Original Article

Ferulago angulata Flower and Leaf Extracts Inhibit Angiogenesis *in vitro* through Reducing *VEGF-A* and *VEGFR-2* Genes Expression

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Abstract

Background: *Ferulago angulata* (Apiacea) has been used in Iranian traditional medicine since ancient times and its various health care and pharmacological benefits have been demonstrated recently. In this study, for the first time, we have investigated the effects of *F. angulata* flower and leaf ethanol extracts on angiogenesis, as the key process in tumor growth, invasion and metastasis.

Methods: Cytotoxic effects of different concentrations (20–140 µg/mL) of each extract were assessed on human umbilical vein endothelial cells (HUVECs) using neutral red uptake assay. After evaluating the less toxic concentrations (up to 80 µg/mL), we performed three-dimensional angiogenesis, tube formation and migration assays to assess the key properties of HUVECs, including the angiogenesis process, in response to the extracts. Finally, quantitative gene expression analysis of *VEGF-A* and *VEGFR-2*, two critical mediators of angiogenesis, was performed using real-time RT-PCR.

Results: Both flower and leaf extracts exhibited concentration-dependent inhibition of sprouting, tube formation and migration capacities of HUVECs. For the flower extract, the respective IC_{50} values were 25.79, 26.52 and 38.92 µg/mL while for the leaf extract, the corresponding IC_{50} values were 34.18, 41.24 and 28.69 µg/mL. Both flower and leaf extracts downregulated *VEGF-A* and *VEGFR-2* genes relative to the *GAPDH* gene as the internal control at concentrations of 60 and 80 µg/mL, respectively.

Conclusion: These findings suggest that both flower and leaf extracts may contain anti-angiogenic compounds and may have the capacity to be utilized in tumor anti-angiogenic therapy strategies.

Keywords: Natural products, quantitative gene expression, scratch assay, 3-D Angiogenesis, tube formation

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Introduction

Angiogenesis, the sprouting and growth of pre-existing vessels, plays a critical role in tumor growth and metastasis.[1] It has been suggested to occur in tumor environment in 1945 and proposed to be a promising target in new therapeutic strategies in 1971.[2–3] Since then, a large number of studies have focused on finding different factors and mechanisms involved in angiogenesis in order to find the best way to impede it and consequently, suppress tumor growth and invasion.[4] Among many different angiogenic factors, the vascular endothelial growth factor (VEGF) family and their relevant receptor tyrosine kinases play a critical role in angiogenesis[5]. In VEGF family, VEGF-A and its main receptor, VEGFR-2 (Flk-1/KDR), have been known as the main mediators in both physiologic and pathologic angiogenesis and are therefore among the most reliable targets for new anti-angiogenic drugs.[6]

There are certain limits on traditional cancer therapies such as poor improvement rate after chemotherapy, surgery, and resistance of some tumors to chemotherapy and radiotherapy.[7–8] Nowadays, combining traditional cancer therapies (such as radiation therapy and chemotherapy) with anti-angiogenic drugs, mainly targeting key molecules in tumor-induced angiogenesis, yields promising outcomes in clinical trials.[8] However, the side effects of chemical-based drugs are inevitable.[9]

Although conventional therapies, including radiotherapy and chemotherapy, have indicated benefit in suppressing different types of tumors, severe side effects reported with such therapies encouraged scientists to examine different plant products, common in herbal medicine, for anti-tumoric and anti-angiogenic activities.[9]

Most plants contain different vitamins, phytochemicals and antioxidants that have been known to represent potent cancer prevention by affecting different biochemical pathways responsible for supporting tumor growth and metastasis via inducing apoptosis or inhibiting angiogenesis or both.[9–10]

Ferulago (Apiacea) comprises 35 to 40 species worldwide, of which seven are native to Iran. *Ferulago angulata* (locally known as chavir) is a shrub indigenous to western Iran, Turkey and Iraq. *F. angulata* has been used in treating digestive pains, hemorrhoids, snake bite, ulcers and as sedative in herbal medicine and also a flavor in oil and dairy by native people.[11]

In this study, the anti-angiogenic activity of *F. angulata* flower and leaf ethanol extracts has been investigated at less cytotoxic concentrations in human umbilical vein endothelial cell line (HUVEC). Two-dimensional and three dimensional *in vitro* assays were used to evaluate angiogenesis, and then gene expression of *VEGF-A*, and its main receptor, *VEGFR-2*, were quantified in

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HUVECs with anti-angiogenic activity, but less cytotoxic concentrations.

Materials and Methods

Preparation of *F. angulata* flower and leaf ethanol extracts

F. angulata were collected in May 2010 from Shahoo Mountains, western Iran and identified by Dr. Seyedmohammad Masoumi, Assistant Professor of botany at Razi University, Kermanshah, Iran. A voucher herbarium specimen (No: 580) was deposited in the herbarium of the Biology Department of Razi University (HBRU). Aerial parts of the plant were dried in dark, at room temperature (20–25°C) for 7 days and dried flowers and leaves were ground in a blender. ca. 50 g of each powder was put in Soxhelt apparatus separately and mixed with petroleum ether (~3 times) until discoloration. Subsequently, the two almost resin-free suspensions were centrifuged (at 3000 × g for 10 min) to extract lipids, pigments and resins. Acquired pellets about 6% w/w of the starting dried flower and leaf were re-mixed with 300 mL ethanol and stirred for 4 days. After centrifugation of the ethanolic flower and leaf samples at 3000 × g for 15 min, using a Heidolph rotary evaporator, the supernatants were evaporated under vacuum to dryness and the resulting powders were dissolved in dimethylsulfoxide (DMSO) at different concentrations. The DMSO concentrations in the culture medium were lower than 1% v/v so as not to have cytotoxic effects on HUVECs.

