

Auraptene Inhibits Migration and Invasion of Cervical and Ovarian Cancer Cells by Repression of Matrix Metalloproteinases 2 and 9 Activity

Khadijeh Jamialahmadi^{1,2}, Sofia Salari⁴, Nafiseh Sadat Alamolhodaei³, Amir Avan⁵, Leila Gholami⁶, Gholamreza Karimi^{4,7*}

¹ Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

² Department of Medical Biotechnology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

³ Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

⁴ Pharmaceutical Research Center, Institute of Pharmaceutical Technology, Mashhad University of Medical Sciences, Mashhad, Iran

⁵ Metabolic Syndrome Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

⁶ Department of Modern Sciences and Technologies, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

⁷ Department of Pharmacodynamics and Toxicology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

Key Words

auraptene, cancer, migration, invasion, matrix metalloproteinases

Abstract

Objectives: Auraptene, a natural citrus coumarin, found in plants of Rutaceae and Apiaceae families. In this study, we investigated the effects of auraptene on tumor migration, invasion and matrix metalloproteinase (MMP)-2 and -9 enzymes activity.

Methods: The effects of auraptene on the viability of A2780 and Hela cell lines was evaluated by MTT assay. Wound healing migration assay and Boyden chamber assay were determined the effect of auraptene on migration and cell invasion, respectively. MMP-2 and MMP-9 activities were analyzed by gelatin zymography assay.

Results: Auraptene reduced A2780 cell viability. The results showed that auraptene inhibited in vitro migration and invasion of both cells. Furthermore, cell invasion ability suppressed at 100µM auraptene in Hela cells and at 25, 50µM in A2780 cell line. Gelatin zymography

showed that for Hela cell line, auraptene suppressed MMP-2 enzymatic activity in all concentrations and for MMP-9 at a concentration between 12.5 to 100µM in A2780 cell line.

Conclusion: Auraptene inhibited migration and invasion of human cervical and ovarian cancer cells in vitro by possibly inhibitory effects on MMP-2 and MMP-9 activity.

1. Introduction

Cervical and ovarian cancers are the fourth and the fifth common cancers among women worldwide, respectively. Interestingly, more than 85% of all cervical cancers and its mortality occur in developing countries [1, 2]. In ovarian cancer, the average time of clinical remission is approximately 2 years. Hence, development in cancer therapy process seems quite necessary [3]. Although these female cancers are treatable in early stages, many cases are diagnosed at an advanced stage after metastasis has occurred so resulting in a poor prognosis and treatment failure.

Matrix metalloproteinases (MMPs) are a large group of zinc-dependent endopeptidases which are responsible for extracellular matrix (ECM) dissociation. MMPs have an important role in angiogenesis [4, 5], tumor

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*Corresponding Author

Gholamreza Karimi, Pharmaceutical Research Center, Institute of Pharmaceutical Technology, Mashhad University of Medical Sciences, Mashhad, Iran.

Tel: +98-511-882-3255 Fax: +98-511-882-3251

E-mail: KarimiG@mums.ac.ir

proliferation and migration or metastasis [6, 7]. Among the various MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are closely related to tumor invasion and metastasis [8]. As a rule, ECM dissociation by MMPs is one way to metastasis; Therefore identification novel chemotherapeutics for the matrix metalloproteinase inhibition may be a promising strategy for blocking migration and tumor metastasis [9].

Auraptene is a well-known oxo-coumarin was first extracted from, *Citrus aurantium*, which is one of the citrus species [10, 11].

Previous studies have shown that auraptene has various valuable properties including anti-inflammatory [12], anti-oxidant [13], anti-coagulant [14], anti-microbial [15], anti-cancer, neuroprotective [16], and immuno-modulatory effects [14,17,18].

Krishnan et al demonstrated that auraptene reduced cell proliferation at concentration of 20- 50 μ M in MCF-7 breast cancer cells [19].

In another study, auraptene decreased the incidence of colon adenocarcinoma at 100, 500 μ g/ml during the initiation and post initiation. Also, auraptene inhibited the development of azoxymethane (AOM)-induced precursor lesions for colorectal carcinoma [20].

