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In vitro Anti-angiogenic Activity of Persian Shallot (*Allium Hirtifolium*) Extract Is Mediated Through Inhibition of Endothelial Cell Proliferation/Migration and Down-regulation of VEGF and MMP Expression

Delnia Arshadi[a], Kamran Mansouri[b], Reza Khodarahmi[b], Parivash Seyfi[a], Yadollah Shakiba[b], Ali Mostafaie[b*]

[a] Faculty of Pharmaceutical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran
[b] Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran

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Development of phytotherapies aimed at angiogenesis inhibition, in combination with classical anti-cancer therapies, is among the most intensively studied approaches for treatment of cancer. Epidemiological and animal studies have indicated that consumption of *Allium* species like shallot is associated with a reduced risk of cancer development. As a continuation of our efforts to study and characterize the effective anti-angiogenic agents from *Allium* species, here, we investigated the effects of aqueous extract of shallot on critical steps and mediators of *in vitro* angiogenesis. The anti-proliferation, -migration, and -tubulogenesis properties of the aqueous extract of shallot (at 100 - 1500 µg/ml) were evaluated using three-dimensional capillary tube formation as well as a wound-healing assay in endothelial cell-based experimental systems. In addition, the effect of the extract on vascular endothelial growth factor (VEGF) secretion and matrix metalloproteinase (MMP-2 and -9) expression was assayed using ELISA, gelatin zymography, and RT-PCR techniques. Treatment with the aqueous extract of shallot at ≥ 500 µg/ml concentrations resulted in significant decreases in endothelial cell proliferation, migration, and tubulogenesis. Moreover, the extract caused a dose-related inhibition of VEGF secretion and MMP-2/-9 expression. Taking all the data into account, the current study indicated that shallot – containing potent anti-angiogenic properties - exerts its inhibitory effect mainly through down-regulation of VEGF and MMP-2/-9; essential angiogenic mediators in many malignant and chronic inflammatory diseases.

[*]**Corresponding Author: Ali Mostafaie, E-mail:** amostafaie@kums.ac.ir

Introduction

There is a strong motivation for discovery and development of apoptosis inducers, anti-proliferative agents and angiogenesis inhibitors as potential anti-cancer drugs [1, 2], although the emergence of drug-resistant tumor cells as well as dose-limiting levels of neurological and bone marrow toxicity are two important limitations for their widespread use [3]. Angiogenesis, the endothelial cell division/migration and growth from pre-existing vessels to form new capillaries, is essential in many physiological processes such as ovulation, menstruation, and wound healing [4]. This multi-step process is tightly controlled by a balance of angiogenesis inducers and inhibitors. Angiogenesis is pathogenic in many chronic inflammatory diseases such as diabetic retinopathy, rheumatoid arthritis, atherosclerosis and neuro-degeneration [5-7]. Similarly, the growth of many solid tumors is not only dependent on angiogenesis, but any uncontrolled (excessive) angiogenesis provides cancer cells a gateway through which they can enter the circulation and metastasize to distant sites [1, 8-10].

Unregulated angiogenesis depends on an increased growth rate of endothelial cells (EC) [11], decreased expression of endogenous inhibitors, and/or increased levels of angiogenic inducers such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) [12]. Today, there is growing evidence that critical events in tumor cell growth and invasion include the degradation of the extracellular matrix (ECM) - a process that is MMP-mediated - and VEGF-induced EC proliferation and tubulogenesis. Currently, there is great interest in discovering anti-angiogenic compounds from synthetic and/or natural sources [13].

A large number of population-based studies have consistently shown that individuals consuming diets rich in fruits and vegetables have a reduced risk of developing a wide variety of cancers. For example, it has been shown that some of *Allium* species are powerful inhibitors of tumor cell proliferation [14]. *Allium hirtifolium* (Persian shallot) is used in Asian diet and is widely believed to be beneficial for health. Like other members of *Allium* family, Persian Shallot contains biologically-active components including organo-sulfur agents, polyphenol compounds, and selenium [15]. In the literature, it has been reported that organo-sulfur agents derived from members of the *Alliaceae* family have potent anti-cancer activities. Moreover, shallots contain considerable amount of polyphenols that are well-known anti-cancer compounds [16]. In an earlier study, we found that an aqueous extract of Persian Shallot inhibited the growth of several tumor cell lines [17]. Furthermore, we noted that this extract could abolish angiogenesis *in vitro* and *in vivo* [18] and that a flavonoid-rich fraction was responsible for the anti-angiogenic properties [19]. These findings suggest that Persian Shallot could be a suitable candidate for cancer prevention and/or treatment. As the mechanisms by which the Persian Shallot extract influences angiogenesis are still unknown, the aims of the present study were both to examine the direct effect of the aqueous extract of Persian Shallot on EC and to elucidate its effect on critical factors in/mediators of angiogenesis, including VEGF and MMP(s). Apart from understanding the possible mechanisms of action, the data generated in the current study may also provide insight to us and other investigators seeking to develop more efficient intervention strategies *in vivo* (especially) against VEGF/MMP-9-dependent tumor cells [20].