Cell culture

HUVECs were obtained from the Medical Biology Research Center of Kermanshah University of Medical Sciences. The cells were cultured in Dulbecco Modified Eagle's Medium (DMEM) (Gibco, Belgium): Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma-Aldrich, USA) with 1:1 ratio, containing 10% Fetal Bovine Serum (FBS) (Biocrom AG), 100 U/mL penicillin (Sigma-Aldrich, USA) and 100 µg/mL streptomycine (Sigma-Aldrich, USA). Incubation was completed at 37°C in a humidified atmosphere of 5% CO_2. HUVECs of passages 10–15 were used in different experiments and the culture media were changed twice a week. Cells were detached using 0.025% trypsin-0.02% EDTA (Sigma-Aldrich, USA) in PBS.

Cell Viability Assay

Neutral Red (Sigma-Aldrich, USA) uptake assay was conducted to assess cell viability of HUVECs after each flower and leaf extract treatments. Briefly, HUVECs were cultured at a concentration of 3×10^4 cells/well on 24-well plates in DMEM: RPMI 1640 (1:1), supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycine. After 24 h, when HUVECs achieved approximately 50–60% confluent monolayer, the medium of each well was replaced with fresh DMEM: RPMI 1640 (1:1) containing 3% FBS, supplemented with appropriate antibiotics and different concentrations of flower and leaf extracts (20, 40, 60, 80, 100, 120, and 140 µg/mL) and the highest concentration of applied DMSO (0.1% v/v) used as control. The cells were incubated for two time intervals, 24 h and 48 h in a humidified atmosphere of 5% CO_2 at 37°C. At the end of the specified time period, the medium and dead cells were washed out using phosphate buffered saline (PBS) (MERCK, Germany). Subsequently, 500 µL of serum free DMEM: RPMI 1640 (1:1) medium containing 33 µg/mL of Neutral Red dye, was provided to the cells and incubated for 2 h in humidified 37°C air containing 5% CO_2. Then, the medium was washed out using PBS and 500 µL of destaining solution including 15% v/v acetic acid and 45% ethanol added to each well and incubated at 37°C in dark shaking incubator for 15 min. The absorbance of each well was measured at 540 nm using destaining solution as reference[12]. Each test was done in triplicate and repeated three times.

Three-dimensional angiogenesis assay

Cytodex 3-microcarrier beads (Amersham Pharmacia Biotech) were prepared according to the manufacturer's instruction. HUVECs were loaded on beads by flicking cell suspension and micro carriers every 20 min in a DMEM: RPMI 1640 (1:1) medium containing 10% FBS supplemented with appreciate antibiotics for 4 h in humidified 37°C air containing 5% CO_2, and then incubated for 16 h under the same conditions without flicking.

The following day, the cell-coated beads were mixed with collagen solution (collagen type I, 10X DMEM: RPMI 1640 (1:1) medium, 23 mg/mL $NaHCO_3$ and FBS with the ratio of 7.5:1:1:0.5, respectively)[13] on ice and 100 µL of the solution loaded into each 96-well plate and allowed to solidify for 20 min at 37°C and 5% CO_2 incubator. Then, 3% FBS containing DMEM: RPMI 1640 (1:1) medium and different concentrations of flower and leaf extracts (30, 40, 50 and 60 µg/mL of the flower extracts and 40, 50, 60 and 70 µg/mL of the leaf extracts) were added to each 96-well plate of HUVECs. Cells were shown (Figure 3) with an inverted microscope (CETI, UK) for 3 days.[14] Using Adobe Photoshop software, sprout formation was measured as the mean number of sprouts in ten beads for each treatment and presented as the percentage of control.

In vitro endothelial tube formation assay

ECM gel (Sigma-Aldrich, USA) was used for tube formation potential of *F. angulata* flower and leaf extracts, following the manufacturer's instruction. Briefly, each well of 24-well plates was coated with 100 µL of gel and after 30 min incubating at 37°C in 5% CO_2 to form a matrix, HUVECs were seeded onto the gel at the concentration of 5×10^4 cells per well in DMEM: RPMI 1640 (1:1) supplemented with 5% FBS, antibiotics and different concentration of flower and leaf extracts (40 and 50 µg/mL of the flower extracts, 50 and 60 µg/mL of the leaf extracts) for 16 hr.[15] To evaluate the tube-like structure formation, the cells were photographed with inverted microscope (CETI, UK) and the average branch points were measured in four randomly chosen microscopic fields using Adobe Photoshop software.

Cell migration assay

The *in vitro* scratch assay was conducted to study the effect of *F. angulata* flower and leaf extracts on the migration capacity of HUVECs. HUVECs were seeded at high density on a 24-well plate in DMEM: RPMI 1640 (1:1), containing appropriate antibiotics and 10% FBS. On the following day, HUVECs adhered and spread to create a confluent monolayer. Cell monolayer were scratched with a spatula. Detached cells were removed and the attached cells were incubated with 500 µL of culture medium containing 5% FBS, supplemented separately with 40 and 60 µg/mL of flower extracts, 60 and 80 µg/mL of leaf extracts and the vehicle control (0.1% DMSO) at 37°C, 5% CO_2 and 90% humidity incubator. The proportion of migrating cells from the edge of the scratch in extract-treated wells was compared to the control wells

for 24 h in five separate fields.[16]

