island, NY, USA). The total RNA extraction kit was purchased from Yekta Tajhiz Azma (Tehran, Iran). The ExcelRT™ Reverse Transcription Kit II for the synthesis of cDNA was obtained from SMOBIO Technology, Inc. (Taiwan). For western blot analysis, all primary antibodies against MMP-2, MMP-9, and β-actin were acquired from ZellBio GmbH (Germany).

Cell Culture

The CT26 cell line was procured from the National Cell Bank of Iran (the Pasteur Institute, Tehran, Iran). The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS and 1% streptomycin/penicillin at 37 °C in 5% CO_2 atmosphere. The cells were passaged utilizing trypsin-EDTA in their exponential phase.

Growth Inhibition Assay

The inhibitory effect of auraptene on cell growth was assessed by the 3–4,5-dimethylthiazol-2-yl-2,5 diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). For this purpose, CT26 cells (10×10^3 cells/well) were incubated overnight. Then, the cells were treated with auraptene

(12.5–500 µM) for 48 h. After that, 10 µl of the MTT reagent stock (5 mg/ml) was added to each well. The respective supernatants were removed after 3 h, and 100 µl of DMSO was added to each well to dissolve the formazan crystals. The microplates were shaken gently for 40 min in the dark at room temperature, and the absorbance was measured at 545 and 630 nm (background) using a Stat FAX303 plate reader. The IC_{50} value was determined using GraphPad Prism software. All treatments were carried out in triplicate.

Cell Migration Assay

The wound healing assay was conducted to determine the effect of auraptene against the migration of CT26 cells according to the method described previously (Khorsand et al. 2022). A total of 2.5×10^5 cells/well were treated with auraptene at a concentration of 200 µM in a 24-well plate. The process of cell migration assay was monitored, and the cells were imaged under a microscope secured with a digital camera (CAMV1200SC, Canada). The imaging procedure was carried out at various time intervals (0, 6, 10, 20, and 24 h). The experiment was performed in triplicate and then repeated three times. In this method, cells were treated with 5-FU as the positive control.

Animal Model of CRC

Female Balb/C mice (6–8 weeks old, $n = 50$) were purchased from the Razi Institute (Karaj, Iran) and kept under standard laboratory conditions (constant humidity, room temperature of 20–25 °C, and a 12-h light/dark cycle). All animal experiments were performed according to the guidelines provided by the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1399.744). CT26 colon adenocarcinoma cells (1×10^6) were implanted subcutaneously (*s.c.*) into the right flank of 18–20-week-old female Balb/c mice (weight range: 19–21 g). The size of the tumor was calculated using the formula $1/2 \times \text{length} \times \text{width}^2$, and when it reached around 85 ± 50 mm^3, the animals were randomly divided into five groups ($n = 8$ per group): (i) the control group (sham control) with intratumoral injection of 100 µl of normal saline and DMSO as an auraptene solvent, (ii) the positive group subjected to the intraperitoneal injection (*i.p.*) of 5 mg/kg 5-FU, and (iii, iv, v) auraptene groups with intratumoral injection of auraptene at the concentrations of 50, 100, and 200 µM. The injection process was conducted every other day for 14 days. After this time period, the animals were sacrificed, and whole tumors were collected and kept at −80 °C for further analyses (Fig. 1).