Furthermore, a recent study showed that auraptene could suppress the Dextran sulfate sodium (DSS)-induced gelatinolytic activity of MMP-7 as well as the expression of MMP-2 and MMP-9 in ulcerative colitis in mice. Although the anticancer effects of auraptene in some cancer cells has been shown, its involvement in growth inhibition and lowering of cervical and ovarian cancer cells invasion remain unknown. In this study, we aimed to investigate the effect of auraptene as notable citrus coumarin on the growth capacity of two cancer cell lines, Hela and A2780 as cervical and ovarian cancers, respectively. Additionally, due to the pivotal role of MMP-2 and MMP-9 in tumor proliferation, migration and invasion, the inhibitory effect of auraptene on MMPs activity was reported [21].

2. Material and Methods

2.1. Cell lines and Reagents

A2780 and Hela cell lines from Pasture Institute (Tehran, Iran); Dimethyl Sulfoxide (DMSO), Triton X-100, penicillin / streptomycin, Sodium dodecyl sulphate, Tris-HCl and Giemsa from Sigma (Saint Louis, MO, USA); RPMI-1640, FBS and phosphate-buffered saline from Gibco; 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) from Milipore (USA); auraptene from Dr. Iranshahi, Iran; Tetramethyl ethylene diamine, Bromophenol Blue and Kumasi Blue R-250 from Merck (Germany).

2.2. Cell culture and treatments

A2780 and Hela cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 100u/ml penicillin and 100 μ g/ml streptomycin at 37°C in the presence of 5% CO₂. After preparation of auraptene stock (10mM)

(preparation with DMSO), the concentration of auraptene at (0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50 100 μ M) were provided by dilution in cell culture medium [3].

2.3. Cytotoxicity assay

The cytotoxicity of auraptene on two cell lines (A2780 and Hela) was evaluated after cell counting and seeding 104 cells in 96 well plates. After overnight incubation, the medium was removed and 100 μ l media supplemented with increasing concentrations of auraptene (0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 μ M) were added to wells with five repetitions.

After 24 hours incubation with auraptene and the medium removal, cells were stained by 20 μ L MTT solutions and were incubated for 3 hours. The medium in each well was carefully removed, and then 100 μ L of DMSO were added to each well. Finally, the absorption of solubilized formazanin each well was measured at 570nm. Values were corrected for background absorbance at 630nm [3].

2.4. Wound healing migration assay

The wound healing migration assay is the study of cell migration and cell interactions. First a straight scratch is made (simulating a wound) and then cell ability to wound healing is detected [22], related to time and auraptene with different concentrations. In this experiment, both cell lines (5 \times 10⁴ cells) were cultured in 24 well plates. After culture confluences, the monolayer cells were scratched, using a sterile (yellow) pipette tip to create a denuded zone (gap) of constant width. Each well was washed with PBS to remove the cellular debris and then exposed to different concentrations of auraptene (6.25, 12.5, 25, 50, 100 μ M). Both cell lines incubated at 37°C in the presence of 5% CO₂. Hela cells migrated to the wounded region was photographed at 0, 6, 24 hours for A2780 cells at 0, 24, 48, 72 hours. Finally, the wound area was measured by the program Image J software [23].

2.5. Matrigel invasion assay

In order to study the effect of auraptene on the cell invasive capacity, a modified Boyden-chamber technique with matrigel-coated membranes was used. Briefly, 105 from each cell line was placed onto the upper chamber and treated with auraptene at concentrations of (6.25, 12.5, 25, 50, 100 μ M) and (6.25, 12.5, 25, 50 μ M) for Hela and A2780 cells, respectively. The complete medium (containing 10% FBS) was applied to the lower chamber as chemoattractant. After overnight incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab.

Finally, the cells that had penetrated the matrigel membrane and invaded to the lower surface of the membrane, were fixed with methanol and stained with 10% Gimsa color solution for 15 min and counted under the microscope [24].