RNA Extraction and RT-PCR

RNeasy Plus Mini kit (Qiagene) was utilized to extract total RNA from HUVECs, according to the manufacturer's protocol. The RNA yield and purity were estimated by 1% agarose gel electrophoresis and the optical density at 260/280 nm ratio. Afterwards, cDNAs were synthesized from total RNA (1 µg) using QuantiTect® Reverse Transcription Kit (Qiagene) according to the manufacturer's instruction. Primers for PCR and real-time RT-PCR were designed using Gene Runner and online primer design softwares such as Primer 3 and primer-BLAST. The sequences of primers are shown below:

GAPDH forward primer, 5'-CCT GCA AAT GGG ACT TAC G- 3'; *GAPDH* reverse primer, 5'-AAA AAC CCT TAT CGC ATT CAA AC-3'; *VEGF-A* forward primer, 5'- CTACCTCCAC-CATGCCAAGT -3'; *VEGF-A* reverse primer, 5'- CACACAG-GATGGCTTGAAGA -3'; *VEGFR-2* forward primer, 5'- GC-GATTGAAAGAAGGAACTAGA -3'; *VEGFR-2* reverse primer, 5'- TAGTCTTTGCCATCCTGCTG -3'.

The lengths of PCR products were 187 bp, 174 bp and 166 bp for *VEGF-A*, *VEGFR-2* and *GAPDH* genes, respectively.

Quantitative real-time RT-PCR

The transcript gene expression level of *VEGF-A* and *VEGFR-2* were measured using Rotor-Gene 3000 System (Corbett Research, Australia) and Quanti Tect™ SYBR® Green PCR kit (Qiagene) in accordance with the manufacturer's protocol. For each amplification experiment, a standard curve was constructed through an initial dilution of 1:1 of cDNA samples and a two-fold serial dilution series to determine the amplification efficiency of reactions. For analyzing data, the $2^{-\Delta\Delta Ct}$ method was used based on the threshold cycle (C_t) values for *VEGF-A* or *VEGFR-2* as target gene and *GAPDH* as an endogenous control gene. Each sample was assessed in duplicate and the assay was repeated at least two times.

Statistical Analysis

One sample Kolmogorov-Smirnov test was used to evaluate parametric characteristics of all data. Thereafter, one-way analysis of variance (ANOVA) with post-hoc (Dunnett *t* (2-sided)) was used to analyze data for each assessment and correlations evaluated by regression. *P*-values less than 0.05 were considered as statistically significant. Results from each assay were presented as the mean ± S.E.M.

Results

Effect of *F. angulata* flower and leaf extracts on HUVECs viability

Results of Neutral Red uptake assay showed a dose and time dependant decrease in HUVECs viability after treating with the flower and leaf extracts in comparison with the controls ($P < 0.05$). The IC50 values of flower extract were 59.37 ± 8.06 µg/mL and 44.4 ± 10.72 µg/mL and the IC50 values of leaf extract were 44 ± 10.36 µg/mL and 46.34 ± 7.2 µg/mL for 24 hr and 48 hr treatment, respectively (Figure 1).

F. angulata flower and leaf extracts decreased three-dimensional in vitro model of angiogenesis

Both flower and leaf extracts decreased three-dimensional *in vitro* angiogenesis in HUVECs seeded on the cytodex-3 micro carriers in a concentration-dependent manner. The flower extracts at the concentration of 50 µg/mL inhibited 98.16% of sprout formation with IC50 of 32.27 ± 16.94 µg/mL. The results of the leaf extracts showed 84.05% inhibition of endothelial cells differentiation and sprout formation at the concentration of 60 µg/mL, and a complete inhibition was observed at higher concentrations, with IC50 of 36.44 ± 16.72 µg/mL (Figure 2).

Both flower and leaf extracts inhibited HUVECs tube formation

Two-dimensional matrigel tube formation assay revealed that both flower and leaf extracts suppressed HUVECs tube formation in a concentration-dependent manner. The flower extracts at the concentrations of 40 µg/mL and 50 µg/mL reduced 79.97% and 91.27% of tube formation, respectively, with IC50 of 42.92 ± 19.11 µg/mL. Similarly, leaf extracts at the concentrations of 50 µg/mL and 60 µg/mL displayed 82% and 93.5% inhibition of tube-like structure formation, respectively, with IC50 of 41.50 ± 19.21 µg/mL (Figure 3).

Cell migration decreased in HUVECs treated with flower and leaf extracts

Flower and leaf extracts remarkably inhibited cell migration of HUVECs in comparison with untreated HUVECs as control