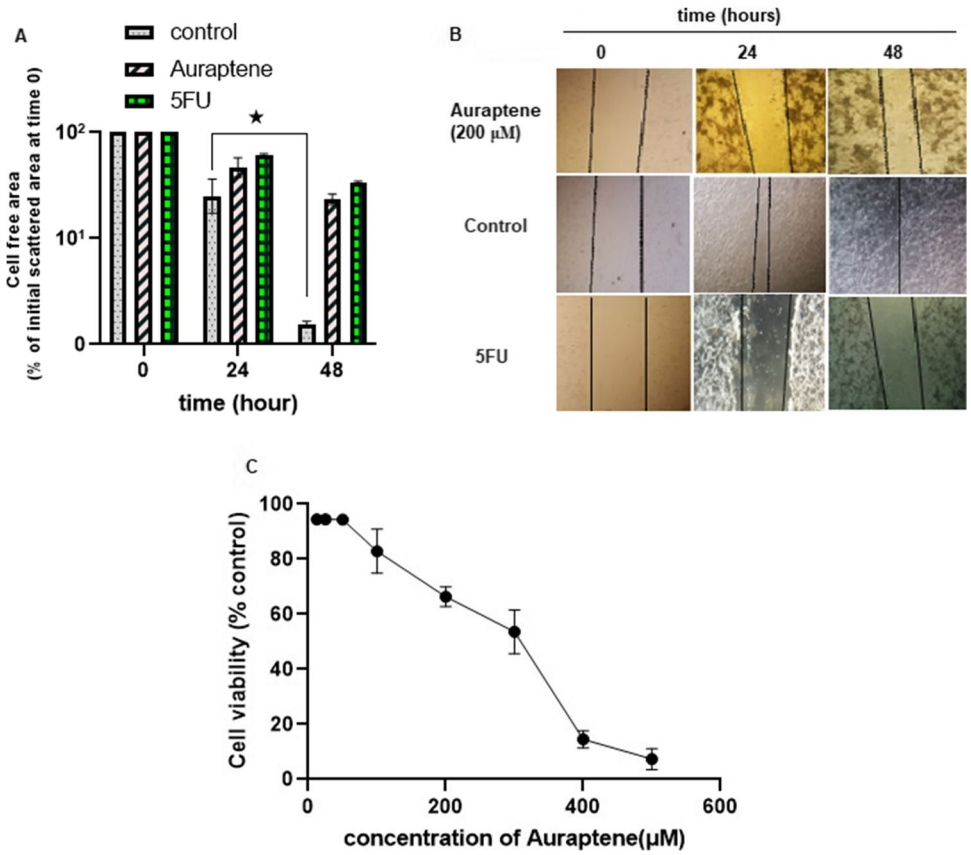

Fig. 1 Anti-proliferative and anti-migratory effects of auraptene on CT26 cells. **A** The graph indicates the percentage of the cell-free area at certain time points during the *in vitro* migration assay. **B** Representative images from the migration assays of CT26 cells. C Growth suppression after 48 h of treatment with auraptene. Data are expressed as the mean ± SEM of three independent experiments (*$p < 0.05$)

Quantitative Real-Time Polymerase Chain Reaction (PCR)

According to the manufacturer's instructions, total RNA was isolated and converted into cDNA using the RNA extraction kit. The expression of all genes was examined by real-time PCR (Table 1). All PCR were performed on Applied Biosystems (ABI) with the following cycling conditions: 95 °C for 15 min followed by 30–40 cycles of 95 °C for 30 s and 55–60 °C for 60 s (Mogharrabi et al. 2020). The relative expression of the genes was normalized against β-actin as an endogenous control. The relative fold change of these mRNAs was determined by the Pfaffl method, as described previously (Pfaffl 2001).

Western Blot

The protein levels of MMP-2 and MMP-9 were assessed using the western blot method according to the study by Mohammadi et al. (2020). Tumor samples were homogenized in ice-cold RIPA buffer, and the bicinchoninic acid method (BCA kit, Pars Tous, Iran) was applied to determine the protein concentration. Electrophoresis was carried out on equal amounts of proteins. The immunoblotting bands were visualized using a Pars Tous chemiluminescence detection kit (Pars Tous Biotechnology, Iran). The relative expression was compared to the β-actin protein expression using ImageJ software (NIH, Bethesda, Rockville, MD, USA).

Histological Analysis

Tumor samples were fixed in freshly prepared 4% formalin. Then, tumor tissues were processed in a graded alcohol series and paraffin-embedded at 56 °C. All tissue blocks were sectioned at a thickness of 5–7 µm and then stained with hematoxylin and eosin (H&E) (Abcam, USA). Finally, samples were evaluated under a light microscope (Cardiff et al. 2014).

Statistical Analysis

Data are expressed as the means and standard error of the mean (mean ± SEM). The differences between the groups were analyzed by one-way analysis of variance (one-way ANOVA), followed by Dunnett's post hoc test. The migration assay results were statistically compared using repeated measures of ANOVA. A p-value of less than 0.05 was considered statistically significant. The obtained values were statistically analyzed using GraphPad Prism version 8.0. All experiments were performed in triplicate and independently.

Results

Auraptene Inhibited Cell Proliferation and Migration in CT26 Cells

In order to investigate the anti-proliferative effects of auraptene, the MTT assay was carried out. The results revealed that auraptene suppressed cell growth in a dose-dependent manner (Fig. 1C). According to the scratch mobility results, auraptene significantly reduced cell migration into the cell-free area after 24 h compared to the control cells (Fig. 1A, B, $p < 0.05$).

