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Dietary compounds galangin and myricetin suppress ovarian cancer cell angiogenesis

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Abstract

Galangin and myricetin are flavonoids isolated from vegetables and fruits which exhibit anti-proliferative activity in human cancer cells. In this study, their anti-angiogenic effects were investigated with *in vitro* (HUVEC) and *in vivo* (CAM) models, which showed that galangin and myricetin inhibited angiogenesis induced by OVCAR-3 cells. The molecular mechanisms through which galangin and myricetin suppress angiogenesis were also studied. It was observed that galangin and myricetin inhibited secretion of the key angiogenesis mediator vascular endothelial growth factor (VEGF) and decreased levels of p-Akt, p-70S6K and hypoxia-inducible factor-1α (HIF-1α) proteins in A2780/CP70 and OVCAR-3 cells. Transient transfection experiments showed that galangin and myricetin inhibited secretion of VEGF by the Akt/p70S6K/ HIF-1α pathway. Moreover, a novel pathway, p21/HIF-1α/VEGF, was found to be involved in the inhibitory effect of myricetin on angiogenesis in OVCAR-3 cells. These data suggest that galangin and myricetin might serve as potential anti-angiogenic agents in the prevention of ovarian cancers dependent on new blood vessel networks.

Keywords

Galangin; Myricetin; p21; Angiogenesis; Ovarian cancer

1. Introduction

Recent studies have focused on the anti-cancer activity of flavonoids isolated from plants (Park et al., 2014; Zhang et al., 2014; Zivkovic et al., 2014). Flavonoids are natural polyphenols present in many different foods, especially fruits and vegetables. Previous studies have suggested that flavonoids display anticancer characteristics and might be able

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to decrease cancer risk through the mechanisms of preventing oxidation and inflammation, diminishing angiogenesis and cell proliferation, and inducing apoptosis (Gates et al., 2009; Kandasamy & Ashokkumar, 2013; Li et al., 2009; Li, Wang, Guo, Zhao, & Ho, 2014; Ma et al., 2014; Yu et al., 2014). Galangin and myricetin are members of the flavonol subclass of flavonoids with antioxidant activities. Galangin was isolated from the galangal rhizome and propolis. Myricetin is more common than galangin and can be found in fruits, vegetables, nuts, berries, tea and red wine (Basli et al., 2012; Ross & Kasum, 2002).

Angiogenesis is a physiologic process through which new blood vessels form from pre-existing vessels (Birbrair et al., 2014). It is also the fundamental step in the transition of tumours, including ovarian tumours, from a benign state to a malignant one. Angiogenesis is typically quiescent in normal adult tissues (Bertl, Bartsch, & Gerhauser, 2006). A microtumour growing beyond 1 to 2 mm^2 faces a limited nutrition and oxygen supply and requires an intact blood system to support its own growth and shed metabolites (Bertl, Bartsch, & Gerhauser, 2006). In contrast, tumour-free adults have no need for angiogenesis in normal situations (Fotsis et al., 1993; Glade-Bender, Kandel, & Yamashiro, 2003). Anti-angiogenesis has been reported to be feasible for treating human cancers and has become one of the most promising strategies in the chemoprevention and treatment of cancers. These early-stage tiny tumours cannot be successfully diagnosed but their continued development demands a new blood vessel network (Sanchez-Munoz, Perez-Ruiz, Mendiola, Alba, & Gonzalez-Martin, 2009). Previous studies have reported that galangin and myricetin decrease angiogenesis in human umbilical vein endothelial cells (HUVECs) (Kim, Liu, Guo, & Meydani, 2006). Myricetin also inhibited angiogenesis in a SKH-1 hairless mouse skin tumourigenesis model induced by UVB (Jung et al., 2010).

Vascular endothelial growth factor (VEGF) plays a central role in the mediation of tumour vascular development and maintenance (Hefler et al., 2007). Therefore, anti-VEGF therapies are important in the treatment of cancers. The VEGF gene is directly regulated by hypoxia-inducible factor 1α (HIF-1α), a heterodimeric basic helix-loop-helix protein (Forsythe et al., 1996). Stabilization and up-regulation of HIF-1α promotes the expression of VEGF by binding to HIF-responsive elements (HREs) in promoters. Ribosomal protein S6 kinase (p70S6K), a serine/threonine kinase that acts downstream of the PI3K/Akt pathway, controls angiogenesis through regulating HIF-1α and VEGF proteins (Bian, Shi, Meng, Jiang, Liu, & Jiang, 2010). Akt is a mediator of VEGF that enhances pathological angiogenesis and tumour growth associated with matrix abnormalities in skin and blood vessels (Chen et al., 2005; Somanath, Razorenova, Chen, & Byzova, 2006). P21, known as a regulator of cell cycle progression, also negatively regulates VEGF protein in some cancer cells including OVCAR-3 cells (Farhang, Goossens, & Haigh, 2013; Luo, Rankin, Juliano, Jiang, & Chen, 2012).

In this study, the effects of galangin and myricetin on reducing angiogenesis were examined in two platinum-resistant ovarian cancer cell lines: A2780/CP70 and OVCAR-3. The mechanisms involved in the effects of galangin and myricetin on angiogenesis were also investigated.

2. Materials and methods

2.1. Cell culture and reagents

Human ovarian cancer cell lines, OVCAR-3 and A2780/CP70, were kindly provided by Dr. B. Jiang, Department of Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, WV, USA. Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% US-qualified fetal bovine serum (Invitrogen, Grand Island, NY, USA). HUVEC cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in a vascular cell basal medium (ATCC) supplemented with Endothelial Cell Growth Kit-VEGF (ATCC). All cells were maintained in a humidified incubator with 5% CO2 at 37 °C. Galangin (purity: 98%) was purchased from Shanghai yuanye Bio-Technology (Shanghai, China). Myricetin (purity: 97%) was purchased from J & K Chemical Technology (Beijing, China). Galangin and myricetin were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions of 100 mM. The primary antibodies against phospho-Akt (Ser473) (p-Akt), total-Akt (t-Akt), phospho-ribosomal protein S6 kinase (p-p70S6K), total-p70S6K (t-p70S6K), and p21 were purchased from Cell Signaling Technology, Inc. (Dancers, MA, USA). The primary antibodies against p53, NFκB (p50), PTEN and GAPDH were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). HIF-1α, mAkt, p70S6K plasmids and VEGF, HIF-1α reporter were purchased from Addgene (Cambridge, MA, USA).

2.2. Cell proliferation

Cell growth inhibition was determined with a “CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay” kit from Promega (Madison, WI, USA). The cells were seeded into 96-well plates at a density of 2×10^4/well and incubated at 37 °C. Cells were allowed to attach to the bottom overnight and then treated with different concentrations of galangin/myricetin. Control cells received an equal volume of DMSO only. After 24 hours, the 100 μL CellTiter 96 AQ_{ueous} One Solution Reagent dilute solution (80 μL PBS + 20μL CellTiter 96 AQ_{ueous} One Solution Reagent) were added to each well. Cells were then incubated at 37 °C for another 1 h and measured at 490 nm. Cell viability was expressed as a percentage of the control from three independent experiments.

2.3. ELISA for VEGF

The levels of VEGF in cell culture supernatants were analyzed by a Quantikine Human VEGF Immunoassay Kit (R&D Systems, Minneapolis, MN, USA). A2780/CP70 and OVCAR-3 cells (2×10^4/well) were seeded into 96-well plates and incubated overnight. Subsequently, the cells were treated with galangin/myricetin or DMSO for 24 h in serum free medium. Culture supernatants were collected. The amounts of VEGF were measured following the manufacturer's instructions, normalized to total protein levels, and expressed as a percentage of the untreated control.