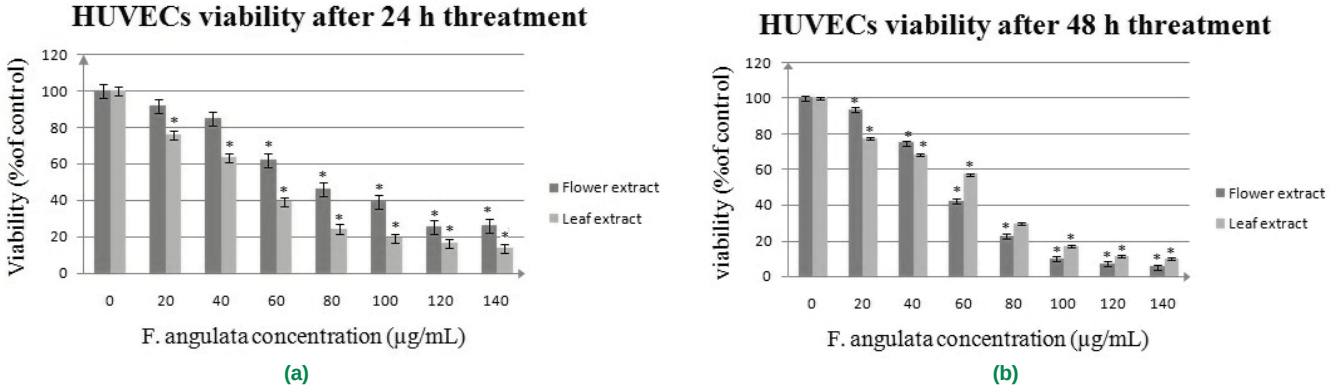
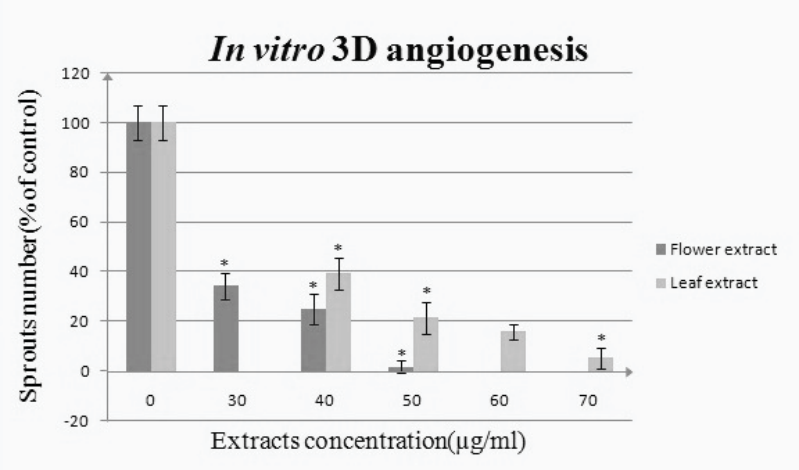


Figure 1. Cell viability effects of *F. angulata* Flower and leaf, on HUVECs. Using Neutral Red uptake assay, cell viability effects of different concentrations of each flower and leaf extracts (0 as control group, 20, 40, 60, 80, 100, 120 and 140 µg/mL) were evaluated on HUVECs, in comparison with the control in **(a)** 24 hr and **(b)** 48 hr. Error bars represent S.E.M of triple samples (*, $P < 0.05$).



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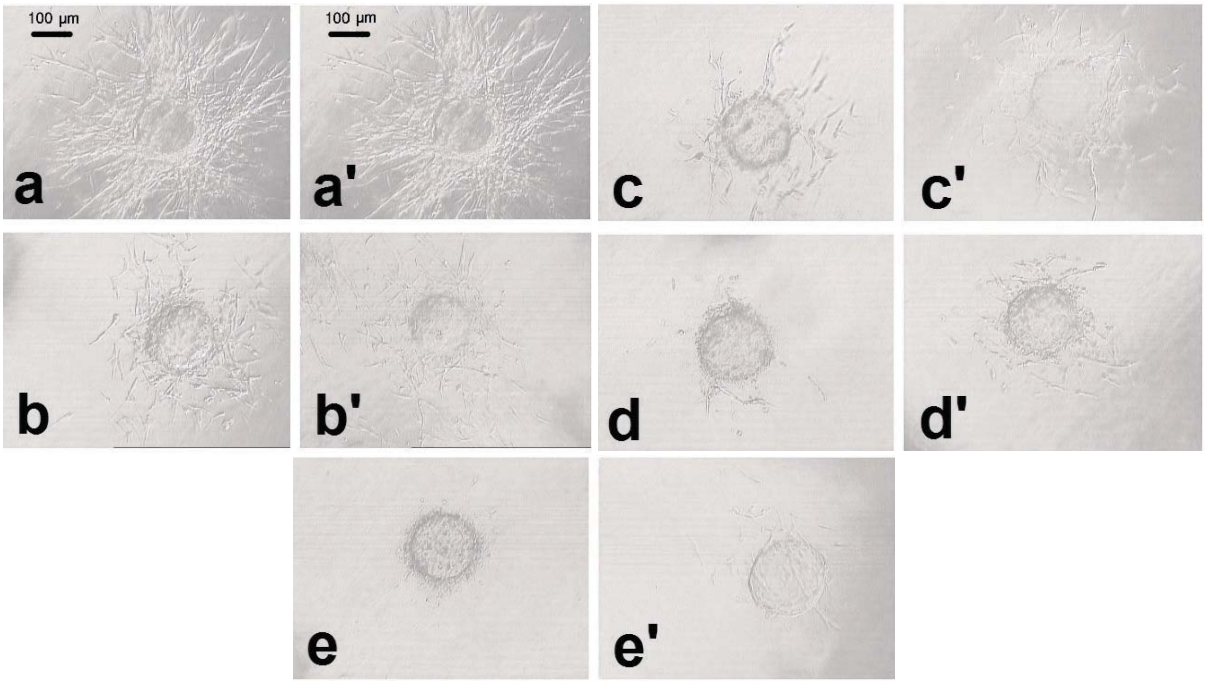

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Figure 2. Sprout formation inhibition with *F. angulata* Flower and leaf extracts in 3-D angiogenesis assay. Cell loaded beads embedded in collagen gel matrix and 3% FBS containing culture medium with different concentrations of flower extract or leaf extract were allowed to cells. **(a)** Sprout formation of HUVECs in different concentrations monitored under the invert microscope after 72 hr (original magnification ×100) a -control (0.5% v/v DMSO), b -30 µg/mL, c -40 µg/mL, d -50 µg/mL, e -60 µg/mL, a' -control, b' -40µg/mL, c' -50 µg/mL, d' -60 µg/mL, e' -70 µg/mL (letters with prime indicate the leaf extract treatment). **(b)** Sprout formation changes of HUVECs in response to different concentrations of extracts, in comparison with the control, were assessed by comparing the number of sprouts per 30 beads in 10 different fields. Assay organized in three independent triplicate experiments. Error bars represent S.E.M, (*, *P <0.05 vs.* control group).