Auraptene Suppressed Colorectal Xenograft Tumor Growth *In Vivo*

As shown in Fig. 2B, C, the size of tumors in mice treated with 200 µM auraptene and 5-FU was significantly smaller than that of the control mice ($p < 0.05$).

Administration of Auraptene Decreased the Expression Levels of MMP-2, MMP-9, E-cadherin, VEGF-A, and VEGFR Genes

The results obtained from the real-time PCR method demonstrated that treatment with 100 and 200 µM auraptene led to the greatest reduction in the expression levels of MMP-2 and VEGF-A genes, by 0.009 and 0.012 times for MMP-2 ($p < 0.0001$) and 0.8 ($p < 0.01$) and 0.6 times ($p < 0.0001$) for VEGF compared with the control group, respectively (Fig. 3A, C). Moreover, the expression levels of MMP-9 and VEGFR genes declined in a dose-dependent manner (Fig. 3B, D). Auraptene at concentrations of 100 and 200 µM elevated the expression level of E-cadherin by 19.5

Table 1 The sequences of specific primers used for the amplification of MMP-2, MMP-9, VEGF-A, VEGFR, E-cadherin, and actin genes

Genes	Forward primers	Reverse primers
MMP-2	5′- AACTGTTGCTTTTGTATGCCCT -3′	5′- CGATGTCAGACAACCCGAGT -3′
MMP-9	5′- GCG TCGTGATCCCCACTTAC -3′	5′- CAGGCCGAATAGGAGC GTC -3′
VEGF-A	5′- AGTGATCAAGTTCATGGATGT -3′	5′- GATGTACTCTATCTCGTCGGGG -3′
VEGFR	5′- TGCTTCTCACAGGATATGGCT -3′	5′- AGAAGAGAGTCTGGCCTGC -3′
E-cadherin	5′- GTCTACCAAAGTGACGCTGA -3′	5′- GGGAAACATGAGCAGCTCTG -3
Actin	5′- CTGTCC CTGTATGCCTCTG -3′	5′- CTGTCC CTGTATGCCTCTG -3′

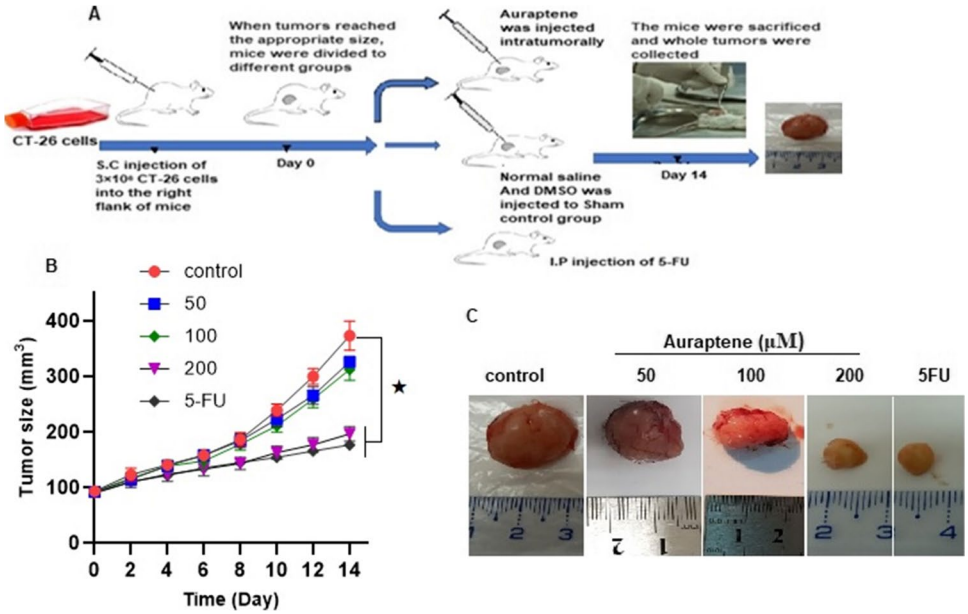

Fig. 2 Therapeutic efficacy of auraptene in mice bearing subcutaneous CT26 cell tumor. **A** Schematic view of the study procedures. **B** Average tumor volume in each group. **C** Images of mice tumors on day 14 (*$p < 0.05$ vs. control)

($p < 0.05$) and 115 ($p < 0.0001$) times in comparison with the control group, respectively (Fig. 3E).