Materials and Methods

Reagents and Cell Lines

Dulbecco's modified minimum essential medium (DMEM), 10X minimum essential medium (MEM), fetal bovine serum (FBS), and MCDB131 were obtained from GIBCO Invitrogen (Carlsbad, CA). The ELISA kit for the assay of VEGF was purchased from R&D Systems (Minneapolis, MN), RNX-Plus reagent was obtained from Sinagen (Tehran, Iran) and Quantitech RT kit was purchased from Qiagen (Valencia, CA). Rat-tail collagen Type I was obtained from Sigma (St. Louis, MO). Dextran-coated cytodex-3 micro-carrier was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The LDH cytotoxicity assay kit was obtained from Roche Chemical Company (Mannheim, Germany). The gel reagents and all other chemicals used herein were of the highest analytical grade/purity available and used without further purification. All solutions were prepared with sterile double distilled water.

Human umbilical vein endothelial cells (HUVEC) and the MCF7 breast adenocarcinoma cell line were obtained from the National Cell Bank, Pasteur

Institute of Iran (Tehran). HUVEC were also isolated/purified from human umbilical veins of newborns using actinidindigestion [21].

Preparation of aqueous extract

Fresh Persian Shallot bulbs were obtained from local markets in Kermanshah, Iran. The bulbs (100 g) were peeled and homogenized in 100 ml deionized water. Homogenization was carried out in a high-speed blender for 15 min and continued using a magnetic stirrer overnight. The mixture was then filtered through three-layer cheesecloth and centrifuged at 10000 × g for 30 min at room temperature. The extract was then freeze-dried and stored at -20°C until use. For the experiments, a Persian Shallot extract stock was prepared by dissolving a fixed amount of powder in cell culture medium and then diluting with medium to desired concentrations.

HUVEC and MCF7 cultures

MCF7/HUVEC cultures were initiated in DMEM containing 10% heat-inactivated FBS, 100U penicillin /ml, and 100 µg streptomycin /ml) in 24-well culture plates and were maintained at 37°C in a humidified incubator containing 95% air/5% CO_2 until they were 90% confluent. Thereafter, all cultures were trypsinized (using standard protocols) and then re-seeded into fresh plates. HUVEC between passages 2 and 6 were used in these experiments. At near-confluence, the cells were treated with Persian Shallot extract at 100, 500, 1000, or 1500 µg/ml (in triplicate for each dose). The morphology of the HUVEC was then inspected using an inverted microscope 24 hr after initiation of the treatment.

Cytotoxicity assay

To determine the cytotoxic doses of the Persian Shallot extract, different concentrations of extract were added to the cultured MCF7 or HUVEC cells. After 48 hr of incubation, the culture medium in each well was removed and centrifuged to obtain cell-free medium for used in and lactate dehydrogenase (LDH) assays [22]. The absorbance of converted dye in the LDH assay was measured at 490 nm (with background subtraction at 630 nm) in a plate-reader (Stat Fax 2100, Awareness Technology Inc. Palm City, USA). The absorbance of the medium from the treated cells was compared with that of the positive controls (i.e, cells that had been treated with 1% Triton-X100 to maximize release of LDH). Each concentration was tested in three independent experiments. The level of cell death/viability among and morphology of, the cells remaining in the wells was determined by trypan blue exclusion [23].

Cell proliferation assay

An anti-proliferation assay was performed on the HUVEC (as representatives of macrovascular EC) in medium supplemented with 10% FBS. Exponentially-growing cells were seeded into 25 cm^2 flasks and allowed to attach overnight. After 24 hr incubation at 37°C (with 95% air/5% CO_2), the culture medium was exchanged with medium containing different doses of Persian Shallot extract (100-1500 µg/ml) and cells were cultured for an additional 72 hr. The HUVEC were then harvested by trypsinization and counted using a KX-21 Hematological Analyzer-Coulter Counter (Sysmex Co, Norderstedt, Germany); these values were then compared against those from control wells that had received fresh medium only. From these results, an IC_{50} value for the extract was calculated (indicative of concentration of extract causing 50% inhibition in cell proliferation).

Wound repair assay by endothelial cells

Cell migration was evaluated by a wound repair assay. HUVEC were cultured in 24-well culture plates. When the cells were confluent, identical straight strip wounds were created using sterile plastic yellow tips. The cells were then washed with phosphate-buffered saline (PBS, pH 7.4) and then treated with fresh DMEM containing 2% FBS (concentration of serum that allows cell survival but not significant proliferation) and different concentrations of Persian Shallot extract (0-1500 µg/ml). After an additional 24 hr of incubation, the cells were washed twice with PBS and then fixed in 4% paraformaldehyde (in PBS) at room temperature. The cells were then stained with Giemsa and photographed (at appropriate magnification) with a camera (TS100, Nikon Corporation, Japan) connected to an inverted microscope.

Preparation of collagen gels

Type I collagen from rat tail tendons was solubilized by stirring for 48 hr at 4°C in a sterile 0.02 M acetic acid solution [24]. For collagen gel formation, 7 vol of cold collagen solution, 1 vol of 10X minimum essential medium (MEM) and 2 vol of sodium bicarbonate solution (11.76 mg/ml) were mixed in a sterile flask kept on ice to prevent immediate gelation.

In vitro HUVEC capillary tube formation assay in collagen matrix

HUVEC cells were grown in medium supplemented with 10% FBS at 37ºC and 5% CO_2 and after 3-5 passages were used for this experiment. The cells were mixed with sterilized cytodex-3 micro-carriers coated beads with gelatin, at a ratio of 30 HUVEC cells/bead in 1 ml of medium supplemented with 10% heat-inactivated FBS. Beads with cells were shaken gently every 20 min for 8 hr at 37 ºC and 5% CO_2 [25]. The mixture were then transferred to four wells of a 24-well tissue culture plate and left for 12-16 hr in 1 ml of medium (37°C, 5% CO_2). The following day, cell-coated beads were re-suspended (cultured) in Type I collagen matrix and placed in a 37°C, 5% CO_2 incubator, as described above. After collagen gel formation, MCDB-131 medium was added to each well. In order to study any anti-tubulogenic effect of the extract, different concentrations of the extract (0 - 1500 µg/ml) were added to the wells. The plates were incubated for 3 d (37°C, 5% CO_2) and effects on formation of capillary-like structures were monitored (under microscope) daily. The capillary-like structures were then photographed with the camera connected to the microscope.

