JYI RESEARCH

Anti-angiogenic activity of auraptene

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ABSTRACT

The objective of this research was to test the *in vitro* anti-angiogenic activity of auraptene, a coumarin from citrus peel oil. We tested the ability of auraptene to inhibit *in vitro* angiogenesis using vascular endothelial growth factor (VEGF)-induced human umbilical endothelial cells (HUVEC) growth stimulation. Auraptene was applied at concentrations between 0 µM and 1µM to HUVEC in the presence of 10 ng/mL of VEGF at 37 ºC in a 5% CO_2 humidified incubator for 6h. The viability, migration, and invasion of HUVEC in the presence of auraptene were evaluated by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation assay and Boyden Chamber, respectively. Our results indicated that auraptene at 50 to 500 nM dose-dependently inhibited VEGF-induced *in vitro* angiogenesis. *In vitro* tube formation was also reduced to less than 20% of that of the control in the presence of 500 nM of auraptene. The viability of HUVEC exposed to 500 nM auraptene for 72 h was reduced to about 15 % of that of untreated cells. Auraptene at 500 nM thus inhibited VEGF-induced HUVEC migration and invasion. This is the first report that supports the use of auraptene as an inhibitor of angiogenesis. Our results may help to explain the inhibitory activity of this bioactive compound against cancer cell growth and proliferation.

INTRODUCTION

Cancer is the leading cause of disease-related death in the US and around the world. Therapeutic approaches including the use of radiation and chemotherapy to treat cancer have resulted in modest long-term effects on cancer cell viability, tumor growth, and improved patient survival. Significant emphasis is therefore being placed on identifying compounds that can delay cancer development. Bioactive compounds from foods are being investigated for their ability to delay the onset and/or progression of cancer.

Auraptene (7-geranyloxycoumarin) is a naturally occurring dietary antioxidant coumarin present in fruits of the citrus family, including sweet orange and Satsuma (Murakami et al., 1997; Takahashi et al., 2002). In citrus fruits, auraptene is more concentrated in the peel oil, although small amounts can be found in commercially available citrus juices (Murakami et al., 1997). Several studies have reported that citrus food compounds including auraptene have many health-enhancing effects including cancer chemoprevention. These citrus bioactive compounds act on pathways related to cell proliferation, differentiation,

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inflammation, obesity, and cell death. Auraptene has been reported to suppress the process of carcinogenesis by modulating the expression of a variety of proteins that give rise to tumor formation, also known as oncogenic proteins (Hara et al., 2005; Kawabata et al., 2006). Auraptene has been shown to inhibit cell proliferation, matrix metalloproteinases-2, -7, and -9 (MMP-2, MMP-7, and MMP-9) activities, activate caspase-8, and induce cancer cell apoptosis (Hara et al., 2005; Kawabata et al., 2006; Jun et al., 2007a). It bears a geranyloxyl side chain (Figure 1) which acts as the carrier group for auraptene incorporation uptake by cells, and has a good life span. These factors may be important to the chemopreventive effects of auraptene in the body (Kuki et al., 2008).

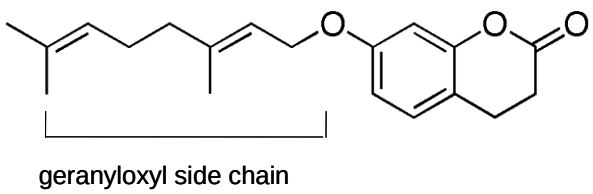

Figure 1. Structure of auraptene showing the geranyloxyl side chain.

Angiogenesis is the formation of new blood vessels from pre-existing ones. There are two types of angiogenesis: the physiological and the pathological angiogenesis. The process of physiological angiogenesis is essential for organ growth and repair including wound healing. During physiological angiogenesis, a balance between stimulators and inhibitors of angiogenesis must be maintained. The stimulators of angiogenesis include hormones, growth factors and their receptors such as the vascular endothelial growth factor (VEGF) and its receptor tyrosine kinases (VEGFR-1 and VEGFR-2), enzymes such as the matrix metalloproteinases-2 and -9 (MMP-2 and MMP-9), and cell-adhesion molecules such as selectin and integrins (Brooks, 1996). An imbalance between the simulators and inhibitors of angiogenesis may lead to the development and progression of chronic degenerative diseases, such as macular degeneration (Folkman, 2007; Folkman, 2006a).