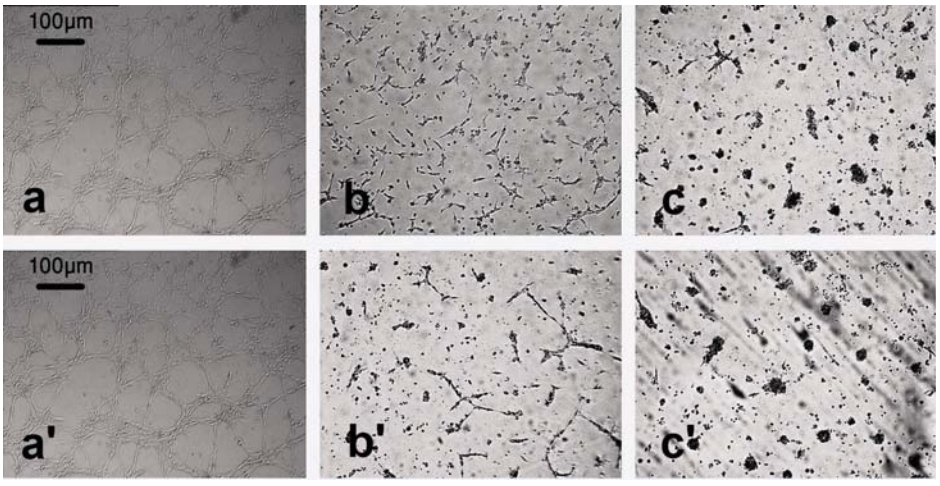
within 24 hr at non-toxic concentrations. The flower extracts suppressed cell migration for 89.14% at concentrations of 60 µg/mL, with IC50 of 40.51 ± 20.5 µg/mL. The leaf extracts at concentrations of 80 µg/mL inhibited cell migration for 93.28%, with IC50 of 42.72 ± 20.81 µg/mL (Figure 4).

Both flower and leaf extracts reduced *VEGF-A* and *VEGFR-2* transcription in HUVECs

Data analysis of the real-time RT–PCR results by normalizing to the *GAPDH* gene in HUVECs treated with 60 µg/mL flower extract showed a decrease in transcript levels of *VEGF-A* and *VEGFR-2* to 70% and 69% for 24 hr, respectively (Figure 5). Transcript levels of VEGF-A and *VEGFR-2* were reduced to 90% and 92%, respectively, with treatment of 80 µg/mL leaf extract by normalizing to the *GAPDH* gene in HUVECs for 24 hr (Figure 5).

Discussion

Cancer, one of the most common causes of death, has challenged many scientists all over the world to find a promising cure.[17–18] Conventional therapies like chemotherapy and radiotherapy represent a short time benefit, which is in part, the consequence of the tumor cells characteristics like genomic instability.[19] In addition, conventional treatments result in increased amounts of free



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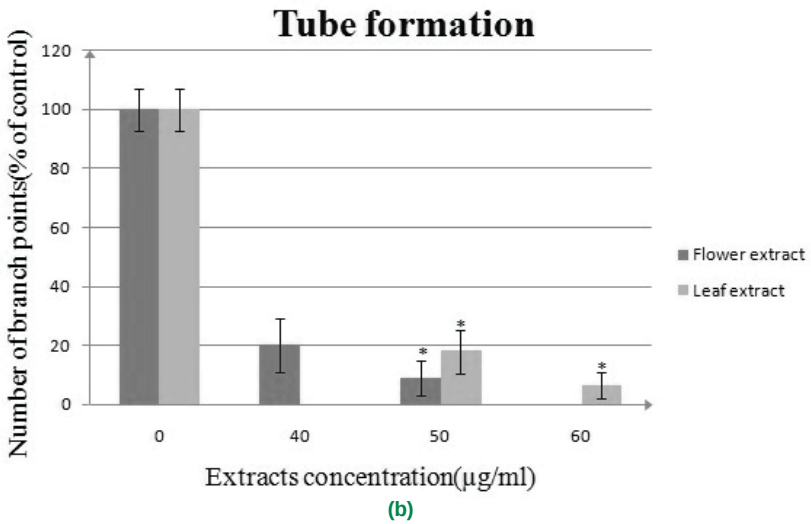


(b)

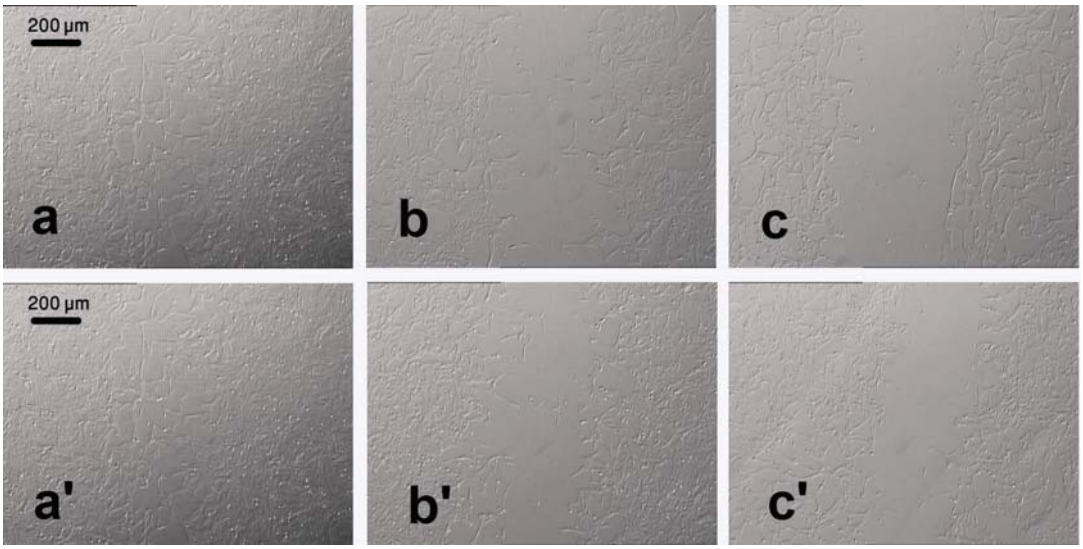
Figure 3. Tube formation inhibition by *F. angulata* Flower and leaf extracts. **(a)** HUVECs were cultured on solidified ECM gel and different concentrations of flower extract (a -0 µg/mL, b -40 µg/mL and c -50µg/mL) or leaf extract (a' -0 µg/mL, b' -50 µg/mL and c' -60 µg/mL) were added to cells in triplicate and assay conducted three times, independently (letters with prime indicate the leaf extract treatment). **(b)** Tube formation changes were evaluated by comparing the number of branch points in 4 different fields after 16 h between treated wells and control. Error bars represent S.E.M, (*, *P <0.05 vs.* control group).