Gelatin Zymography

The effects of the aqueous extract on the activity of MMP-2 and MMP-9 were assessed using gelatin zymography[26]. Confluent HUVEC were isolated and immediately incubated in the absence of serum (FBS) with different concentrations of the extract for 16 hr. Firstly; protein content of the supernatant from HUVEC treated with Persian Shallot extract was measured by the Bradford method using bovine serum albumin as the standard. Then, the supernatant of treated and control wells were mixed with sample buffer and loaded onto a 7.5% polyacrylamide gel containing 1% SDS and 1 mg/ml gelatin under non-reducing condition. Following electrophoresis, the gels were washed twice with washing buffer containing 2.5% Triton X-100 for one hour at room temperature (to remove SDS) followed by a brief rinsing in washing buffer without Triton X-100. The gels then incubated at 37°C for 24 hr in substrate buffer containing 25 mM Tris (pH 7.5) and 5 mM $CaCl_2$ for the development of enzyme activity bands. Thereafter, the gels were stained with Coomassie Brilliant Blue R-250 in 50% methanol and 10% glacial acetic acid for 30 min and de-stained in methanol-acetic acid mixed solution. The gelatinolytic activities of each MMP were then detected as clear/transparent bands against a background of Coomassie Brilliant Blue-stained gelatin. Protein standards were run concurrently to aid in determining the molecular weights of the MMP bands.

Determination of VEGF level with ELISA

HUVEC/MCF7 in logarithmic growth phase were cultured in 25 cm^2 flasks at the density of 2×10^5 in DMEM supplemented with 10% FBS for 24 hr. For the measurement of VEGF, the cells were treated with different concentrations of Persian Shallot extract (in serum-free medium) for another 24 hr. Thereafter, cell-free culture supernatants were collected and VEGF concentrations assessed using a quantitative ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The sensitivity of the kit was 5 pg/ml.

MMP-2 and MMP-9 gene expression analysis using RT-PCR

The effect of Persian Shallot extract on the MMP gene expression was examined by RT-PCR analysis. HUVEC were treated with different concentrations of Persian Shallot extract (100-1500 µg/ml) for 12 hr. To evaluate the possible inhibitory effect of the extract on expression of MMP-2 and MMP-9, total cellular RNA was extracted from treated and control cells, using the RNX-Plus reagent, according to the manufacturer instructions. For RT-PCR, the extracted RNA (1 µg) was reverse transcribed into cDNA for 1 hr at 37°C using the Qiagen Quantitech RT kit. The PCR was then performed using the primers:

For housekeeping β-actin gene (internal reference):

5′-CTACAATGAGCTGCGTGTGG-3′ (sense)
5′-AGCTCTTCTCCAGGGAGGA-3′ (anti-sense)

For MMP-2:

5′-CAGGCTCTTCTCCTTTCACAAC-3′ (sense)
5′-AAGCCACGGCTTGGTTTTCCTC-3′ (anti-sense)

For MMP-9:

5′-TGGGCTACGTGACCTATGACAT-3′ (sense)
5′-GCCCAGCCCACCTCCACTCCTC-3′ (anti-sense)

Each PCR cycle consisted of a denaturation step at 94°C for 1 min, an annealing step for 30 sec (at 60°C for MMP-2 and β-actin and 55°C for MMP-9), and an extension step at 72°C for 1 min. The PCR reaction utilized 30 cycles for β-actin and 40 cycles for MMP-2 and MMP-9. The PCR products were then resolved over a 2% agarose gel and photographed with UV trans-illumination. The densities of MMP-2, MMP-9, and β-actin DNA bands were then determined/quantitated (in arbitrary units) using Lab Works 4.0 gel documentation software (UVP, Upland, CA), and the expression of the genes were then normalized based on expression of the housekeeping β-actin gene (by calculation of intensity ratios of MMP/β-actin band areas for each sample). To obtain values > 1, these ratios were multiplied by 100 and defined as the relative expressions of MMP-2 and MMP-9.

Statistical Analyses

Statistical analyses were performed using SPSS software (Version 11.5 for Windows; Chicago, IL). The significance of values was determined using a two-tailed Student's *t*-test. All results are expressed as mean (± SD). P-values ≤ 0.01 were considered statistically significant.

Results

We showed, in earlier work [19], that aqueous extract (as well as ethyl acetate) fraction of shallot's ethanolic extract had the highest flavonoid content, based on quercetin calibration curve. Since the water solubility (and bioavailability) can be considered as an advantage for potentially effective anti-angiogenic/anti-tumor substances, the aqueous fraction was selected to study in details and the other fractions (except for ethyl acetate fractions), which were not only flavonoid-deficient but also had no significant anti-proliferative/angiogenic activity at their non-toxic concentration ranges, were excluded from further evaluations, in the scope of the present work.