2.6. Gelatin zymography

To measure enzymatic activity of MMP-2 and 9 as the key enzymes in the cell invasion and metastasis, gelatin zymography were examined. Cells (5×10^5 cells/ml) were seeded in 6-well plates. After overnight incubation, HeLa cells were treated with auraptene at concentrations of (6.25, 12.5, 25, 50, 100 μ M) and A2780 cells at concentration of (6.25, 12.5, 25, 50 μ M). At the end of incubation periods, upper medium was centrifuged at 400 g for 5 min. Then, 12 μ l of supernatant aliquots of culture medium from each concentration was mixed with 3 μ l of loading buffer 5x. Samples were then loaded on to 8% acrylamide gels containing 0.1% gelatin as a substrate and the electrophoresis was done at 110v for 2 hours.

For revitalizing structure and activity of the MMP enzymes, gels were inserted into developing buffer at 37°C for 42 hours and then stained with Coomassie brilliant blue R-250 for 1 hour at room temperature.

After decolorization and gel photographing, Image J software was used for counting the color density at band formation area [25].

2.7. Statistical analysis

Each experiment was repeated three to four times. Data were expressed as mean \pm standard deviation and analyzed by one-way analyses of variance with ANOVA and Dunnett's posttest. Differences were considered statistically significant when p-values were <0.05 .

3. Results

3.1. The effects of auraptene on HeLa and A2780 cells viability

As shown in (Fig.1), cell viability was examined by MTT assay. Exposure to auraptene for 24 hours significantly reduced cell viability at concentration of 25-100 μ M in HeLa cells (IC₅₀: 47.93 μ M) and 6.25-100 μ M in A2780 cells (IC₅₀: 31.49 μ M). Moreover, maximum toxicity was observed at concentration of 100 μ M in both cell lines.

3.2. Inhibitory effect of auraptene on HeLa cell migration

The anti-migratory effects of auraptene on cervical and ovarian cancer cells was evaluated by Wound healing migration assay. In HeLa cell line, significant change in cell migration was found at 50, 100 μ M auraptene after 6 hours of incubation and at 100 μ M after 24 hours, compared to control. Also, the percent of migration was 100% in other concentrations, after 24 hours (Fig.2B).

3.3. Inhibitory effect of auraptene on A2780 cells migration

After incubation with various concentrations of aurap-

tene for 48 hours, auraptene significantly inhibit the cell migration at 12.5, 25, 50 μ M. Also, after 72 hours incubation, cell migration significantly decreased in all concentrations (Fig.3B).

3.4. Inhibitory effect of auraptene on cell invasion

The inhibitory effect of auraptene on the invasion of HeLa and A2780 cells were demonstrated by cell penetration through the Matrigel-coated polycarbonate filter in the Boyden chamber. The results showed that in HeLa cell line, auraptene reduced cell invasion at concentration of 100 μ M (Fig.4A), while for A2780 cell line, inhibitory effect of auraptene on cell invasion was found at 25 and 50 μ M, compared to control (Fig.4B).

3.5. Effect of auraptene on MMP-2, MMP-9 enzymes activity

Matrix metalloproteinases are important enzymes that degrade extracellular matrix (ECM) components and so play important roles tumor invasion and metastasis. In this regard, to investigate the inhibitory effects of auraptene on MMP-2 and MMP-9 enzyme activities, gelatin zymograph assay was applied. In our study, MMP-9 activity was significantly reduced at 12.5-100 μ M auraptene in HeLa cell line.

These results showed that reduction in MMP-2 enzymatic activity by auraptene was in a concentration-dependent manner (Fig.5A).

In A2780 cell line, MMP-2 and MMP-9 activity was significantly reduced at all examined concentrations compared to control. Maximum decrease occurred at 50 μ M auraptene. Reduction in enzymatic activity was in a concentration-dependent manner (Fig.5B).

Notably, the inhibitory effect of auraptene seems to be more on MMP-2 than MMP-9, at same concentrations of auraptene in both cell lines.

4. Discussion

Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. Increased cell migration, reduction in cell adhesion, intracellular matrix disintegration and invasion to other organs are correlated with metastasis [26].

Matrix metalloproteinases are the best enzymes candidates for metastasis. Among MMPs, MMP-2 and MMP-9 have pivotal role in angiogenesis, tumor proliferation and invasion. Hence, they could be promising in developing chemotherapeutic drugs and preventing metastasis [27].