Intratumoral Administration of Auraptene Reduced the Protein Expression of MMP-2 and MMP-9

As displayed in Fig. 4A, the protein expression of MMP-2 in the control group was high and visualized as an intense band. Protein bands were also observed in samples treated with 50 µM and 100 µM auraptene, whereas no bands were detected in samples treated with 5-FU or 200 µM auraptene. Consistent with our findings in gene expression analysis, the protein expression level of MMP2 was diminished by 0.63 times in samples treated with 50 µM auraptene ($p < 0.001$) and by 0.13 times in those treated with 100 µM auraptene ($p < 0.0001$) compared with the control group (Fig. 4B).

According to Fig. 4A, auraptene at the concentration of 200 µM and 5-FU significantly reduced the expression of the MMP-9 protein by 0.17 and 0.04 times compared with the control group ($p < 0.0001$, Fig. 4B). These results suggested that auraptene had a higher impact on the expression of MMP-2 than MMP-9.

Pathological Findings

The stained slides were evaluated by an expert pathologist. There was detectable tumor calcification and necrosis in the tumor tissue in groups treated with 100 and 200 µM auraptene, as well as 5-FU, compared to the control group, whereas tumor cells in other groups did not exhibit morphological changes. These findings indicated that auraptene can inhibit tumor growth and induce necrosis in colorectal tumor cells (Fig. 5).

Discussion

In CRC, as in many types of cancer, metastasis has a significant impact on patient survival and reduces the effectiveness of treatment (Engstrand et al. 2018). In the current study, the anti-metastatic properties of auraptene were evaluated. It was found that auraptene dramatically reduced the tumor size in mice bearing CT26 cells. Moreover, auraptene inhibited the growth and migration of colon cancer cells in monolayer cells. Afshari et al. (2019) showed that the anti-proliferative potential of auraptene was because of its ability to induce cell cycle arrest and apoptosis. In their study, auraptene inhibited the migration of human malignant glioblastoma cells. Another study reported that auraptene prevents the proliferation of MCF-7 cells by targeting genes related to the S-phase of the cell cycle, such as cyclin D1 and insulin-like growth factor 1 (IGF-1) as the stimulators of this phase (Krishnan and Kleiner-Hancock 2012). The results of the study by Jamialahmadi et al. (2018) indicated that the anti-migratory effects of auraptene were due to its ability to suppress the activity of MMP-2 and MMP-9. In order to determine the anti-metastatic potential of auraptene, we evaluated the effects of auraptene on the expression of MMP-2 and MMP-9 in tumor samples. Our study demonstrated that auraptene diminished metastasis by decreasing the expression of MMP-2 and MMP-9 at both gene and protein levels. Our findings showed that the suppression of MMP-2 mRNA was more pronounced than MMP-9 suppression at both gene and protein levels. These results corroborated those of previous studies suggesting that the dysregulation of MMP-2 could be the first sign of CRC tumorigenesis, while MMP-9 dysregulation could be attributed to neovascularization (Araújo et al. 2015). According to the study by