Effect of Persian Shallot extract on HUVEC morphology

To examine the direct effect of the aqueous extract of shallots on vascular EC, we examined its effects on cell morphology/viability and also on steps involved in the process of angiogenesis (including, EC proliferation, migration, and tubulogenesis). First, the effect of the extract on HUVEC morphology was examined; the extract had no adverse effect up to 1500 µg/ml (Figure 1).

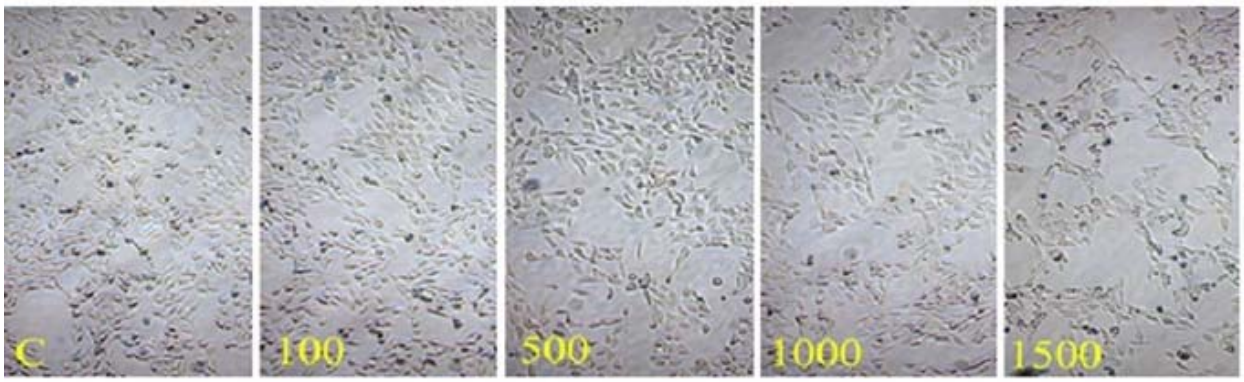

Fig. 1. Effects of various amounts of Persian Shallot extract on HUVEC morphology. Photomicro-graphs shown are representative example of three independent experiments. 10× magnification.

Persian Shallot extract directly inhibits EC proliferation

Proliferation of vascular EC is an important event during angiogenesis [27]. To explore mechanisms underlying the anti-angiogenic behavior of the aqueous extract of shallots, we investigated whether the material was able to inhibit HUVEC proliferation. To test this, HUVEC were incubated with extract at concentrations ranging from 0.0 to 1500 µg/ml. As indicated in Figure 2, the extract directly (and strongly) inhibited EC proliferation in a dose-related manner, with a half-maximal inhibition (IC_{50}) at 500 µg/ml. A significant inhibition was even noted at 100 µg extract/ml. However, increasing the concentration of extract to 1000 and 1500 µg/ml had not additional inhibitory effect above that seen with the 500 µg/ml dose. To determine whether the reduced cell growth in general was due to cell death or not, LDH assays were performed (data not shown). The LDH release from the extract-treated cells was increased slightly compared to that by untreated control cells, confirming the specific inhibitory effects of the Persian Shallot extract on EC proliferation were not due to outright effects on cell viability.

Effect of Persian Shallot extract on endothelial cell migration and tubulogenesis

Migration of EC represents another critical step in angiogenesis, allowing the cells to disseminate from pre-existing vessels and form new vessels. To assess the effect of Persian Shallot extract on HUVEC motility, an *in vitro* wound-healing assay was performed. In the absence of extract (untreated wells), HUVEC migrated to the center of the initial wound (gap zone) and mostly filled it in the indicated time (Figure 3). Extract at 100 µg/ml did not significantly modify the initial area of the gap (wound repair) by the HUVEC. However, when cells were treated with increasingly higher concentrations of the extract, the wound area (width) increased in a dose-related manner; this outcome suggested that the extract has a potential to prevent EC migration without considerable toxic effects.

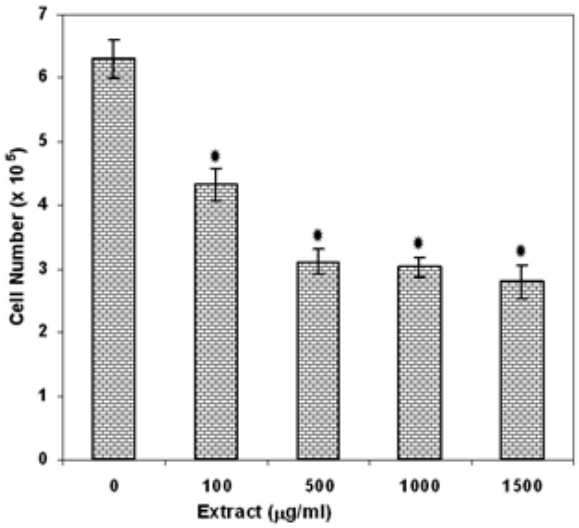

Fig. 2. Inhibition of HUVEC proliferation due to Persian Shallot aqueous extract. Cell viability was estimated by the analyzing the content of LDH in the culture medium (data not shown). Data are the mean ± SD of three independent experiments, P* < 0.01), vs control

We then investigated the effect of the extract on the morphological differentiation of EC into capillary-like structures and whether the extract affected tube formation on cytodex micro-carriers. After 3 d of culture in a collagen matrix, untreated control wells showed a branching pattern of capillary tube structures (Figure 4A). The Persian Shallot extract (at 100 µg/ml) yielded partial anti-angiogenic effects; at higher doses, the extract prevented development of tubular structures and inhibited EC budding from the micro-carriers (in a dose-related manner). Altogether, Persian Shallot extract showed potent anti-tubulogenic effects and tubulogenesis was completely abolished at 1000 µg/ml (Figure 4).