During pathological angiogenesis, there is either an excess of blood vessel formation or insufficient blood vessel formation. Pathological angiogenesis is associated with more than seventy chronic degenerative diseases (Losso, 2003). Excessive angiogenesis is associated with degenerative diseases such as cancer, AIDS, psoriasis, and macular degeneration (Losso, 2003). Insufficient angiogenesis is associated with pathological conditions including impaired chronic wound healing, stroke, infertility, and scleroderma. The growth of solid tumors is dependent on vascularization, because they require large supplies of oxygen and nutrients. Furthermore, newly formed blood vessels create a route for tumor cells to migrate and metastasize. The clinical significance of angiogenesis inhibition has been recognized and validated in preclinical and clinical trials (Carmeliet & Jain, 2011; Folkman, 2006b; Carmeliet, 2005; Ferrara & Kerbel, 2005). Current methods that aim to inhibit angiogenesis have focused on targeting endothelial, mural and stromal, haematopoietic, and neoplastic cells (Carmeliet, 2005; Banerjee & Gore, 2009). Although several studies have reported that auraptene has shown inhibitory effects on tumor cells (Murakami, 2009; Krishnan et al., 2009), whether auraptene inhibits tumor angiogenesis and suppresses further tumor growth remains unclear. Endothelial cell migration, proliferation, invasion, adhesion, and tube formation are key steps in angiogenesis initiation and progression (Jain, 2003; Kaur et al.,

2004). Inhibitors of vascular endothelial growth factor and tyrosine kinases target the vascular endothelial growth factor (VEGF), its receptors (VEGFR-1 and VEGFR-2) and tyrosine kinase receptors, and endothelial cell signaling pathways that regulate endothelial cell migration, proliferation, growth, and survival.

Numerous studies have indicated that the consumption of phytochemicals contained in fruits and vegetables may inhibit, delay, and even reverse the process of cancer development in humans. In this study, we investigated whether auraptene inhibited angiogenesis through suppression of intracellular signaling pathways.

METHODS AND MATERIALS

Materials

Auraptene was purchased from LKT Laboratories (St. Paul, MN). The CellTiter96 One Aqueous Solution was acquired from Promega (Madison, WI). The *in vitro* angiogenesis kit was obtained from Chemicon (Temecula, CA). VEGF was obtained from Peprotech (Rocky Hill, NJ). Cell migration and invasion assay kits were purchased from CellBiolabs (San Diego, CA). All other reagents were of analytical grade.

Cell lines and culture conditions

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics, a division of Lonza (Walkersville, MD). Cells were grown at 37°C in a 5% CO_2 atmosphere in a humidified incubator. Medium used was EGM-2 Bulletkit (Cambrex-Lonza). Procedures used for maintaining and culturing cells were supplied by Lonza.

Tube formation assay

HUVEC tube formation on Matrigel (ECM matrix) was conducted following the manufacturer's protocol. Briefly, 50 µl of ECM matrix (Matrigel) solution at 4 °C was added to 96-well plates (Nunc, Nalge Nunc International, Rochester, NY) and allowed to solidify and polymerize at 37°C, 5% CO_2 for 1 hour. HUVECs were suspended at the concentration of 1.0 x 10^4 cells per 100 µl in culture medium (RPMI) containing 10 ng/ml of VEGF. Auraptene (at concentrations ranging from 0 to 1µM) was dissolved in DMSO with the final DMSO concentration being less than 0.1%. Cells were carefully layered on top of the polymerized gel and the plates were incubated for 6 hours at 37°C and 5% CO_2 in a humidified incubator. Tube formation was inspected and photographed using a Leitz phase-contrast inverted microscope at 40X magnification.

Cell viability assay

HUVECs (1 x 10^4cells/well) were seeded in 96-well plates (Nunc, Nalge Nunc International, Rochester, NY) in a total volume of 0.1 ml in serum-containing medium and allowed to adhere overnight. Replicates of culture plates were prepared and incubated for 24, 48, or 72 hours in a humidified incubator containing 5% CO_2 at 37 °C. Cell viability was determined by the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay as follows. 20 µl of the reagent were added to both control and treated cells. Plates were incubated for 1 hour at 37°C and the absorbance was measured at 490 nm using a Spectra Max *Plus* (Molecular Devices, Sunnyvale, CA). Values obtained from the cell viability and proliferation assays were normalized to the levels in untreated control cells to determine the percentage of viable cells.