2.4. *In-vitro* angiogenesis assay

OVCAR-3 cancer cells were seeded into 96-well plates at 2×10^4/well and incubated overnight before treatment with different concentrations of galangin/myricetin for 24 h. The

conditioned medium was collected. Growth factor reduced Matrigels (BD Biosciences, San Jose, CA, USA) were added into 96-well plates at 50 μL/well and incubated at 37 °C for 30 min to gel. HUVEC cells were harvested in vascular cell basal medium and seeded into Matrigel beds at a concentration of 1.5×10^4/90 μL medium. Afterwards, 10 μL of collected conditioned medium were added to each well and then incubated at 37 °C for 6 h. Each well was photographed under a microscope. Each picture of 1388×1040 pixels was further divided into 6 rectangular areas by gridlines to obtain the tube length using the NIH ImageJ software. Angiogenesis was evaluated by normalizing tube length to that of the control.

2.5. *In-vivo* angiogenesis assay

Specific pathogen-free fertile chicken eggs (Charles River Laboratories, North Franklin, CT, USA) were incubated at 37.5 °C and slowly turned by an automatic egg turner (G.Q.F. Manufacturing Company, Savannah, GA, USA). The OVCAR-3 cells (1.2×10^6 cells in a 20 μL FBS-free medium) were mixed with 80 μL of Matrigel (BD Bioscience), supplemented with different concentrations of galangin/myricetin, pre-gelled on an autoclaved silicone mat for 30 min, and implanted into the chorioallantoic membrane (CAM) of the 9-day-old chicken embryo. After incubating another 5 days, tumour implants and blood vessels were photographed and counted for branching blood vessels by three investigators blinded to the treatment. Angiogenesis was evaluated by normalizing the number of branching vessels to that of control CAM.

2.6. Western blot

Ovarian cancer cells (10^6) were seeded in 60-mm dishes and incubated overnight before treatment with galangin/myricetin or DMSO for 24 h. The cells were washed with PBS, lysed in 100 μL mammalian protein extraction reagent including 1 μL Halt Protease, 1 μL phosphatase inhibitor and 2 μL eathylenediaminetetraacetic acid (EDTA) (M-PER, Pierce, Rockford, IL, USA), as per manufacturer's instructions. Total protein levels were assayed with a BCA Protein Assay Kit (Pierce). Cell lysates were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane with a Mini-Protean 3 System (Bio-Rad, Hercules, CA, USA). The membranes were blocked in 5% nonfat milk in Tris-buffer saline containing 0.1% Tween-20 for 1 h at room temperature. The membranes were incubated with the appropriate dilutions of the primary antibodies and secondary antibodies. After washing with TBST, the antigen-antibody complex was visualized with the SuperSignal West Dura Extended Duration Substrate (Pierce). Protein bands were quantitated with NIH ImageJ software, normalized by corresponding GAPDH for analysis.

2.7. Transfection with small interfering RNA (siRNA)

OVCAR-3 cells were seeded in 60-mm dishes at 5×10^5/dish and incubated overnight before transfection with p21 siRNA or control siRNA (Santa Cruz Biotechnology) using jetPRIME™ DNA and siRNA Transfection Reagent (VWR International, Radnor, PA, USA) according to the manufacturer's protocol. After 24 hours, cells were treated with myricetin or DMSO. Cell lysates were collected for Western blot to test p70S6K, Akt, and HIF-lα proteins.

2.8. Plasmid transfection and luciferase assay

OVCAR-3 cells were seeded in 96-well plates at 10,000 cells/well and incubated overnight. The OVCAR-3 cells were transfected with Akt, p70S6K/HIF-lα, or SR-α (as vehicle) plasmids, and HIF-1α/VEGF luciferase reporter using jetPRIME™ DNA and siRNA Transfection Reagent (VWR International) according to the manufacturer's protocol. Four hours after transfection, the mediums were removed and followed by a 24 h treatment with galangin/myricetin. The cells were harvested and analyzed for luciferase activities with ONE-Glo Luciferase Assay System (Promega) and detected by Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany). Total protein levels were analyzed with a BCA Protein Assay Kit (Pierce), and the activities of the reporter were normalized by corresponding total protein levels for statistical analysis.

2.9. Small interfering RNA (siRNA) transfection luciferase assay

OVCAR-3 ovarian cancer cells (5000 cells/well) were seeded in 96-well plates and incubated overnight. The cells were transfected with siRNA p21 or control siRNA, and then transfected with HIF-1α/VEGF luciferase reporter for 24 hours using jetPRIME™ DNA and siRNA Transfection Reagent (VWR International) according to the manufacturer's protocol. The mediums were removed and followed by a 24 h treatment with galangin/myricetin. The cells were harvested and analyzed for luciferase activities with ONE-Glo Luciferase Assay System (Promega) and detected by Lumat LB9507 (Berthold Technologies). Total protein levels were analyzed with a BCA Protein Assay Kit (Pierce), and the activities of the reporter were normalized by corresponding total protein levels for statistical analysis.

2.10. Statistical analysis

All experiments were performed at least three times independently. Results were expressed as mean ± standard error of mean (SEM) using Microsoft Excel (2007). Statistical assessment was carried out with the program system of SPSS (Version 18.0 for Windows). The results were analyzed using one-way analysis of variance (ANOVA) and post hoc test (2-sided Dunnett's test) to test both overall differences and specific differences between each treatment and control. A p value of less than 0.05 was considered significant.

3. Results

3.1. Effect of galangin and myricetin on ovarian cancer cells viability

CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay was performed after treatment of ovarian cancer cells with galangin and myricetin to investigate their effects on the viability of A2780/CP70 and OVCAR-3 cells. It showed that galangin had little effect on A2780/CP70 and OVCAR-3 cell viability at concentrations of 20 μM galangin and below. Cell viability was decreased from 74.4 and 66.8% at a concentration of 40 μM galangin to 14.2 and 15.6% at a concentration of 160 μM galangin in A2780/CP70 and OVCAR-3 cells. ($p<0.05$) (Figure 1A). Myricetin had more cytotoxicity to the ovarian cancer cells than galangin. Compared with controls (DMSO), cell viability with myricetin treatment (5-80 μM) for 24 h ranged from 101.6 and 100.8% to 9.1 and 6.6% ($p<0.05$) for A2780/CP70 and OVCAR-3 cells, respectively (Figure 1B).

3.2. Galangin and myricetin reduce VEGF secreted by A2780/CP70 and OVCAR-3 cells

Vascular endothelial growth factor (VEGF), an important growth factor that is involved in tumour vascular development and maintenance (Duyndam et al., 2002), was investigated. As shown in Figure 2A, VEGF levels were 68.7%, 17.3% ($p<0.05$) in A2780/CP70 cell and 67.2%, 24.6% ($p<0.05$) in OVCAR-3 cell inhibited by 10 and 60 μM galangin treatment, respectively. Similarly, Figure 2B shows that VEGF levels were 74.4 and 51.9% ($p<0.05$) in A2780/CP70 cell and 74.9 and 46.9% ($p<0.05$) in OVCAR-3 cell inhibited by 5 and 20 μM myricetin treatment.

3.3. Galangin and myricetin inhibit *in vitro* angiogenesis induced by OVCAR-3 cells

The effective inhibition of VEGF secretion by galangin and myricetin was expected to inhibit angiogenesis *in vitro*. Therefore, *in vitro* tube formation by HUVEC cells induced by the culture medium of ovarian cancer cells treated with different concentrations of galangin and myricetin was tested. It was found that culture media conditioned by ovarian cancer cells OVCAR-3 promoted *in vitro* angiogenesis and a well-established network formation of HUVEC cells (Figure 3). HUVEC networks, however, were truncated after galangin and myricetin treatment. A significant reduction in tube length was observed at a concentration of 10 μM galangin and 5 μM myricetin when compared to the corresponding controls ($p<0.05$) (Figure 3).