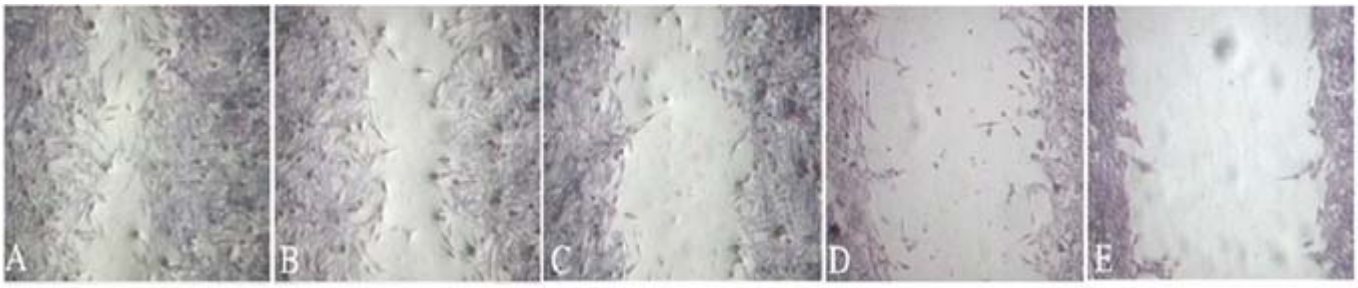

Fig. 3. The inhibitory effect of Persian Shallot extract on HUVEC migration. The representative photomicrographs show the effect of various concentrations of extract on endothelial cell migration in the wound-healing model after 48 hr. (A) control, (B) 100 µg/ml, (C) 500 µg/ml, (D) 1000 µg/ml, (E) 1500 µg/ml. 10× magnification.

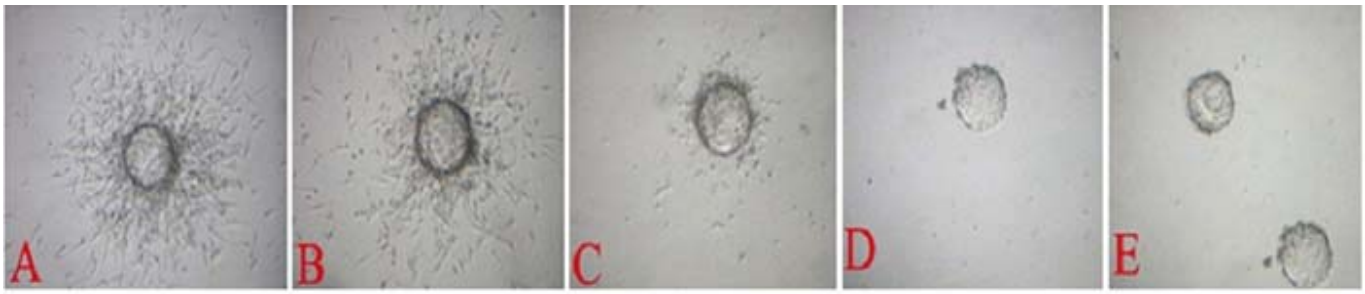

Fig. 4. Inhibitory effect of Persian Shallot extract on *in vitro* endothelial cell tube formation in collagen matrix. Spontaneous formation of capillary-like structures by HUVEC on dextran-coated cytodex-3 microcarriers was used to assess anti-angiogenic potential. (**A)** Angiogenesis of endothelial cells in untreated wells (control). **(B-E)** Angiogenesis by endothelial cells treated with different extract concentrations (100, 500, 1000, or 1500 µg/ml, respectively). Photomicrographs shown are representative example of three independent experiments. 10× magnification.