All experiments were carried out in triplicates.

Cell migration assay

The assay was carried out using a CytoSelect™ 96-well Cell Invasion Assay (Cell Biolabs Inc, San Diego, CA) following the manufacturer's instructions. HUVECs (suspended at 1.0 x 10^6 cells/0.1 ml in serum free media and treated with or without auraptene (0-500 nM)) were seeded in the upper chamber. The wells of the feeder tray (lower chamber) were loaded with 150 µl of media containing 10 ng/ml of VEGF. After incubation for 6 hours at 37 ºC in 5% CO_2 atmosphere, the cells that migrated through 8 µm pore size to the lower chamber were dislodged from the underside of the membrane, lysed in the presence of CyQuant® GR dye solution, 150 µl of the mixture was transferred to 96-well plates (Nunc, Nalgen Nunc International, Rochester, NY). Plates were read at 480nm/520 nm using Perkin Elmer LS 50B spectrofluorometer. Each assay was conducted in triplicate and repeated at least three times.

Cell invasion assay

The assay was carried using a CytoSelect™ 96-well Cell Invasion Assay (Cell Biolabs Inc, San Diego, CA) following the manufacturer's instructions. HUVEC (suspended at 2.0 x 10^6 cells/0.1 ml in serum free media) and treated with or without auraptene (0-500 nM) were seeded in the upper chamber. The wells of the feeder tray (lower chamber) were loaded with 150 µl of media containing 10 ng/ml of VEGF. After incubation for 24 hours at 37 ºC in 5% CO_2 atmosphere, the cells that migrated to the lower chamber were dislodged from the underside of the membrane, lysed in the presence of CyQuant® GR dye solution, 150 µl of the mixture was transferred to a 96-well plate suitable for fluorescence measurement and the plate was read at 480nm/520 nm using Perkin Elmer LS 50B spectrofluorometer. Each assay was conducted in triplicate and repeated at least three times.

RESULTS

Inhibition of tube formation

An *in vitro* angiogenesis model was used to determine the effect of auraptene on tube formation, a critical step in the process of angiogenesis. Tube formation was assessed with HUVEC on EC matrix. In the presence of 10 ng/ml of VEGF, HUVEC plated on the EC matrix aligned and formed capillary-like structures within 6 hours (Fig. 2). Auraptene dose-dependently inhibited tube formation within 6 hours with an IC_{50}= 200 nM. At both 300 and 500 nM auraptene, the VEGF-stimulated capillary-like network of the EC matrix was completely disrupted, suggesting that auraptene strongly inhibited *in vitro* angiogenesis (Fig. 2). Quantitative analysis showed that auraptene inhibited tube formation by 15%, 65%, and 86% at 100 nM, 300 nM, and 500 nM, respectively (Fig. 2).