3.4. Galangin and myricetin inhibit *in vivo* angiogenesis induced by OVCAR-3 cells

This experiment was performed to test the hypothesis that effective inhibition of VEGF secretion by galangin and myricetin treatment can be translated into anti-angiogenic effects *in vivo*. Chicken chorioallantoic membrane (CAM) assay was used to detect their effects on angiogenesis induced by ovarian cancer cells. As shown in Figure 4, galangin (40 μM) and myricetin (20 μM) significantly reduced formation of blood vessels which were induced by OVCAR-3 cells in CAM ($p<0.05$).

3.5. The pathway of anti-angiogenesis in ovarian cancer cells treated with galangin and myricetin

An investigation into which proteins are involved in the expression of VEGF in A2780/CP70 and OVCAR-3 cells was conducted. We detected the levels of HIF-1α, PTEN, NFκB (p50), p-Akt (Ser473), t-Akt, p-p70S6K and t-p70S6K proteins in A2780/CP70 and OVCAR-3 cells. Figure 5A and 5B show that galangin and myricetin strongly down-regulated levels of HIF-1α, inhibited phosphorylation of Akt and p70S6K, but had no effect on the expression of PTEN and NFκB (p50) in A2780/CP70 and OVCAR-3 cells.

HIF-1α is one of the key factors for the regulation of VEGF expression. Transfected plasmid HIF-1α concentration-dependently attenuated galangin's inhibitory effects on expression of VEGF in OVCAR-3 cells (Figure 5C). Next, OVCAR-3 cells were transfected with the HIF-1α reporter together with p70S6K and Akt plasmids. Galangin treatment significantly inhibited HIF-1α transcriptional activity; however, this inhibition was significantly attenuated by overexpression of p70S6K and Akt proteins (Figure 5C). Similarly, myricetin also significantly inhibited HIF-1α and VEGF transcriptional activities in OVCAR-3 cells.

Concentration-dependent overexpression of p70S6K and Akt proteins significantly reversed this inhibition (Figure 5D). These results indicate that galangin and myricetin inhibit angiogenesis in OVCAR-3 cells, at least partly, through decreasing levels of HIF-1α, Akt and p70S6K.

3.6. Role of p21 on the myricetin-inhibited angiogenesis in OVCAR-3 cells

P21, as a regulator of cell cycle progression, has been reported to inhibit VEGF expression in OVCAR-3 cells (Luo, Rankin, Juliano, Jiang, & Chen, 2012). Therefore, some proteins which are associated with p21 were examined to clarify whether myricetin inhibits viability in OVCAR-3 cells via the p21 protein (Figure 6A). It was found that myricetin up-regulated levels of the p21 and p53 proteins and down-regulated levels of the oncogene cmyc protein.

To determine the role of p21 on the anti-angiogenesis effect of myricetin, OVCAR-3 cells were transfected with HIF-1α and VEGF-promoter with p21 siRNA (50 nM). Figure 6B shows knockdown of p21 by specific siRNA (50 nM) resulted in the abrogation of myricetin-inhibited HIF-1α and VEGF transcriptional activities. As the western blot result shows, transfection with p21 siRNA increased the levels of HIF-1α protein in OVCAR-3 cells treated with and without myricetin. From these results, it was inferred that p21/HIF-1α/VEGF is one of the pathways involved with myricetin-inhibited angiogenesis in OVCAR-3 cells. However, the results from western blot showed that knockdown of p21 didn't abrogate the effect of myricetin on the levels of p-Akt and p-p70S6K proteins in OVCAR-3 cells (Figure 6C).

4. Discussion

While early diagnosis of ovarian cancer is still difficult, chemoprevention with dietary compounds might be a good strategy for the vast majority of healthy women who are at risk of developing ovarian cancers. Previous studies have reported that galangin and myricetin inhibited cell growth in several cancer cells, such as hepatoma, pancreatic, esophageal, melanoma, gastric, and colon carcinoma cells (Kim, Jeon, & Nam, 2012; Kim, Ha, Yoon, & Lee, 2014; Phillips, Sangwan, Borja-Cacho, Dudeja, Vickers, & Saluja, 2011; Zang et al., 2014; Zhang et al., 2010; Zhang, Lan, Huang, & Hua, 2013; Zhang, Chen et al., 2013). However, their effects on ovarian cancer cells are currently unknown. Galangin and myricetin, which have similar structure (same parent structure, ring A and ring C), are members of flavonol. However, compared to galangin, myricetin has 3 adjacent hydroxyl groups in ring B that results in different chemical properties such as polarity. The difference of structural characteristics between galangin and myricetin may lead to different efficacy in inhibiting the development of ovarian cancers. In this research, myricetin have more cytotoxicity to ovarian cancer cell lines A2780/CP70 and OVCAR-3 than galangin. It is in accordance with previous work which exhibited the inhibitory effects of flavonoids on leukemia cell line HL-60 as follows: myricetin > quercetin (2 adjacent hydroxyl groups in ring B) > galangin. The ortho-substituting hydroxyl group in ring B is associated with enhanced inhibitory effects of flavonol on the cancer cells proliferation (Chang, Mi, Gu, Yuan, Ling, & Lin, 2007).

Tumours need an intact blood system to supply nutritients and oxygen and shed metabolites (Bertl, Bartsch, & Gerhauser, 2006). Angiogenesis is a physiologic process through which new blood vessels form from pre-existing vessels (Birbrair et al., 2014). It is a necessary condition for sustained tumour growth and plays a central role in the development and progression of cancers. Ovarian tumours are richly vascularized, and the degree of neovascularization and angiogenesis is associated with biological aggressiveness (Alvarez, Krigman, Whitaker, Dodge, & Rodriguez, 1999). In this research, galangin and myricetin effectively inhibited formation of blood vessel networks induced by ovarian cancer cells. In the CAM model, galangin and myricetin treatment significantly shrunk blood vessel counts, confirming their potency in countering angiogenesis.

Vascular endothelial growth factor (VEGF) plays a central role in the mediation of tumour vascular development and maintenance. It is connected to various steps of ovarian carcinogenesis (Hefler et al., 2007). The VEGF gene is a direct target of hypoxia-inducible factor 1α (HIF-1α), a heterodimeric basic helix-loop-helix protein (Forsythe et al., 1996). Stabilization and up-regulation of HIF-1α promotes the expression of VEGF by binding to HIF-responsive elements (HREs) in promoters that contain the sequence NCGTG. Previous studies show that another flavonoid compound with similar chemical structure, kaempferol, inhibited angiogenesis in ovarian cancer cells through down-regulating HIF-1α expression (Jiang & Liu, 2008; Luo, Rankin, Liu, Daddysman, Jiang, & Chen, 2009). This is consistent with the result obtained here, indicating that galangin's and myricetin's inhibitory effects on angiogenesis in ovarian cancer cells can be traced back to suppression of HIF-1α expression. Combined with our previous work (Luo, Rankin, Liu, Daddysman, Jiang, & Chen, 2009), we found that galangin, myricetin and keampferol all significantly inhibited HIF-1α protein in A2780/CP70 and OVCAR-3 cells at a low concentration (< 10 μM). As members of flavonol, the three compounds have exactly similar chemical structure except ring B which is substituted with different amounts of hydroxyl group. We speculate that the parent structure of flavonol is related with the high inhibitory effects on the HIF-1α expression in A2780/CP70 and OVCAR-3 cells.