radicals, which are one of the leading causes of mutations and tumor formation.[9] Considering these disadvantages and by paying special attention to angiogenesis as a key process in malignancies, a new aspect was born in cancer-therapy strategies by targeting tumor induced angiogenesis mainly via affecting the endothelial cells.[3,20] This new approach seems more beneficial since it follows relatively similar mechanisms in different types of solid tumors[4]. Specialized cells responsible for angiogenesis in solid tumors are endothelial cells which have a stable genome.[21–22] In response to rapid growth of tumor cells and progressive hypoxia in central parts of the tumor mass, nutrition requirements and production of the angiogenic factors will be increased.[23–24] As a result, endothelial cells in adjacent vessels become active and begin to proliferate, differentiate, migrate, invade extracellular matrix, and sprout toward the inducing signal source.[25–26] Anti-angiogenic drugs affect different molecules in angiogenesis signaling pathways.[27] Despite many reports on effectiveness of these drugs in treating cancer, severe side effects and some other drawbacks in using anti-angiogenic therapy have been reported in patients.[28] Therefore, researchers are encouraged to seek approaches with less side effects and higher efficacy.[29] Natural products in plants show promising characteristics; plants contain flavonoids, anthocyanins, antioxidants and many different compounds and metabolites, the therapeutic effects of which have been proved predominantly.[10,30–31]

In this study, we have investigated the anti-angiogenic effects of *F. angulata*, a native plant used as folk remedy by ancient Iranians. First, evaluating the cytotoxic effects of ethanol extracts of flower and leaf on HUVECs demonstrated a higher cytotoxicity for the flower extract compared to the leaf extract at concentrations higher than 40 µg/mL in 48 hr; however, the flower extract showed less cytotoxicity than the leaf extract at all concentrations in 24 hr.

Results of angiogenesis assays suggest that extracts influence endothelial cells' viability and angiogenic capacities, tube formation and sprouting, through distinct mechanisms. 3-D citodex angiogenesis assay, at concentrations more than 50% viabilities, indicated a dose-dependent decrease in HUVECs' sprouting; and 2-D tube formation assay in these concentrations exhibited a dose-dependent decrease in the number of branch points.

We planned a wound healing assay to assess the anti-migratory



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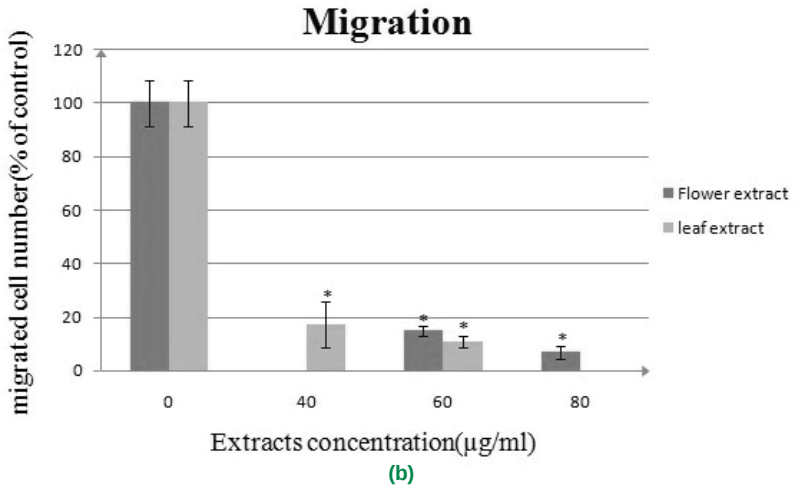

(b)

Figure 4. Declining the migration capacity of HUVECs in response to *F. angulata* Flower and leaf extracts. **(a)** After creating a scratch in a confluent monolayer of HUVECs, cells were incubated for 24 hr with extracts. Results were monitored under the invert microscope. a and a' -control, b -40 µg/mL of flower extract, c -50 µg/mL of flower extract, b' -50 µg/mL of leaf extract and c' -60 µg/mL of leaf extract (letters with prime sign indicate the leaf extract treatments). **(b)** The number of migrated cells from the edge of the scratch counted in five separate fields for each well and compared in treated wells with the controls. Three independent assays performed in triplicate. Error bars represent S.E.M, (*, *P <0.05 vs.* control group).

effects of flower and leaf extracts on endothelial cells migration potency as one of the main steps in the angiogenesis process. The migration assay obviously prevented endothelial cells' migration in exposure to both extracts.