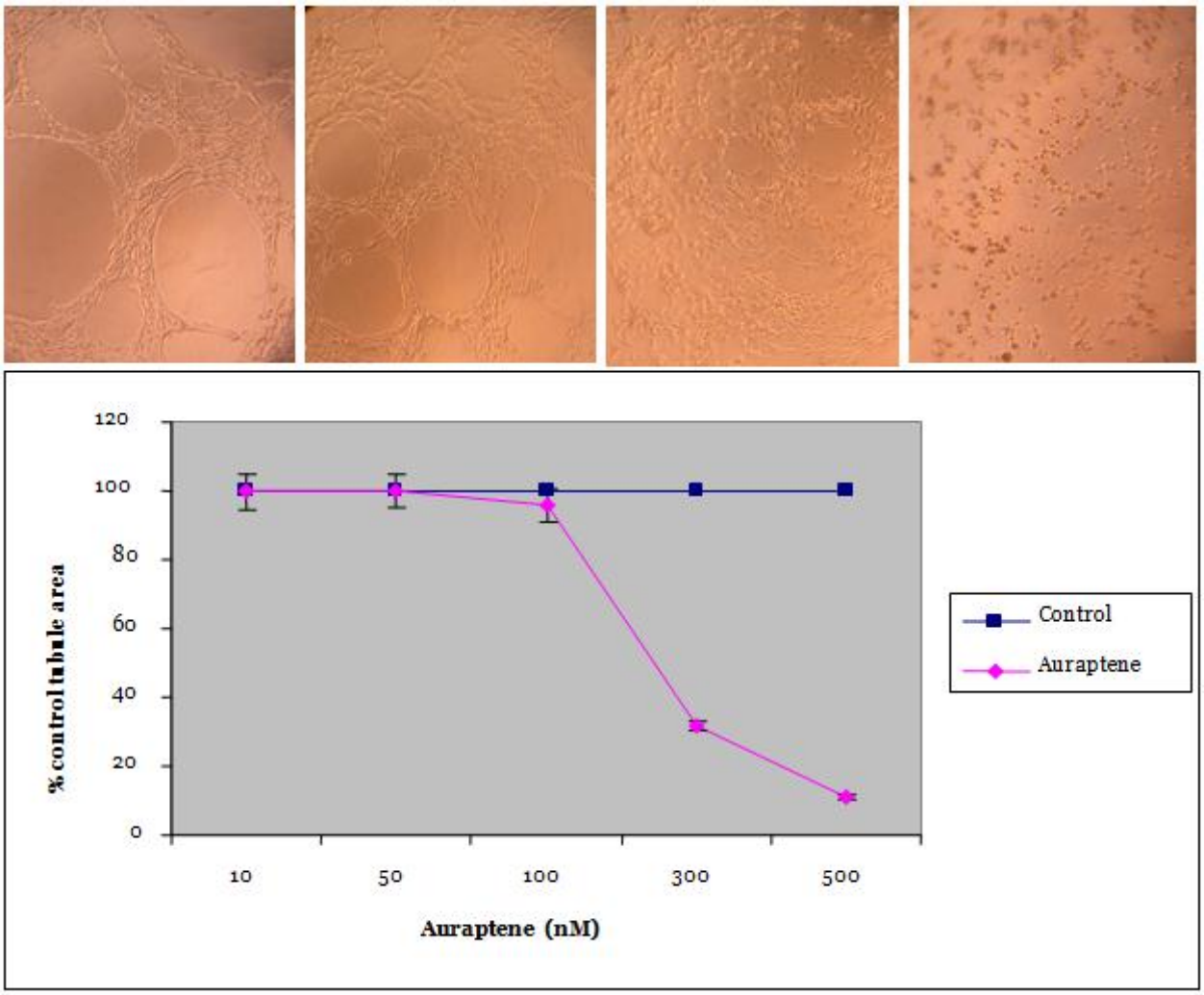

Figure 2. Inhibition of VEGF-induced endothelial tube formation by auraptene in absence (A) or presence of 100 nM (B), 300 nM (C), or 500 nM (D) of auraptene (magnification x10).

Inhibition of HUVEC cell viability

HUVECs were treated with various concentrations of auraptene for 72 hours. Auraptene dose-dependently inhibited HUVEC viability with an IC_{50} value of 200 nM (Fig. 3). The viability- inhibiting effect of auraptene on HUVEC was evident after exposure of cells to 100 nM of auraptene. The viability of HUVEC was inhibited by 20%, 70%, and 85% after treatment with 100, 250, and 500 nM of auraptene, respectively. At 500 nM auraptene, the number of viable and proliferating cells was very low.

Inhibition of HUVEC migration and invasion

Using VEGF as a chemoattractant, auraptene dose-dependently inhibited the migration of HUVEC in the presence of 10 ng/ml of VEGF (Fig. 4). There was a significant inhibition of cell migration at an auraptene concentration of 250-500 nM after 6 hours incubation. Auraptene also dose-dependently inhibited HUVEC invasion in the presence of VEGF as a chemoattractant with the largest inhibition occurring at a 500 nM auraptene concentration of (Fig. 5). Cancer endothelial cell migration is an