A previous study performed in OVCAR-3 cells showed that p70S6K had an effect on angiogenesis via regulating HIF-1α and VEGF proteins (Bian, Shi, Meng, Jiang, Liu, & Jiang, 2010). Akt has also been involved in angiogenesis and tumour development. In several *in vivo* studies, Akt, known as a mediator of VEGF, enhanced pathological angiogenesis and tumour growth associated with matrix abnormalities in skin and blood vessels (Chen et al., 2005; Somanath, Razorenova, Chen, & Byzova, 2006). Previous works indicate that HIF-1α and p70S6K proteins are also regulated by Akt (Blancher, Moore, Robertson, & Harris, 2001; Chung, Grammer, Lemon, Kazlauskas, & Blenis, 1994). In this present work, VEGF, HIF-1α, p-Akt and p-p70S6K proteins were all decreased after treatment with galangin and myricetin in A2780/CP70 and OVCAR-3 cells. Overexpression of HIF-1α protein reversed galangin's and myricetin's inhibitory effects on VEGF transcription in OVCAR-3 cells. Meanwhile overexpression of both p70S6K and Akt proteins in the presence of galangin and myricetin neutralized their inhibitory effects on HIF-1α transcription. We also found that the effect of myricetin on VEGF was neutralized by overexpression of either p70S6K or Akt proteins. These results confirm speculation that

galangin and myricetin inhibit angiogenesis in OVCAR-3 cells, at least partly, through the Akt/p70S6K/HIF-1α/VEGF pathway. Previous works found that myricetin had effects on the Akt/p70S6K pathway in HepG2 and SKH-1 cells (Jung et al., 2010; Zhang, Chen et al., 2013) and galangin inhibits the phosphorylation of Akt in HepG2 cells (Zhang, Li et al., 2013). This agrees with the results obtained in this study. NFκB, a direct modulator of HIF-1α expression (van Uden, Kenneth, & Rocha, 2008), is associated with angiogenesis (Luo, Rankin, Juliano, Jiang, & Chen, 2012). It is regulated by Akt at the expression, activation, and translocation levels. It was reported that myricetin inhibited IkappaB kinase (IKK) activity and NFκB levels in ECV304 cells (Tsai, Liang, Lin-Shiau, & Lin, 1999). However, in the present work, galangin and myricetin had no effect on the expression of NFκB protein in A2780/CP70 and OVCAR-3 cells. PTEN, which acts as a tumour suppressor gene through the action of its phosphatase protein product such as p-Akt, sensitized human ovarian cancer cells to cisplatin-induced apoptosis and inhibited angiogenesis (Takei et al., 2008). Therefore, an investigation was made to determine whether galangin and myricetin inhibit the phosphorylation of Akt through promoting PTEN expression. However, no effect on the expression of PTEN was found.

P21, known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1, is a regulator of cell cycle progression. Some previous works indicate that p21 is a negative regulator of VEGF protein in cancer cells including OVCAR-3 cells (Farhang, Goossens, & Haigh, 2013; Luo, Rankin, Juliano, Jiang, & Chen, 2012). The effect of p21 protein on the myricetin-inhibited angiogenesis in OVCAR-3 cells was determined in this study. The result agrees with previous works in that p21 negatively regulated transcription of VEGF protein in OVCAR-3 cells. It was also found that p21 is a negative regulator of HIF-α at the transcriptional level, but has no effect on the phosphorylation of p-Akt and p-p70S6K. These results indicate that an Akt and p70S6K-independent pathway of p21/HIF-1α/VEGF, at least partly, was one of the pathways involved in myricetin-inhibited angiogenesis in OVCAR-3 cells. It is a new pathway, different from previous works that show HIF-1α as a regulator of p21 expression (Cho et al., 2008; He et al., 2013).

It is clear that p21 is tightly controlled by the tumour suppressor protein p53 (Gartel & Radhakrishnan, 2005). p53 is a multifunctional tumour suppressor that regulates transcription, DNA repair, cell cycle arrest, genomic instability, differentiation, senescence, apoptosis, angiogenesis and glucose metabolism (Darcy et al., 2008). Another study indicates that p53 inhibits angiogenesis through suppressing expression of VEGF (Farhang et al., 2013). HIF-1α, a direct regulator of VEGF expression, has been reported to be degraded by accumulation of p53 (Chen, Li, Luo, & Gu, 2003). Moreover, p53 has been shown to suppress transcriptional activity of HIF-1α (Ravi et al., 2000). Cmyc, a multifunctional nuclear protein, plays a role in several cell processes such as cell cycle progression, apoptosis, and cellular transformation, and it is regulated by the p53 protein (Ho, Ma, Mao, & Benchimol, 2005). Our previous study indicates that cmyc regulates VEGF expression via a p21-dependent pathway (Luo, Rankin, Juliano, Jiang, & Chen, 2012). In this current research, p53/cmyc/p21/HIF-1α/VEGF might be a pathway associated with myricetin-inhibited angiogenesis in OVCAR-3 cells. The two pathways associated with myricetin-inhibited VEGF secretion by OVCAR-3 cells are both involved in HIF-1α protein

expression, indicating that HIF-1α is the key protein on the regulation of VEGF secretion by OVCAR-3 cells which is inhibited by myricetin.

5. Conclusion

This research indicates that galangin and myricetin are good candidates for the chemoprevention of ovarian cancer in women. *In vitro* (HUVEC) and *in vivo* (CAM) models demonstrate that galangin and myricetin inhibit angiogenesis induced by ovarian cancer cell lines. In OVCAR-3, the inhibitory effects of galangin and myricetin on angiogenesis, at least partly, are involved in the Akt/p70S6K/HIF-1α/VEGF pathway. It was also found that myricetin inhibits angiogenesis in OVCAR-3 cells through a new pathway: p21/HIF-1α/VEGF. As an *in vitro* model, cell cultures cannot take absorption, distribution, metabolism, and excretion of galangin or myricetin into consideration. Further studies in animal models and human trials are needed to further determine the efficacy of this natural compound as an agent for chemoprevention of ovarian cancer.

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- Two dietary compounds inhibited in vitro (HUVEC) angiogenesis.
- Two dietary compounds inhibited in vivo (CAM) angiogenesis.
- The effects on the secretion of VEGF is involved in Akt/p70S6K/HIF-1α pathway.
- Angiogenesis inhibition is through p21/ HIF-1α/VEGF pathway.