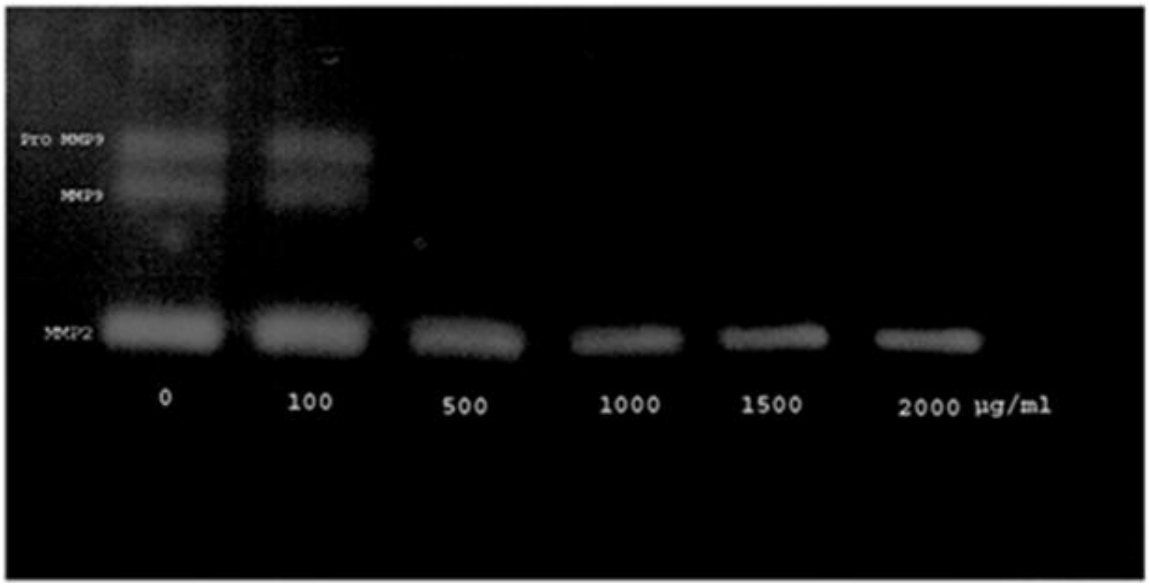

Fig. 5. Gelatin zymography assay of secreted MMP-9 and MMP-2 activity/expression from HUVEC treated with increasing concentrations of Persian Shallot extract. Note the partial inhibition of MMP-2 activity (dose-related manner; 100-2000 µg/ml) and complete suppression of MMP-9 and pro-MMP-9 activity (at 500 µg/ml) relative to that seen with the control (C). Data shown are the representative zymogram from three independent experiments. Unreduced molecular weight standards are not shown.

Effect of Persian Shallot extract on MMP-2 and MMP-9 gelatinolytic activity

Since MMP secreted by EC play a critical role in the process of ECM remodeling and EC sprouting/ invasion/tube formation, we examined the effect of the extract on the activity and as levels of MMP. In order to study the effect of Persian Shallot extract on MMP-2 and -9 activities, equal volumes of supernatants from HUVEC treated with Persian Shallot extract and control were subjected to gelatin zymography. The zymogram of control samples yielded major bands with gelatinolytic activity that corresponded to MMP-2 and MMP-9 (Figure 5). In contrast, there was a down-regulation in MMP-2 and -9 secretion in the test groups (in comparison with controls). Extract at ≥ 500 µg/ml inhibited MMP-9 activity completely. In contrast, a dose-related reduction was noted with respect to MMP-2 activity.

Effect of Persian Shallot extract on VEGF

Because VEGF has a critical role in angiogenesis, the inhibitory effects of Persian Shallot extract on VEGF expression by HUVEC and MCF7 was examined. As indicated in Figure 6A, treatment of the EC with the Persian Shallot extract resulted in decreased VEGF secretion in a dose-related manner (this reduction was significant at ≥ 100 µg/ml). In a parallel study to further examine whether the extract inhibited tumor (cell)-induced angiogenesis, the effect of the extract on VEGF production by MCF7 breast cancer cells was evaluated. As expected, the potential for tumor-induced angiogenesis (as reflected by VEGF production by the MCF7 cells) was suppressed by treatment with extract (in dose-dependent manner; Figure 6B).

Effect of Persian Shallot extract on MMP-2 and MMP-9 mRNA expression

A semi-quantitative RT-PCR approach was employed to assess the inhibitory effect of the Persian Shallot extract on MMP expression (at mRNA level; Figure 7). Changes in the ratio of expressed MMP genes to that of β-actin expression was used to reflect (semi-quantitatively) the changes induced in gene expression. The data revealed significant down-regulation of MMP-9 in HUVEC treated with the extract (Figure 7A). At 500 µg/ml, MMP-9 mRNA expression was significantly inhibited compared to that of the control cells; inhibition was near-complete at ≥ 1000 µg/ml. On the other hand, the effect of the

extract on MMP-2 mRNA was not as potent as that against MMP-9; inhibition was not as great at 500 µg/ml nor near-complete at 1000 µg/ml, although the inhibitory effect of the extract on MMP-2 expression at doses ≥ 1000 µg/ml was still significant in comparison with that presented by the control cells (Figure7B).

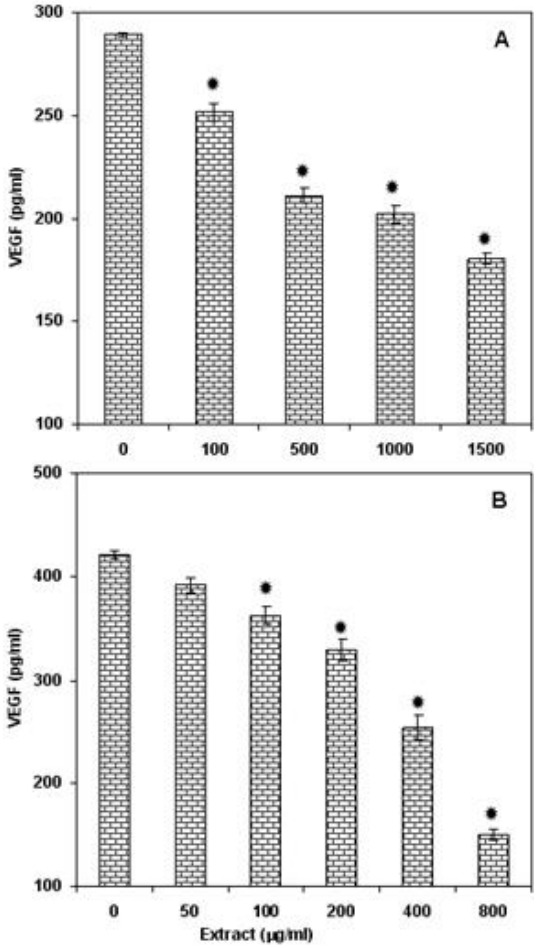

Fig. 6. Effect of Persian Shallot extract on VEGF secretion/ production. Results for **(A)** HUVEC and **(B)** MCF7 mammary gland adenocarcinoma cells are shown. Persian Shallot extract decreased VEGF secretion from cultured endothelial/malignant cells dose dependently (100-1500 µg/ml). The inhibitory effect appears to be more pronounced in MCF7 cells. Data are the mean ± SD of three independent experiments, P* < 0.01), vs control.