Cervical and ovarian cancers are the fourth and the fifth common cancers among women worldwide, respectively. Thus, there is an urgent need for major research to shed further light on these cancers [3].

Auraptene is a natural citrus coumarin found in many plants of Rutaceae, Apiaceae and Composite. However, the effect of auraptene on metastasis in cervical and ovarian cancers and on MMPs activity is still unknown. The aims

of this study were to investigate the effect of auraptene on metastasis in cervical and ovarian cancers and MMP-2 and MMP-9 activity, as well. In order to study of auraptene on cell viability, MTT test was done. Auraptene significantly reduced cell viability in both cell lines, compared to control and its inhibitory effect was in dose- dependent manner.

In another experiment, after 72 hours of incubation with increasing concentration of auraptene in three prostatic cell lines (LNCaP, DU145, PC3) cell viability was inhibited in dose-dependent manner [28]. Additionally, Zheng et al demonstrated the effect of auraptene at two different concentrations in colon cancer cell line. In this regard, auraptene inhibited cell viability, DNA cleavage induction and cell proliferation. The inhibitory effect of auraptene, was probably related to G1/S arrest and induction of apoptosis [29].

The effect of auraptene on cell migration was studied by wound healing assay. After 24 hours of incubation, there were no differences between control and auraptene in both cell lines and wound healing occurred.

In A2780 cell line, after 72 hours of incubation, wound healing occurred only in control group. It was revealed that all concentrations of auraptene have inhibitory effect on cell migration and it was dose-dependent.

Further investigation illustrated that in HUVEC cell line, auraptene inhibited cell migration and proliferation, compared to control [30]. Also, in a recent study, in oral epithelial cells, wound closure was improved by 100% in the presence of >1 μ M auraptene [31].

In our study, in lower concentrations, auraptene needed more times to inhibit cell migration in A2780 cell line. In fact, cell migration ability in A2780 cell was lower than Hela cell line, and inhibitory effect of auraptene initiated after 48 hours. In Hela cell line, treatment with auraptene was significantly different from control group, after 6 hours of incubation.

In order to study of cell invasion, matrigel invasion assay was performed. In both cell lines, auraptene had dose dependent inhibition on cell migration and invasion.

In our study, gelatin zymography as complementary test was done to detect MMP-2 and MMP-9 activity. Significant change in MMP-2 and MMP-9 activity was represented in both cell lines after treatment with auraptene.

Recently, a study reported the effect of auraptene on MMP-2 and MMP-9 activity induced by dextran sulfate sodium in tumor cells. In this study, auraptene inhibited pro-MMP-2 and pro-MMP-9 expression compared to control. While, dextran sulfate sodium increased MMP enzyme activity to amount of 13 fold for pro-MMP-2 and 31 fold for MMP-9 [21]. In fact, MMPs activation are through junction to urokinase-type plasminogen activator receptor (uPAR) and then plasminogen is converted to plasmin by uPA. Afterwards, plasmin converts pro-MMP to MMP. Therefore, the relationship between uPA and uPAR are an initial step in tumor invasion [27].

Increasing evidence implied that auraptene reduced the production of pro-MMP-7 protein without influence on mRNA expression in HT-29 cell line. This phenomenon was related to mTOR inhibition activity of auraptene. Furthermore, reduction of pro-MMP-7 translation was corre-

lated with inhibition of 4EBP1 and eIF4B phosphorylation which activated by ERK1/2 pathway [30]. Further investigation showed that intraperitoneal injection of osthole as natural coumarin, reduced infarct volume and matrix-metalloproteinase-9 activity after transient focal cerebral ischemia in rats [32].

Other studies on anticancer effect of some coumarins have been reported. Among terpenoid coumarins, examined in Raji cell line, including auraptene, umbelliprenin, badrakemone, methyl galbanate, galbanic acid, feselol, mogoltacin, conferone and ferukrinone, Galbanic acid and umbelliprenin were showed to have inhibitory effect on matrix metalloproteinase enzymes. Also, it was concluded that auraptene and umbelliprenin, would have precious anti-tumor-promoting effects in many carcinogenic cycles [33].