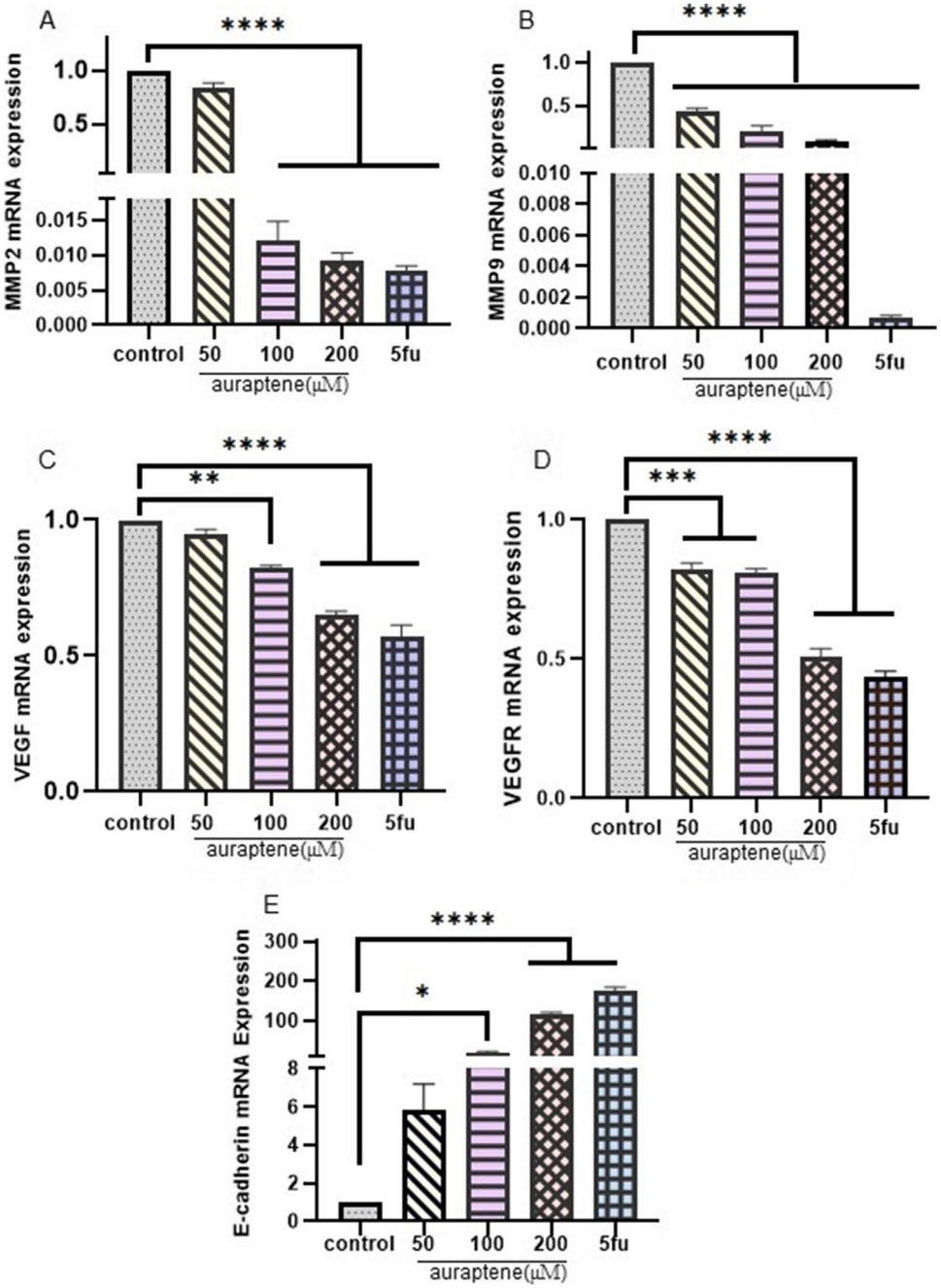

Fig. 3 The impact of auraptene on the expression of anti-metastatic and anti-angiogenic genes. The expression rates of **A** MMP-2, **B** MMP-9, **C** VEGF-A, **D** VEGFR, and **E** E-cadherin genes; data are represented as the mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ denote significant differences compared with the control group

Kawabata et al. (2006), when mice received dextran sulfate sodium (DSS), which causes ulcerative colitis, the activity of MMP-2, MMP-9, and MMP-7 was decreased in the mucosa in response to auraptene treatment. In their study, the relative activity of MMPs was assessed by gelatin zymography. They indicated that in mice receiving auraptene for 2 weeks, the activity of MMP-2 and MMP-9 was decreased by 100 and 80%, respectively. In addition, they showed that DSS administration increased MMP-2 and MMP-9 activities by 13 and 31 times, respectively. They also reported that auraptene might have anti-metastatic properties and be beneficial in the treatment of CRC. In another study, the effect of auraptene on the invasion and metastasis of cervical (HeLa) and ovarian (A2780) cancer cells was assessed. In that study, auraptene suppressed the activity of MMP-2 and MMP-9 in a dose-dependent manner at all concentrations (Jamialahmadi et al. 2018). Similar to the results of our study, Charmforoshan et al. (2019) observed that auraptene down-regulated the mRNA expression of MMP-2 and MMP-9 in the human breast cancer cell line MCF-7. Moreover, they reported that auraptene had anti-metastatic effects and could be used as adjuvant chemotherapy.