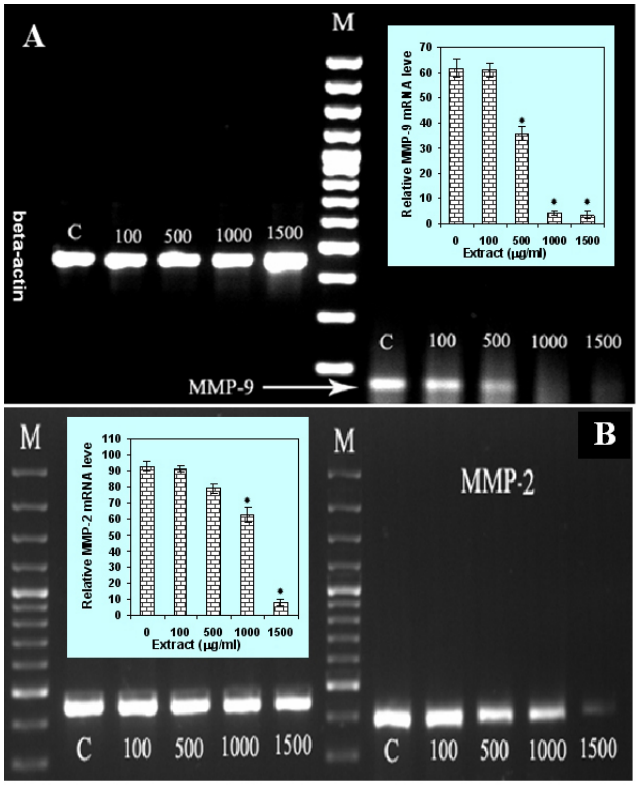

Fig. 7. RT-PCR products of as determined by semi-quantitative RT-PCR analysis and indicated by agarose gel electrophoresis. **(A)** MMP-9, **(B)** MMP-2, and **(A and B)** β-actin mRNAs are shown. Note decrease in MMP-9 and MMP-2 expression as extract concentration increases. No inhibitory effect on the expression level of β-actin was observed at 100-1500 µg/ml of the extract. **(*Insets*)** Normalized relative expression intensity ratio of the MMP genes upon the expression of housekeeping gene and the inhibitory effects of the different amounts (0-1500 µg/ml) of Persian Shallot extract. Persian Shallot extract (significantly at 500 and 1500 µg/ml) decreased MMP-9 and MMP-2 expression, respectively. Data are the mean ± SD of three independent experiments, P* < 0.01), vs control.

Discussion

The results of several population-based studies have indicated that intake of *Allium* vegetables is inversely associated with the risk of cardiovascular, diabetes and infectious diseases as well as certain types of cancers [16]. Additionally, several experimental studies triggered intense research in the past two decades aimed not only to identify the putative phytochemicals responsible for the aforementioned health benefits of *Allium* vegetables but also to elucidate the mechanism of their action. There is also increasing evidence that the chemopreventive properties of fruits and vegetables result from the additive and synergistic effects of several phytochemicals present in these foods [14]. Several potential mechanisms such as direct cytotoxic effect on tumor cells as well as interference with tumor angiogenesis have been suggested for cancer prevention property of known bioactive molecules (e.g, phenolic compounds). Since growth and metastasis of most tumors accelerated by angiogenesis, anti-angiogenesis therapeutics offers a promising approach for inhibiting tumor growth and metastasis.

Recent investigations have shown that *Allium* species and their constituents have anti-cancer effects. For example, Boivin et al. [14] showed that most commonly vegetables consumed in Asian countries (including *Allium*species) had in general a strong inhibitory effect on proliferation of various sensitive/resistant cancer cells. These effects are supported by epidemiological data from population-based studies [28, 29]. Persian Persian Shallot (*Allium hirtifolium*), is used in Asian diet and is widely believed to be beneficial to health. This bulb is darker than garlic and contains considerable amounts of sulfide content. Furthermore, Persian Shallot extracts contain flavones and polyphenolic derivatives suggesting that it also may have anti-cancer and anti-angiogenic properties [30, 31].

Previously, we reported that aqueous extract of Persian Shallot could inhibit angiogenesis in a ring aorta model [18]. In this study, we first showed that the aqueous extract of Persian Shallot inhibited growth of the HUVEC (see Figure 2). Since no cell toxicity and considerable harmful effects on EC morphology was observed at the concentration range used in this study (up to 1500 µg/ml), the anti-proliferative behavior may not be due to EC apoptosis or necrosis but, rather, to the inhibition of cell growth. In addition, we investigated the potential mechanism underlying the anti-angiogenic effect of Persian Shallot extract. The results of HUVEC migration assay suggested that the extract showed considerable inhibitory effect on EC migration. Considering the paused EC proliferation at 2% FBS, we are convinced that the depressive effect of the Persian Shallot extract on EC migration was not due to its inhibitory effect on EC proliferation, but instead to a direct suppression of EC motility. Additionally, the extract showed strong inhibitory behavior on morphogenic differentiation of EC into capillary-like structures (tubulogenesis step of angiogenesis) *in vitro*.