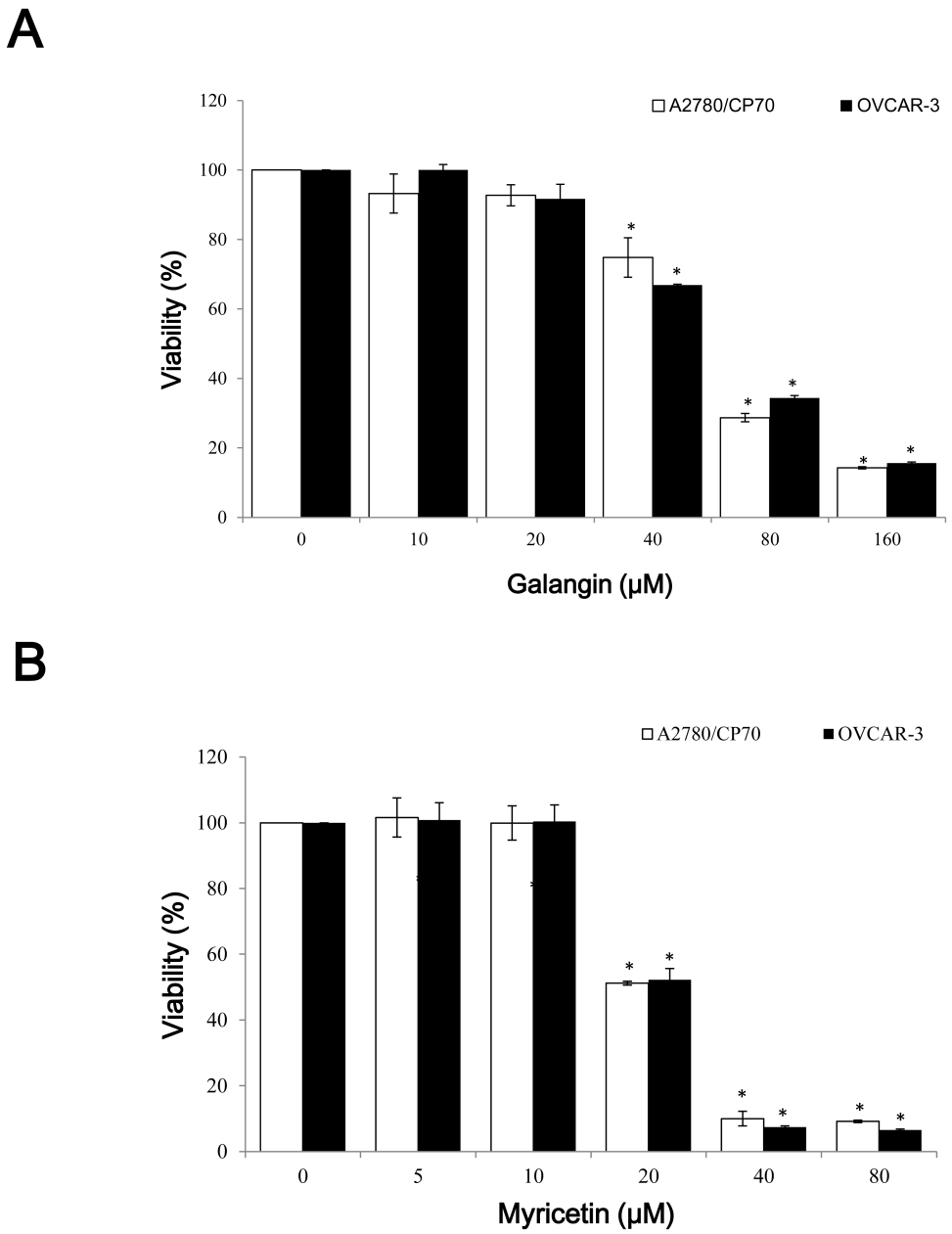


Fig. 1. The cytotoxic effects of galangin and myricetin on ovarian cancer cells A2780/CP70 and OVCAR-3

(**A**) Galangin decreased cell viability in ovarian cancer cells. (**B**) Myricetin decreased cell viability in ovarian cancer cells. Cells (2×10^4 /well) were seeded in 96-well plates, incubated overnight, and then treated with galangin or myricetin for 24 h. Cell viability was determined by CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay and expressed as percentages of control. *$p < 0.05$ as compared to control.

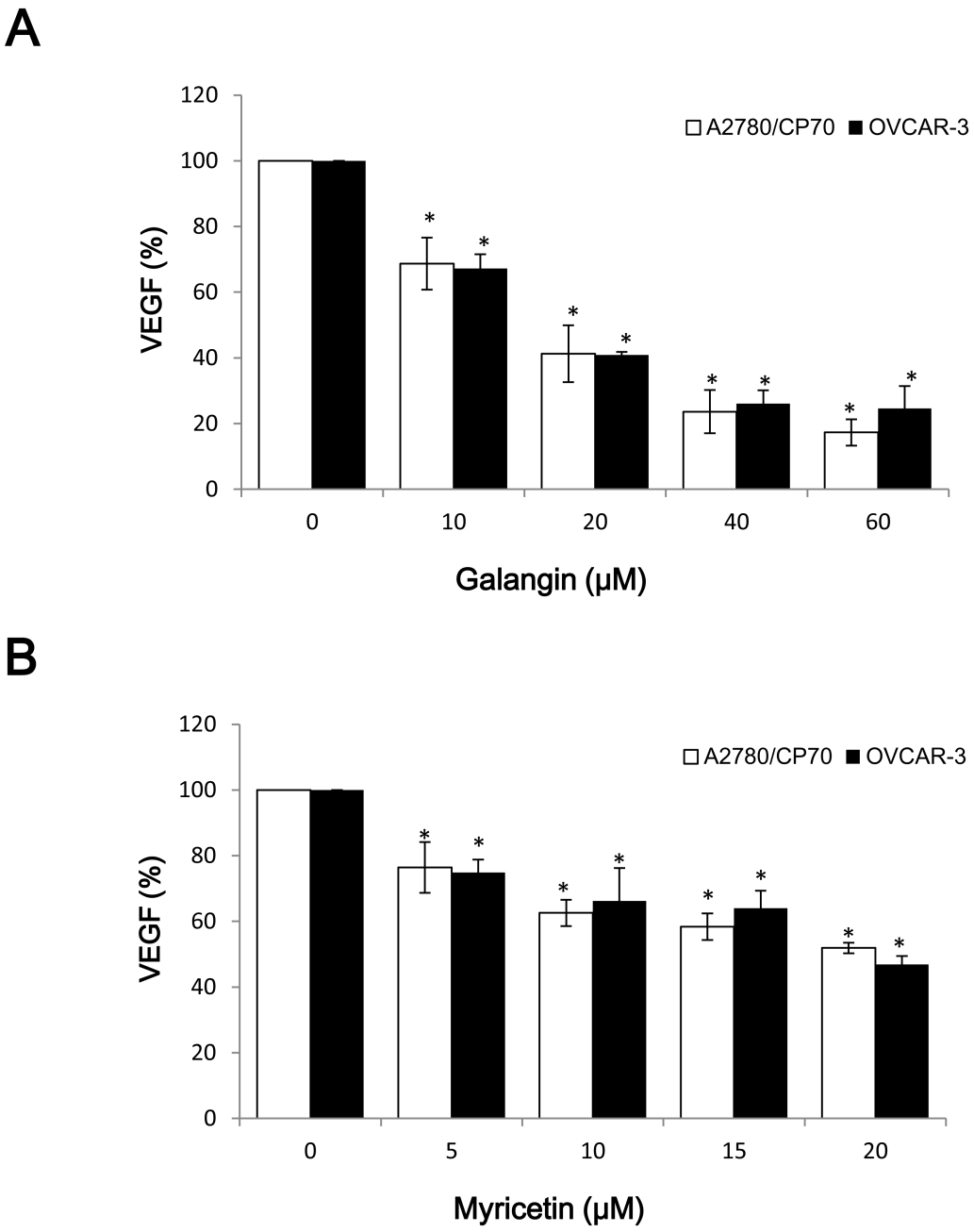


Fig. 2. The inhibitory effects of galangin and myricetin on VEGF secretion

(**A**) Galangin inhibited secretion of VEGF by A2780/CP70 and OVCAR-3 cells. (**B**) Myricetin inhibited secretion of VEGF by A2780/CP70 and OVCAR-3 cells. The human ovarian cancer cell line A2780/CP70 and OVCAR-3 were treated with varying concentrations of galaing and myricetin for 24 hours. Subsequently, the VEGF levels in the supernatant were measured by ELISA. *$p < 0.05$ as compared to control.