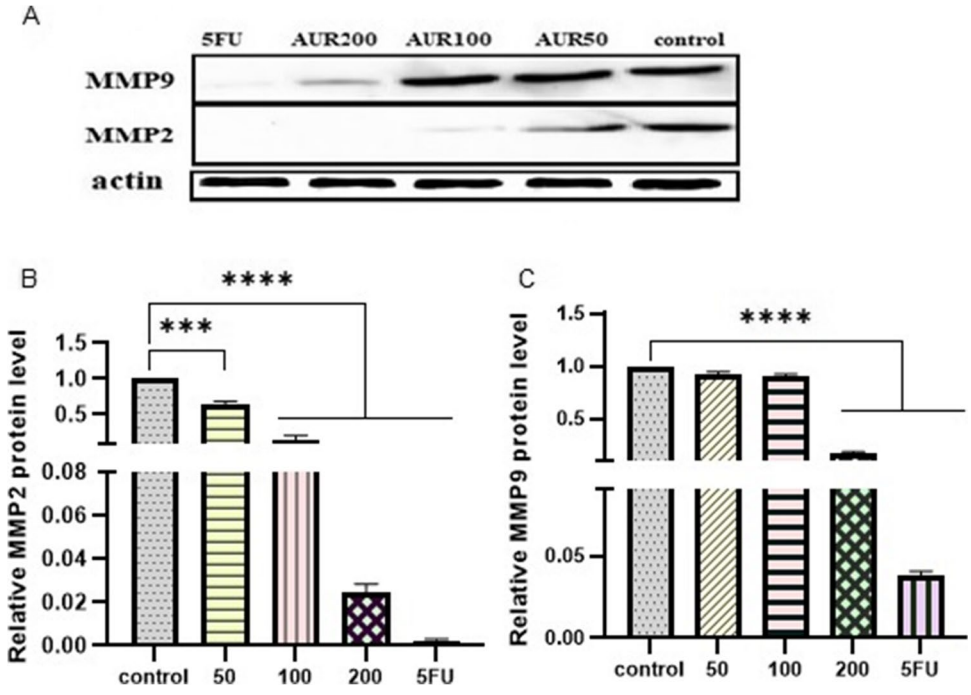

Fig. 4 **A** Representative blots indicate the protein expression levels of MMP-2 and MMP-9 in mice bearing subcutaneous CT26 tumor cells. **B** Quantitative analysis of MMP-2 and MMP-9 protein levels (***$p < 0.001$ and ****$p < 0.0001$ vs. control)