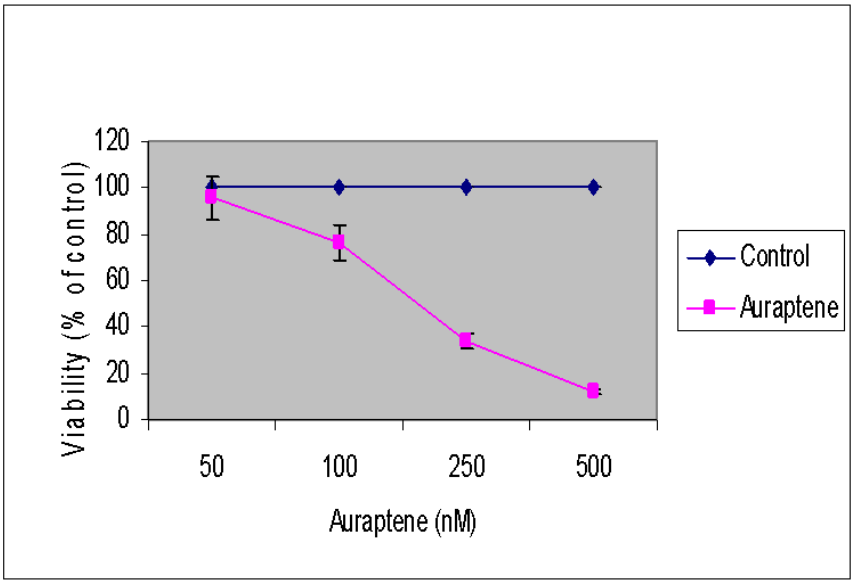

Figure 3. Inhibition of VEGF-induced HUVEC viability. Auraptene (0-1μM) was incubated with HUVEC in the presence of 10 ng/ml of VEGF at 37 °C, 5% CO_2 in a humidified incubator. Cell viability was analyzed after 72 h of incubation. Results are mean ± SD of three determinations. *Indicates statistically different from control ($p < 0.05$).

intermediate step toward metastasis. Thus, a bioactive compound that inhibits any of the steps associated with cancer progression and proliferation may slow cancer spread and metastasis.

DISCUSSION AND CONCLUSION

Auraptene was first isolated in 1953 as a constituent of orange oil (Kariyone and Matsumo, 1953). Citrus products including orange and grapefruits and other plants are major sources of auraptene. Over the last few years, auraptene was found to have anti-inflammatory, anti-proliferative, and pro-apoptotic effects on neoplastic cells, including human colon and prostate cancer cells (Kawabata et al., 2006; Tang et al., 2007; Tanaka et al., 2010).

In this study, we evaluated the antiangiogenic activity of auraptene *in vitro*. VEGF is a major stimulator of angiogenesis. The results of our study showed that auraptene dose-dependently inhibited VEGF-induced HUVEC tube formation *in vitro*. Auraptene inhibited *in vitro* angiogenesis by suppressing the activation of VEGF-induced tube formation. There are several *in vitro* assays for measuring angiogenesis or anti-angiogenesis including tube formation, aortic ring formation, and vessel outgrowth from organ culture (Auerbach et al., 2003). However, the *in vitro* tube formation assay has some advantages over the other *in vitro* techniques because it is reproducible, quantifiable, technically straightforward, and amenable to large scale screening of bioactive compounds. The limitations of the tube formation assay using the HUVECs have been associated with the large variation of tube forming ability among endothelial cells, the limited passage times that do not exceed 10 to 12 passages in culture, and the ability to quantify the tubes. However, the use of tube formation assay for screening anti- or pro-angiogenic bioactive compounds has been widely approved within the research community (Auerbach et al., 2003; Staton et al., 2009). Auraptene also dose-dependently inhibited the viability and VEGF-induced migration and invasion of endothelial cells. This is the first report to suggest inhibition of angiogenesis as possible mechanism by which auraptene inhibits cancer progression.

Angiogenesis inhibitors constrain one or more steps of angiogenesis by targeting endothelial cells. Our results showed that auraptene effectively inhibited endothelial cells migration, invasion, proliferation, and tube formation suggesting that auraptene is an inhibitor of angiogenesis. Several regulators including protein kinase B (AKT), Extracellular Signal-Regulated Kinase (ERK) that are VEGF dependent or VEGF-independent are involved in cell cycle, proliferation, survival, migration, and apoptosis of endothelial cells. In summary, we systemically showed that auraptene, a biologically active, major component of citrus peel oil, inhibited endothelial cell migration, invasion, proliferation, and tube formation, effectively inhibited angiogenesis *in vitro* and prevented tube formation by endothelial cells.