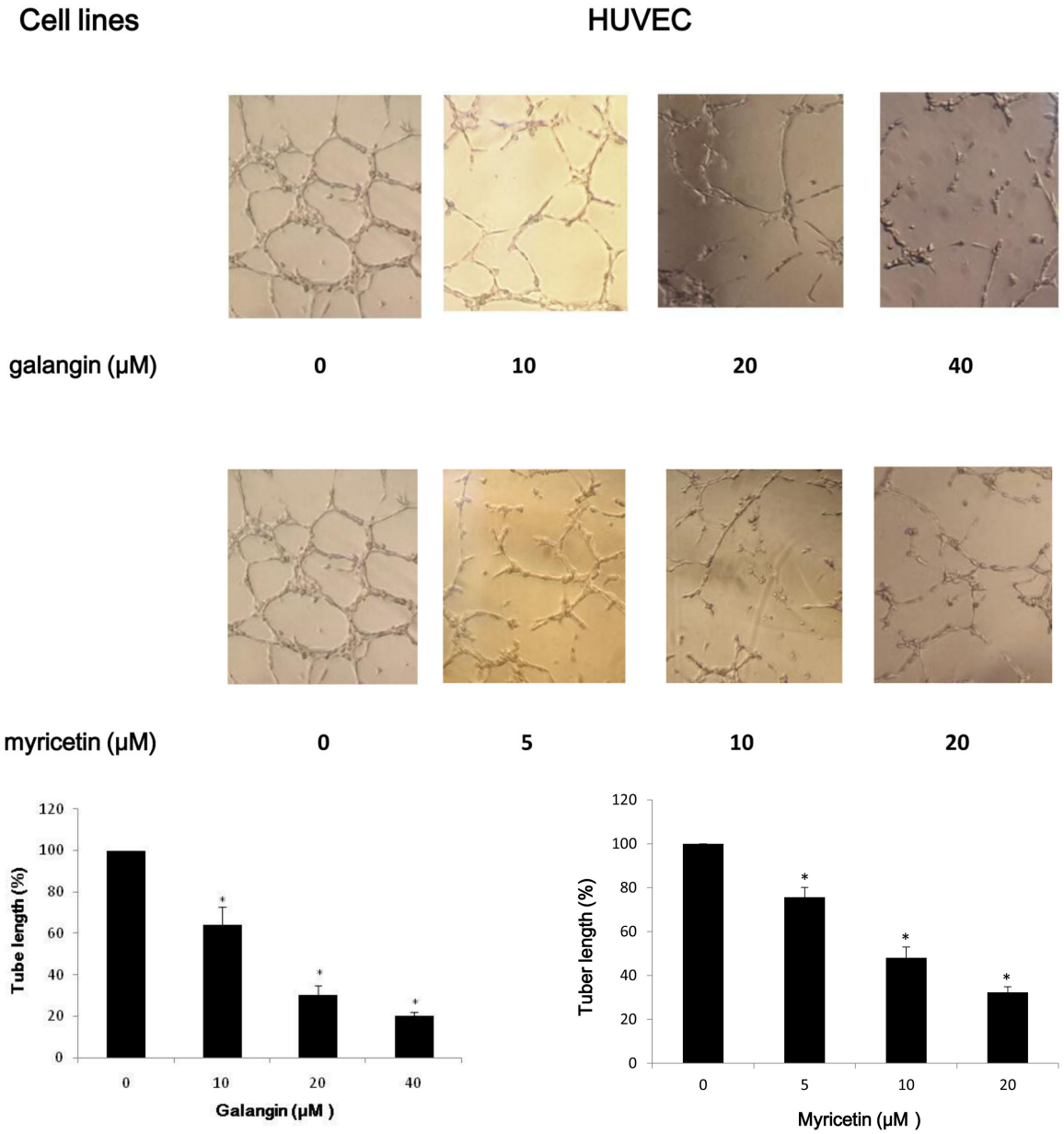


Fig. 3. Galangin and myricetin inhibited in vitro (HUVEC) angiogenesis induced by OVCAR-3 cells

OVCAR-3 cells were seeded (2×10^4), treated with vary concentrations of galangin or myricetin for 24 hours, and then the media were collected. HUVEC cells were harvested, counted and seeded onto the gelled Matrigel beds. The collected conditioned cell culture media were added and the cells were incubated for 6 hours.

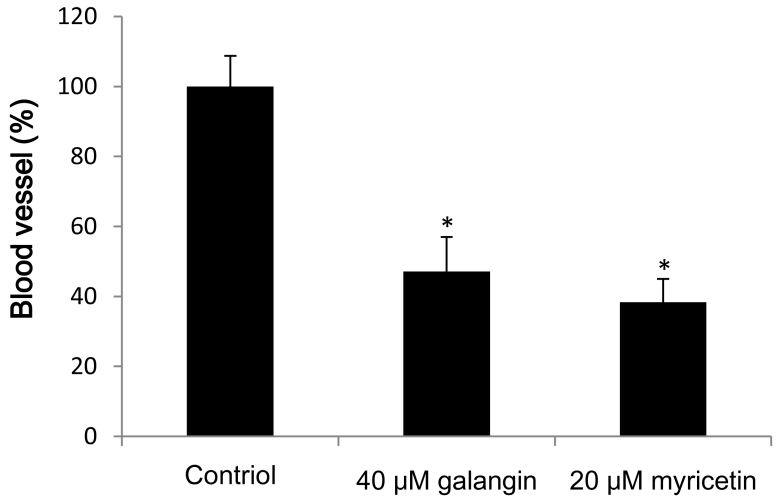
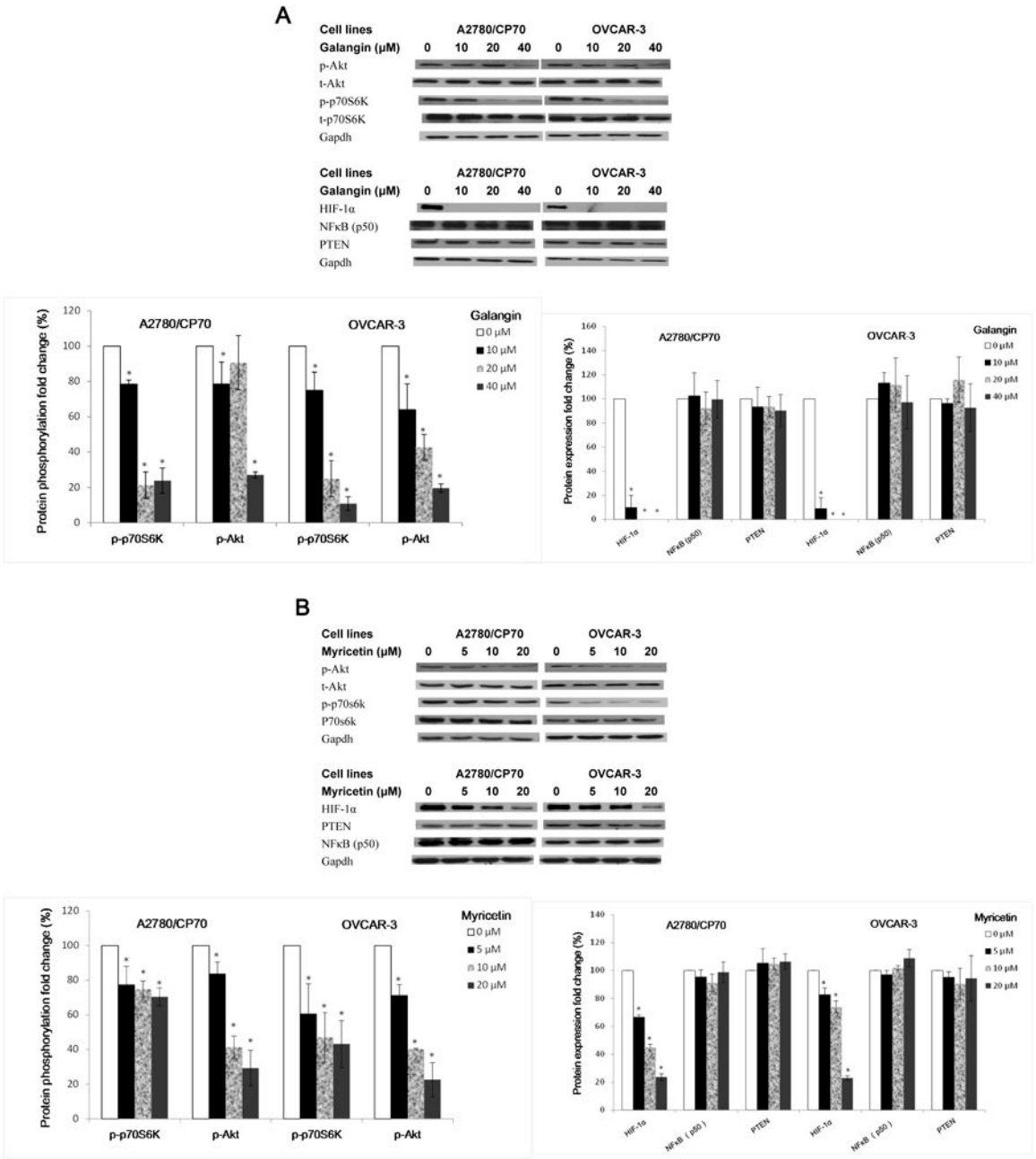


Fig. 4. Galangin and myricetin inhibited in vivo (CAM) angiogenesis induced by OVCAR-3 cells

Angiogenesis was evaluated by normalizing number of branching vessels to that of control CAM. The data are represented as mean ± SEM (n=6 eggs/group). *$p < 0.05$ as compared to control.