5. Conclusion

In conclusion, our findings indicated that auraptene efficiently suppresses the migration and invasion capacity of human cervical (Hela) and ovarian (A2780) cancer cells by reduction of MMP-2 and MMP-9 activity. Considering that there are some unreliable results for performed tests about high concentration (100 μ M) of auraptene, our data suggest that the possibility of auraptene as a potential anti-invasive and anti-migratory candidate should be further investigated in required concentrations in future studies to reveal their detailed mechanisms and functions.

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Conflict of interest

The authors declared that there are no conflicts of interest.

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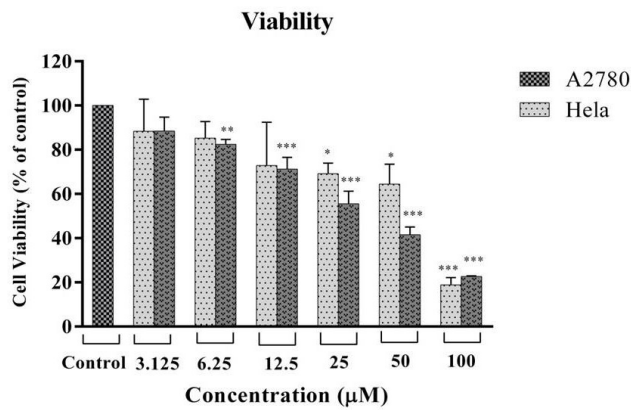


Figure 1 The effects of auraptene on the viability of HeLa and A2780 cell lines. Cells were exposed to various concentrations of auraptene at 37°C. (n=3)

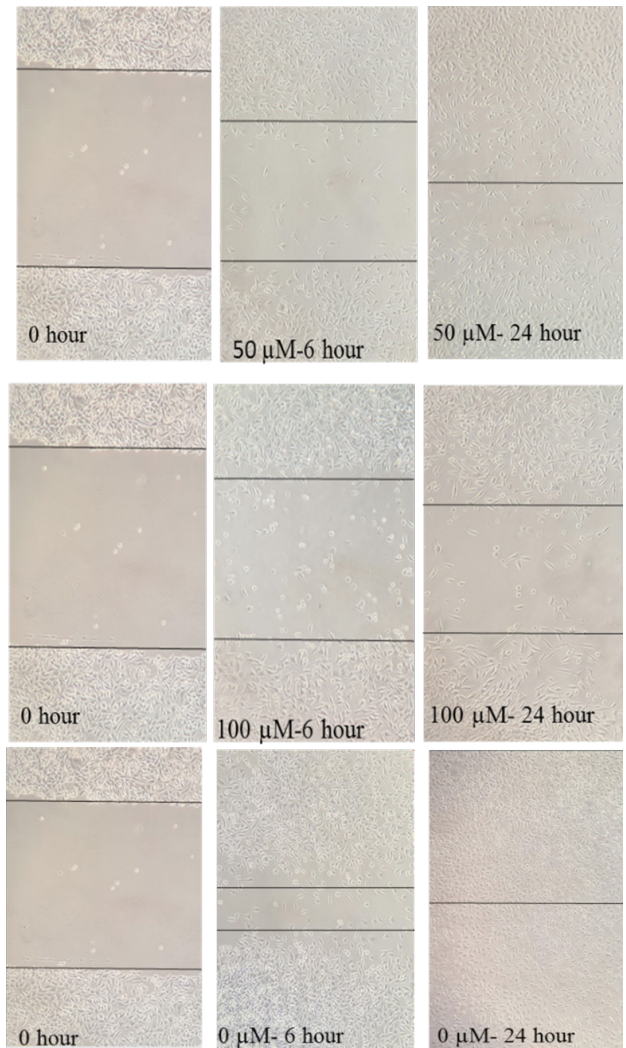


Figure 2A Effect of aurapfetene on HeLa cell migration. Cell migration was significantly reduced by auraptene at 100µM. (n=4)