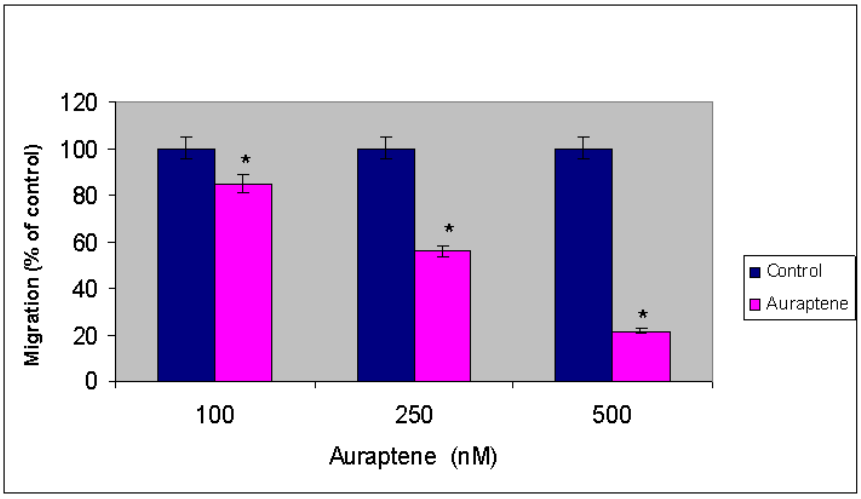

Figure 5. Inhibition of HUVEC migration by auraptene. HUVEC were treated with 0-500 nM of auraptene and evaluated for migration in a modified Boyden Chamber using 10 ng/mL of VEGF as a chemoattractant. Untreated HUVEC were used for comparative purposes. Results are mean ± SD of three determinations.

*Indicates statistically different from control ($p < 0.05$).

Auraptene is a bioactive compound consumed with citrus. The bioactivity of auraptene suggests that citrus products may serve as a cancer-preventative supplement when consumed in appropriate amounts. However, *in vivo* evidence is needed to validate these preliminary findings *in vitro*. Additional experiments that may determine whether auraptene can inhibit the biomarkers of angiogenesis *in vitro* in hormone-dependent and independent breast cancer cells are in progress.

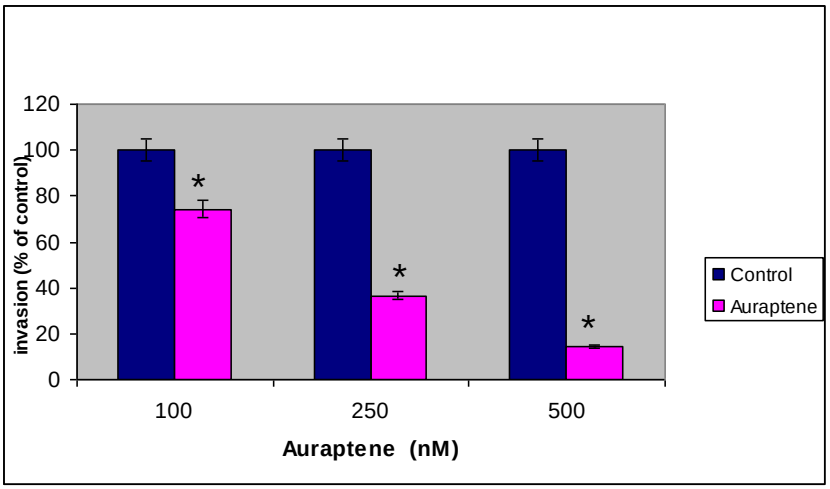

Figure 4. Inhibition of HUVEC invasion by auraptene. HUVEC were treated with 0-500 nM of auraptene and evaluated for invasion in a modified Boyden Chamber using 10 ng/mL of VEGF as a chemoattractant. Untreated HUVEC were used for comparative purposes. Results are mean ± SD of three determinations.

*Indicates statistically different from control ($p < 0.05$).

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