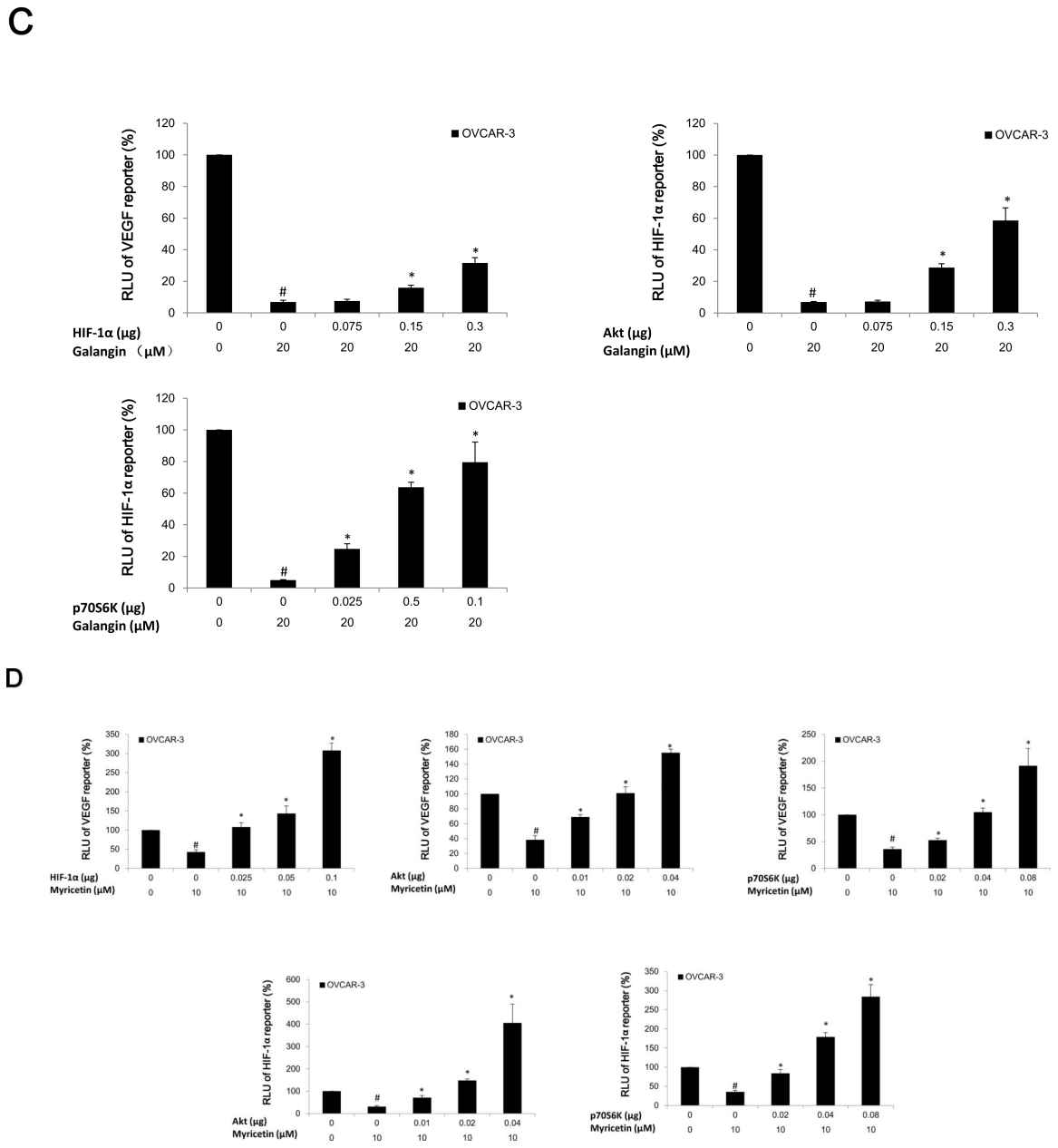
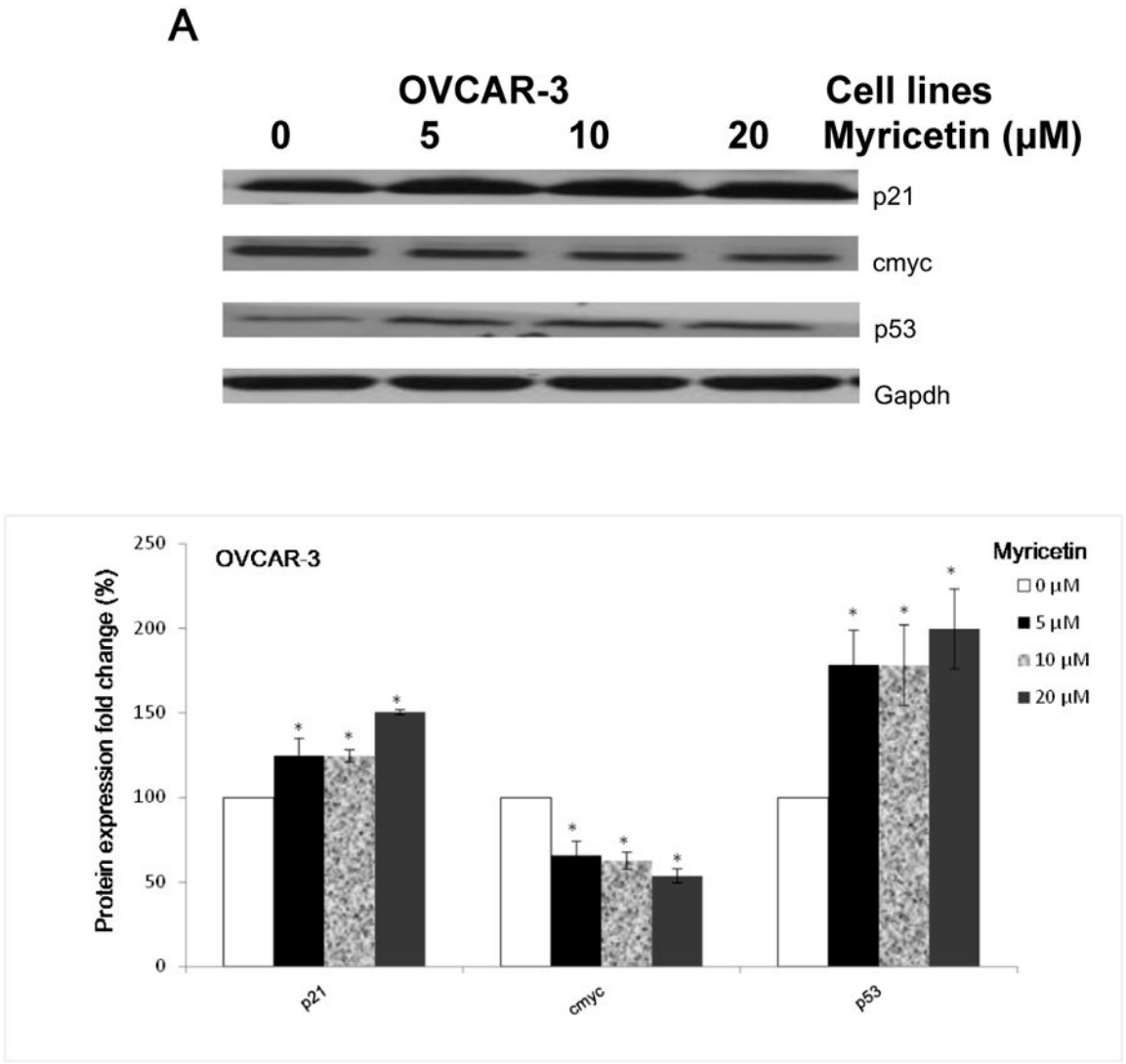