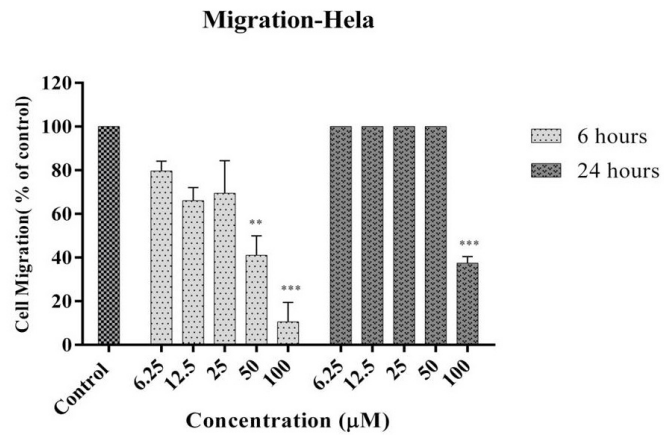


Figure 2B Effect of auraptene on cell migration in a time dependent manner. (n=4)

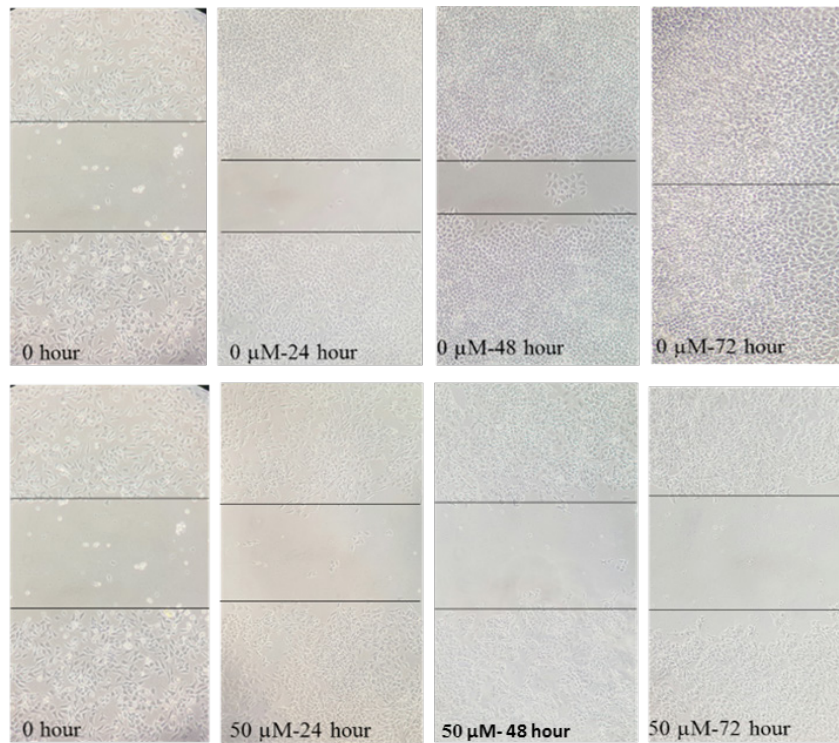


Figure 3A Effect of auraptene on A2780 cell migration. Cell migration was significantly reduced by auraptene at 50μM. (n=4)

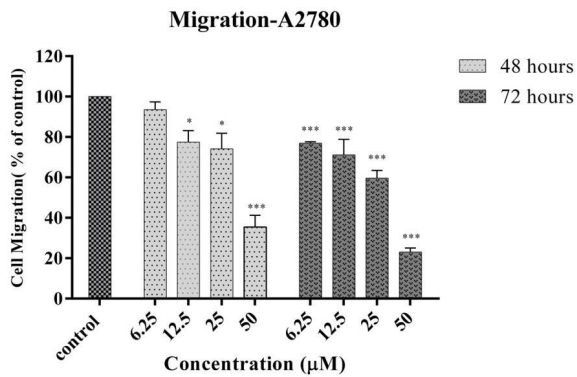


Figure 3B Effect of auraptene on cell migration in a time dependent manner. (n=4)