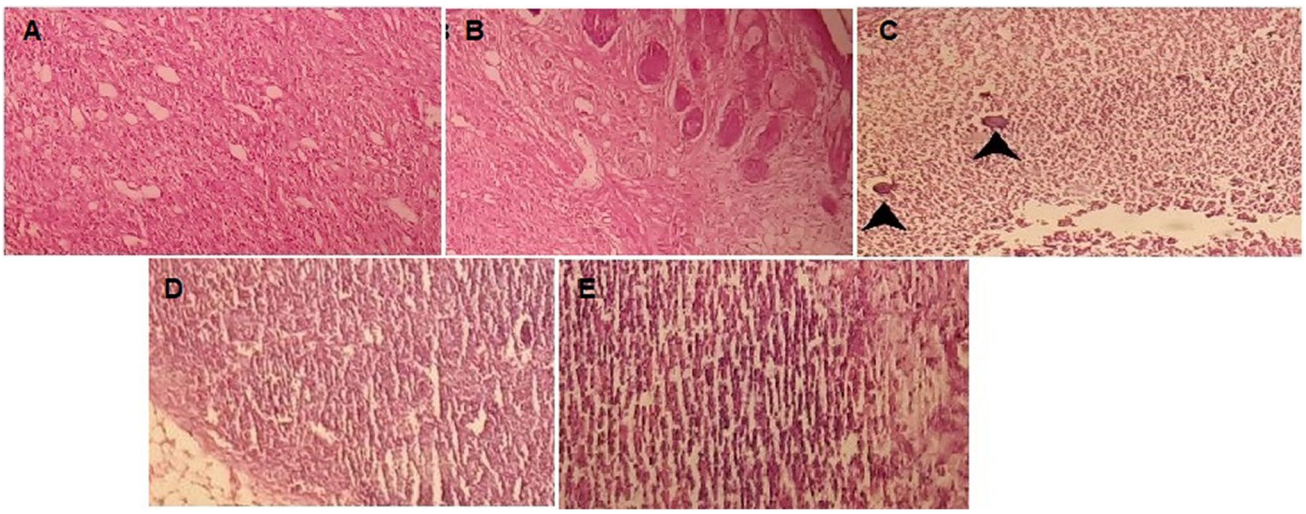

Fig. 5 Representative H&E staining of subcutaneous tumors in different groups: **A** control (×100 magnification), **B** 50 µM auraptene (×40 magnification), **C** 100 µM auraptene (arrowheads show tissue calcification) (×40 magnification), **D** 200 µM auraptene (×40 magnification), and **E** 5FU (×200 magnification)

E-cadherin is a vital protein in cell-cell adhesion, and its downregulation results in the invasion of tumor cells (Christou et al. 2017). Based on our results, the mRNA expression of E-cadherin was increased by auraptene at concentrations of 100 and 200 µM ($p < 0.05$). Our findings were in line with the results reported by Chen et al. (2013), which showed that curcumin inhibited metastasis in a mouse model of CRC by increasing E-cadherin expression. During tumor progression, in addition to metastasis, there is a huge formation of blood vessels around and within tumors. VEGF and its cognate receptor (VEGFR) are essential regulators of angiogenesis (Melincovici et al. 2018). Our results showed that auraptene reduced the gene expression of VEGFR in a dose-dependent manner. Moreover, the expression of VEGF was markedly diminished in response to auraptene treatment at the concentrations of 100 and 200 µM. In a similar study, Jang et al. (2015) found that auraptene inhibited neovascularization in nude mice with renal cancer by lowering VEGF mRNA expression by 70% under hypoxic conditions. In mice treated with 100 µM auraptene subcutaneously, new vessels had a five-fold lower hemoglobin content, and the degree of neovascularization was decreased in comparison

with the control group. Pathological findings from the H&E staining of the vessels in tumor tissues were consistent with their results. Nevertheless, Toliver et al. (2011) revealed the ability of VEGF to induce angiogenesis by tube formation in human umbilical vein endothelial cells (HUVEC) and assessed the impact of auraptene on the prevention of this function. According to their results, auraptene exhibited angiogenic activity by targeting VEGF. On the contrary, our results showed that auraptene inhibited colorectal tumor metastasis by decreasing the expression levels of VEGF and VEGFR genes.

As revealed by H&E staining, there were morphological changes and necrosis in the samples treated with 100 and 200 µM auraptene. Furthermore, notable tissue calcification was observed in these two groups, which is related to extensive tumor cell death. Typically, intratumoral calcification results from degenerative changes in the tissue, such as necrosis or hemorrhage, except in a few cases of bone-forming tumors (Karwowski et al. 2012; Yu et al. 2017). In one study, H&E staining results indicated that the rate of renal tumor cell growth was about one-third lower in mice receiving 100 µM auraptene (*s.c.*) compared with the control mice (Jang et al. 2015). The use of auraptene as an adjunctive therapy and achieving the optimum therapeutic efficacy in CRC patients is a complex issue requiring further *in vivo* and clinical research in the future. There are several limitations to the current research including the use of an angiogenic CRC model and the evaluation of factors involved in angiogenesis. However, further studies including the simultaneous use of auraptene and 5-FU or immunohistochemical analysis of these proteins are needed to clarify the precise mechanism of action of auraptene in metastasis. It would also be beneficial to study the angiogenesis model in the future.

Conclusion

Our findings showed that auraptene can prevent metastasis by reducing the protein levels of MMP-2 and MMP-9 as well as the gene expression levels of VEGF-A, E-cadherin, and VEGFR1 in CRC and this compound might be considered an alternative medication in adjuvant therapy.

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Author Contribution MS, ZMP, SE, and MK contributed to the study conception and design. SE, SEN, and MK carried out all the animal experiments. SE performed all the molecular experiments and contributed to the interpretation of the results. STJ carried out the pathological study. Drafts of the manuscript were prepared by SE, and revisions were completed by SK and ZM for publication in the journal. The corresponding author declares that all the listed authors meet the criteria.

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Declarations

Ethics Approval All animal experiments were performed in accordance with the guidelines of the NIH and the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1399.744).

Conflict of Interest The authors declare no competing interests.

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