Fig. 5. Galangin and myricetin inhibited angiogenesis in ovarian cancer cells were involved in Akt- and p70S6K-dependent pathways

(**A**) Galangin decreased the levels of HIF-1 α, phosphor-Akt, phosphor-P70S6K and had no effect on NFκB and PTEN in A2780/CP70 and OVCAR-3. (**B**) Myricetin decreased the levels of HIF-1α, phosphor-Akt, phosphor-p70S6K and had no effect on NFκB and PTEN in A2780/CP70 and OVCAR-3. Protein bands were normalized by corresponding GAPDH bands (p-Akt/p-p70S6K were normalized by t-Akt/t-p70S6K), and expressed as percentages of control. *$p < 0.05$ as compared to control. (**C**) Overexpression of HIF-1α and Akt (p70S6K) protein attenuated the inhibitory effect of galangin on VEGF and HIF-1α transcriptional activation respectively. (**D**) Overexpression of HIF-1α, Akt and p70S6K protein reversed the inhibitory effect of myricetin on VEGF and HIF-1 α transcriptional

activation. OVCAR-3 cancer cells were seeded in 96-well plate and incubated overnight. The cells were then transfected with VEGF (HIF-1α) luciferase reporter, HIF-1α (Akt, p70S6K) or SR-α plasmids for 4 hours, followed by 24 hours treatment with or without galangin or myricetin. The cells were harvested and analyzed for luciferase and total protein levels, and the levels of VEGF and HIF-1α reporters were normalized by corresponding total protein levels. $^{\#}p < 0.05$ as compared to control; $^{*}p < 0.05$ as compared to galangin (myricetin)-treated control. (RLU: relative luminescence units)



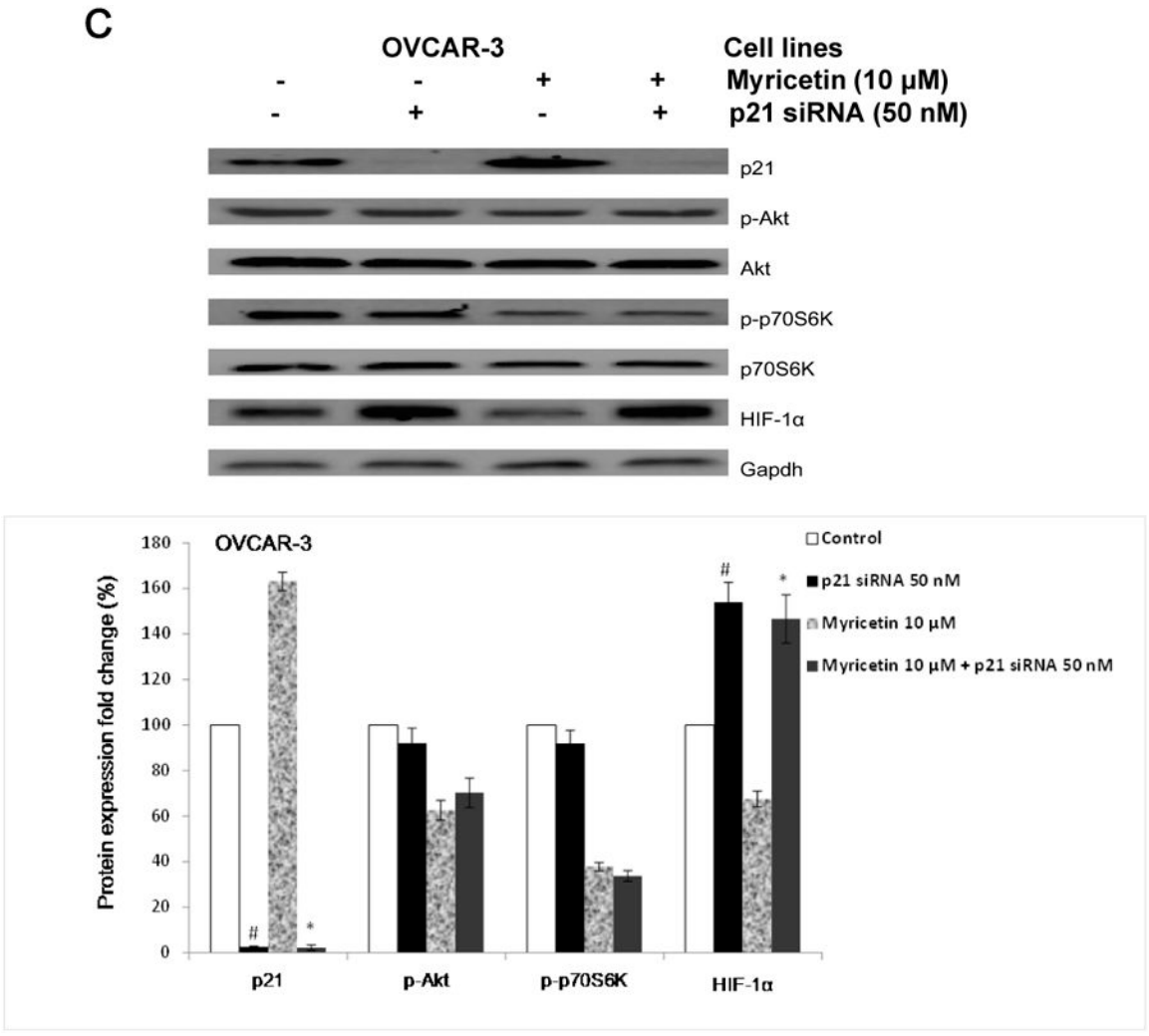


Fig.6. Myricetin inhibited angiogenesis in OVCAR-3 cells with a p21-dependent pathway

(**A**) Myricetin decreased the levels of cmyc and increased expression of p21 and p53 in OVCAR-3. Protein bands were normalized by corresponding GAPDH bands, and expressed as percentages of control. *$p < 0.05$ as compared to control. (**B**) Knockdown of p21 resulted in reversing inhibitory effect of myricetin on VEGF and HIF-1α transcriptional activation. OVCAR-3 cells were transfected with VEGF (HIF-1α) luciferase reporter with p21 siRNA or control siRNA for 24 hours, followed by 24 hours treatment with or without myricetin. The cells were harvested and analyzed for luciferase and total protein levels, and the levels of VEGF and HIF-1α reporters were normalized by corresponding total protein levels. (**C**) Knockdown p21 resulted in abrogation of myricetin-decreased levels of HIF-1α but not p-Akt and p-p70S6K protein. OVCAR-3 cells were seeded in 60-mm dishes, incubated overnight, and then transfected with p21 siRNA or control siRNA. After 24 hours, cells were treated with myricetin or DMSO. Cell lysates were collected for Western blot. Protein bands were normalized by corresponding GAPDH bands, and expressed as percentages of control. $^{\#}p < 0.05$ as compared to control; *$p < 0.05$ as compared to myricetin-treated control.