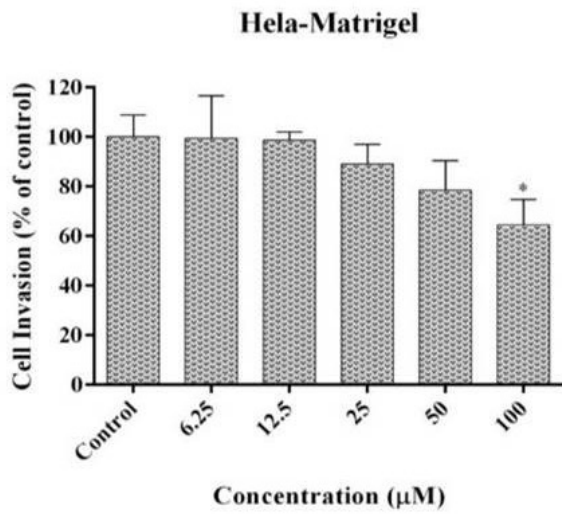


Figure 4A Inhibitory effect of auraptene on Hela cell invasion, after 24 hours incubation. (n=2)

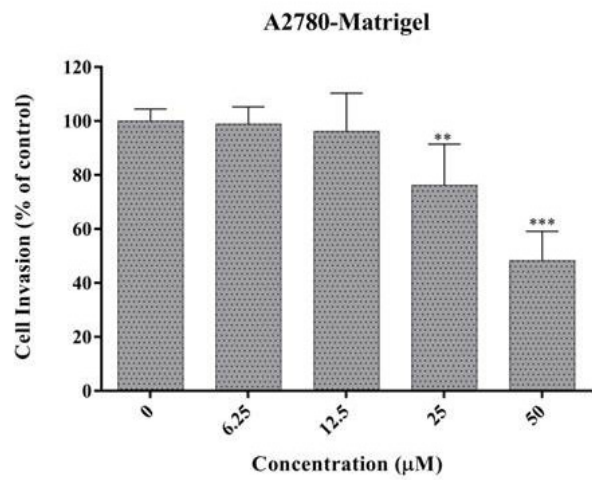


Figure 4B Inhibitory effect of auraptene on A2780 cell invasion, after 24 hours incubation. (n=2)

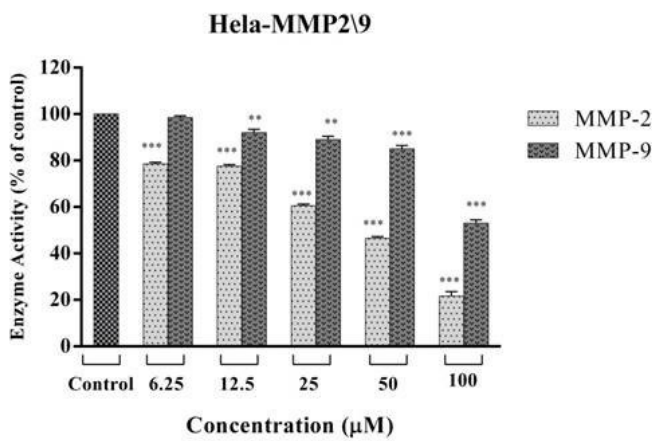


Figure 5A Effect of different concentrations of auraptene on MMP-2 and -9 activity in Hela cells. (n=2)

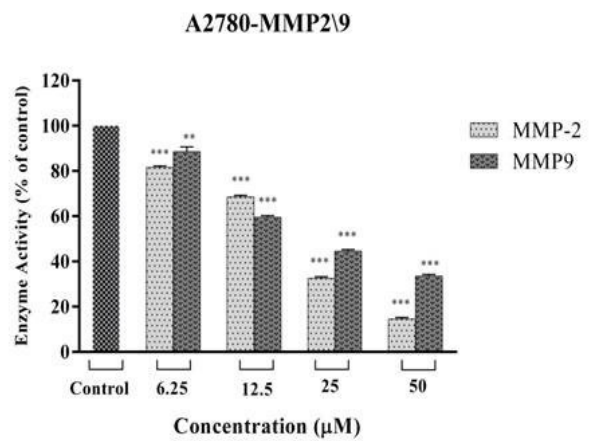


Figure 5B Effect of different concentrations of auraptene on MMP-2 and -9 activity in A2780 cells. (n=2)