VEGF-A (also known as VEGF) and its tyrosine kinase receptors, mainly VEGFR-2 (KDR), are known as the main mediators in both physiologic and pathologic angiogenesis. VEGF-A through binding VEGFR-2 induces a cascade of signaling pathways that result proliferation, migration, survival and vascular permeability.[32–33] As a result, VEGF-A and its receptors have been the most commonly targeted molecules found in anti-angiogenic drugs in different types of tumors.[22,34–35] Therefore, we evaluated *VEGF-A* and *VEGFR-2* gene expression levels in response to the flower and leaf extracts using real-time RT-PCR assays. Comparing gene expression data demonstrated that both extracts significantly reduced *VEGF-A* and *VEGFR-2* transcript gene expression. These data indicate that probable mechanism inhibition of tube formation, sprouting and migration in HUVECs may be via *VEGF-A* and *VEGFR-2* gene expression knockdown in response to the extracts.

F. angulata, is one of the thirty five species of Ferulago genus of Apiaceae (Umbelliferae) worldwide. This plant is indigenous to Iran, eastern Turkey and northern Iraq; as mentioned before, it has been used in native remedies and as flavor in foods. According to GC mass chromatography data, *F. angulata*'s aerial parts contain monoterpene hydrocarbons (cis-ocimene and α-pinene) as the major constituent.[11,36–38] *F. angulata* demonstrate high antioxidant property, anti microbial efficacy and can decrease the NO release in mouse macrophages by downregulating IL-1β; so far, there has been no document on anti-angiogenic properties of *F. angulate*.[36,38–39]

Limonene and perillyl alcohol are two members of Monoterpens, which have been shown to exhibit both preventive and therapeutic characteristics for different types of cancer.[40–44] Considering these information, we propose that the observed anti-angiogenic activity of *F. angulata* might be a consequence of the high monoterpen content of its aerial parts. Of course, further studies will be necessary to evaluate the involvement of specific compounds in this

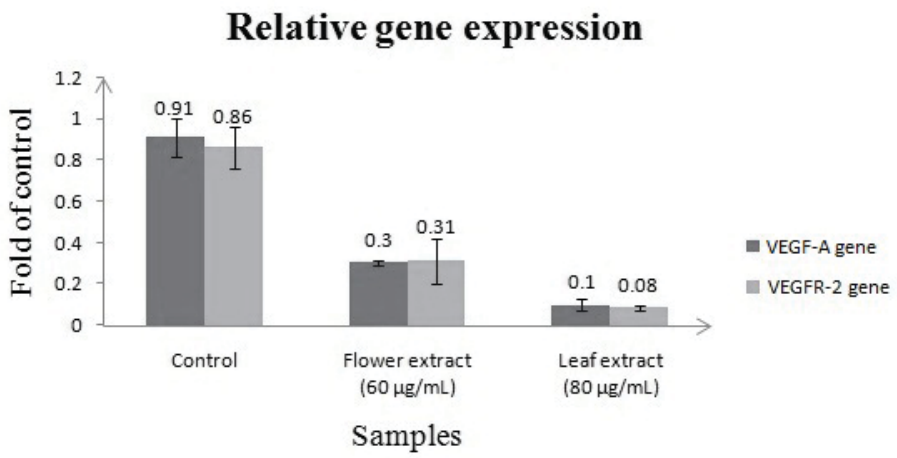

Figure 5. Effects of *F. angulata* Flower and leaf extracts on *VEGF-A* and *VEGFR-2* gene expressions. HUVECs treated with completely angiogenesis inhibiting concentrations of each extracts. After 24 hr, real-time RT-PCR performed to evaluate *VEGF-A* and *VEGFR-2* genes expression changes relative to the internal control gene, *GAPDH*, in response to the extracts. Error bars represent S.E.M. Assays were organized in three independent experiments in duplicate.

mechanism and confirming the *in vivo* anti-angiogenic activity of *F. angulata*.

To our knowledge, this is the first study on anti-angiogenic activity of *F. angulata* ethanolic flower and leaf extracts. Our study indicates that *F. angulata* flower and leaf extracts have angiogenesis and migration inhibitory effects on HUVECs, which may be due to downregulation of *VEGF-A* and *VEGFR-2*. We suggest that *F. angulata* may be considered as a new anti-angiogenic drug and as well in future strategies for treating and preventing malignancies.

Acknowledgments

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References

1. Carmeliet P. Angiogenesis in health and disease. *Nat Med*. 2003: **9:** 653 – 660.
2. Algire GH, Chalkley HW, Legallais FY, Park HD. Vascular reactions of normal and malignant tissues in vivo. I. Vascular reactions of mice to wounds and to normal and neoplastic transplants. *J Natl Cancer Inst.* 1945: **6:** 73 – 85.
3. Folkman J. Tumor Angiogenesis: Therapeutic implications. *N Engl J Med.* 1971*:* **285:** 1182 – 1186.
4. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med.* 1995: **1:** 27 – 30.
5. Nilsson M, Heymach JV. Vascular Endothelial Growth Factor (VEGF) pathway. *J Thorac Oncol.* 2006: **1:** 768 – 770.
6. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol.* 2001: **280:** C1358 – C1366.
7. Ohtsu A. Chemotherapy for metastatic gastric cancer: past, present, and future. *J Gastroenterol.* 2008: **43:** 256 – 264.
8. Senan S, Smit EF. Design of clinical trials of radiation combined with antiangiogenic therapy. *Oncologist.* 2007: **12:** 465 – 477.
9. Borek C. Dietary antioxidants and human cancer. *Integr Cancer Ther.* 2004: **3:** 333 – 341.
10. Sagar SM, MH DY, Wong RK. Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer—Part 1. *Curr Oncol.* 2006: **13:** 14 – 26.
11. Sodeifian G, Ansari K, Bamoniri A, & Mirjalili BF. Study of chemical composition of the essential oil of *Ferulago angulata* (Schelcht) Boiss. from Iran using supercritical fluid extraction and nano scale injection. *Dig J Nanomater Bios.* 2011: **6:** 161 – 168.
12. Fotakis G, Timbrell JA. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters.* 2006: **160:** 171 – 177.
13. Igura K, Ohta T, Kuroda Y, Kaji K. Resveratrol and quercetin inhibit angiogenesis *in vitro*. *Cancer let* 2001: **171:** 11 – 16.
14. Yarani R, Mansouri K, Mahnam A. Anti-angiogenic/anxiolytic effect of Chlordiazpoxide on dual activity for cancer patients. *IJHOSCR.* 2011: **5:** 32 – 35.
15. Borthwick GM, Johnson AS, Partington M, Burn J, Wilson R, Arthur H M. Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Coxindependent mechanism. *FASEB J*. 2006: **20:** 2009 – 2016.
16. Chen H-H, Zhou H-J, Fang X, Inhibition of human cancer cell line growth and human umbilical vein endothelial cell angiogenesis by artemisinin derivatives *in vitro*. Pharmacol Res. 2003: **48:** 231 – 236
17. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. (). Global cancer statistics. *CA Cancer J Clin.* 2011: **61:** 69 – 90.
18. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin.* 2012: **62:** 10 – 29.
19. Terman BI, Stoletov KV. VEGF and tumor angiogenesis. *Einstein Quart J Biol Med.* 2001: **18:** 59 – 66.
20. Lamalice L, Le Boeuf F, Huot J. Endothelial cell migration during angiogenesis. *Circ Res.* 2007: **100:** 782 – 794.
21. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature.* 1997: **390:** 404 – 407.
22. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: A critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol.* 2002: **20:** 4368 – 4380.
23. Folkman J. Anti-angiogenesis: new concepts for therapy of solid tumors. *Ann Surg.* 1972: **175:** 409 – 416.
24. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med.* 2003: **9:** 677 – 884.
25. Böhle AS, Kalthoff H. Molecular mechanisms of tumor metastasis and angiogenesis. *Langenbeck's Arch Surg.* 1999: **384:** 133 – 140.
26. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol.* 2007: **8:** 464 – 478.
27. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature.* 2005: **438:** 967 – 974.
28. Harris AL. Angiogenesis as a new target for cancer control. *EJC Supplements.* 2003: **1:** 1 – 12.
29. Ebos JML, Lee ChR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell.* 2009: **15:** 232 – 239.
30. Fotsis T, Peper MS, Aktas E, Briet S, Rasku S, Adlercreutz H, Wähälä K, Montesano R, Scheaigerer L. Flavonoids, dietary-derived inhibitors of cell proliferation and *in vitro* angiogenesis. *Cancer Res.* 1997:

57: 2916 – 2921.
31. Wang L-S, Stoner GD. Anthocyanins and their role in cancer prevention. *Cancer let.* 2008: **269:** 281 – 290.
32. Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene.* 2000: **19:** 5598 – 5605.
33. Kerbel RS. Tumor Angiogenesis. *N Engl J Med* 2008: **358:** 2039 – 2049.
34. Borgström P, Hillan KJ, Sriramarao P, Ferrara N. Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: Novel concepts of angiostatic therapy from intravital videomicroscopy. *Cancer res.* 1996: **56:** 4032 – 4039.
35. Ylä-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J. Vascular Endothelial Growth Factors: Biology and Current Status of Clinical Applications in Cardiovascular Medicine. *J Am Coll Cardiol.* 2007: **49:** 1015 – 1026.
36. Khanahmadi M, Janfeshan K. Study on antioxidant property of *ferulago angulata* plant. *Asian J Plant Sci.* 2006: **5:** 521 – 526.
37. Ghasempour Hr, Shirinpour E, Heydari H. Analysis by gas chromatography-mass spectrometry of essential oil from seeds and aeral parts of *Ferulago angulata* (schlecht.) Boiss Ghathered in navakoh and Shahoo, Zagross Mountain, West of Iran. *Pak J Biol Sci.* 2007: **5:** 814 – 817.
38. Taran m, Ghasempour HR, Shirinpour E. Antimicrobial activity of essential oils of Ferulago angulata subsp. carduchorum. *JJM.* 2010: **3:** 10 – 14.
39. Amirghofran Z, Malek-Hosseini S, Golmoghaddam H, Kalantar F, Shabani M. Inhibition of nitric oxide production and proinflammatory cytokines by several medicinal plants. *Iran J Immunol.* 2011: **8:** 159 – 169.
40. Gould MN. Cancer chemoprevention and therapy by monoterpenes. *Environ Health Perspect.* 1997: **105:** 977 – 979.
41. Reddy BS, Wang C-X, Samaha H, Lubet R, Steele VE, Kelloff GJ, Rao ChV. Chemoprevention of colon carcinogenesis by dietary perillyl alcohol. *Cancer res.* 1997: **57:** 420 – 425.
42. Stayrook KR, McKinzie JH, Burke YD, Burke YA, Crowell PL. Induction of the apoptosis-promoting protein Bak by perillyl alcohol in pancreatic ductal adenocarcinoma relative to untransformed ductal epithelial cells. *Carcinogenesis.* 1997: **18:** 1655 – 1658.
43. Belanger JT. Perillyl alcohol: applications in oncology. *Altern Med Rev.* 1998: **3:** 448 – 457.
44. Crowell PL. Prevention and Therapy of Cancer by Dietary Monoterpenes. *J Nutr.* 1999: **129:** 775 – 778.