Our results also showed that this extract down regulates vascular endothelial growth factor (VEGF), a multi-functional peptide that is secreted from both tumor cells and EC and through its receptor, sends a classical angiogenic signal to EC. This growth factor (both a potent mitotic and migratory factor for EC; [32]) is also secreted from many types of parenchymal and immune cells on activation by various stimuli, including oxidative stress, angiotensin II and cytokines [33-37]. VEGF also has effects on both the epithelial and EC proliferation/behavior. VEGF encompasses a family of structurally related proteins that includes placental-derived growth factor (PlGF), and VEGF (-A, -B, -C, -D, and -E). These agents are key regulators of blood and lymphatic vessel development during embryogenesis and play an important role in promoting new vascular and lymphatic growth during several physiological and pathological processes/conditions.

As mentioned above, several VEGF are expressed and secreted by EC (autocrine) and many normal and malignant cells. For instance, over expression of VEGF-A (in tumor cells, EC, smooth muscle cells and fibroblasts) has been associated with tumor progression/expansion in several human malignancies including carcinomas of the breast, colon, kidney, liver, lung, pancreas and prostate, and stomach. During cancerous growth, activation of the VEGF/VEGFR axis triggers multiple signaling pathways that result in increased vascular permeability, EC mitogenesis, migration, survival, and mobilization [34, 37, 38]. Owing to central role of VEGF in tumor angiogenesis, the VEGF/VEGFR pathway continues to be a major focus of cancer research and in the development of new therapies for

this process and a large body of experimental evidences has subsequently shown that interfering with VEGF (or VEGFR) function/secretion can potently inhibit tumor growth and angiogenesis. Based on these facts (and as suggested by data shown in Figure 6), it seems that the inhibition of proliferation, migration, and tubulogenesis of EC in a collagen matrix is a result, in part, of the reduced VEGF secretion from EC as well as tumor cells.

Another critical event in tumor cell invasion and tumor angiogenesis is degradation of the extracellular matrix (ECM) that acts as a barrier to both the spread of cancer cells and development of a nourishing capillary network. Matrix metalloproteinases (MMP), a family of zinc- and calcium-dependent proteolytic enzymes are suggested to digest various components of ECM and allow the EC to migrate into the interstitial matrix [20]. Based on the primary sequence as well as protein tertiary structure and substrate specificity, over 20 different MMP have been classified. Two well-known MMP, i.e, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), play a key role in degradation of gelatin and Type IV collagen, the two main components of ECM and basement membranes [20]. Under the inducing effect of VEGF and FGF-2, both cancer cells and EC express MMP [39]. These proteolytic enzymes have attracted significant interest because they are involved in the process of leukocyte activation and trans-endothelial migration [40] and have been also shown to be important extrinsic regulators of growth factor signaling and cell adhesion, and the release pro- and anti-angiogenic fragments of ECM proteins during cancer progression [20]. Many studies have shown that increased expression of MMP (especially MMP-2 and -9) is associated with cancer cell invasion phenotype and is correlated with "angiogenic switch" in many human tumors [41]. In this way, inhibition of expression or function of these proteases may block angiogenesis or postpone tumor cell invasion and metastasis.

There is the possibility that Persian Shallot extract exerts its angiogenic activity via inhibition of MMP activity/expression. In this case, and to pursue the effect of the extract on the expression of MMP-2 and MMP-9 (in HUVEC) in the presence of various concentrations of Persian Shallot extract, the treated (and untreated control) cells were subjected to gelatin zymography (see Figure 5) and mRNA analysis by RT-PCR (see Figure 7). The results demonstrated that aqueous extract of Persian Shallot effectively inhibits the MMP-9 (and relatively MMP-2) expression at the mRNA level. Since MMP have a key role in cancer cell invasion as well as angiogenesis, thus their down regulation by *Allium* plant extract could be a great step in cancer management via inhibiting ECM degradation by EC and impeding theirfollowing migration and proliferation. Furthermore, based on the literature, it is possible that the extract effects may be due to a suppression of MMP-9 promoter activity or a decrease in the amount of known transcription factors controlling MMP/VEGF gene expression [42]. In this respect, further mechanistic studies on this field are needed and underway in our laboratory.

On the other hand, there have been derived from different cancers, e.g, hepatocellular carcinoma, malignant astroglioma, and pancreatic cancer, representative cell lines that express high basal MMP-9 levels [20]. In this regard, since MMP-9 plays an important role in mediating invasion and angiogenic processes, an inhibition of MMP-9 mRNA expression by the Persian Shallot extract might provide evidence for a novel therapeutic/preventive modality to control the growth/invasiveness of MMP-9-dependent malignancies. The observations of suppressed MMP-2 and -9 activity/expression also appear to be important because a negative correlation between expressions of the mentioned MMP (in various cancer cells) has been previously reported [20].

Additionally, it has been previously observed that proteolytic activity of some MMP (MMP-2 and MMP-9) decreases cancer cell apoptosis via different mechanisms [43]. Then, inhibition of MMP, in this context, may also have an additional apoptosis-inducing effect regarding to abnormal inhibition of apoptosis by cancer cells. On the other hand, The ECM sequesters a variety of growth factors and cytokines, some in latent forms. Another mechanism in which MMP may contribute to tumor growth is through the release and/or activation of these matrix-associated factors. For example, MMP-9, but not MMP-2, specifically has been implicated in the (enhanced) release of VEGF from the ECM [44]. These findings demonstrate MMP-mediated release of pro-angiogenic growth factors that mediate tumor-induced angiogenesis and enhance tumor growth.

With this fact in mind, possible decreased level of ECM-derived growth factors, EC proliferation/migration and increase in tumor cell apoptosis, can be, in part, attributed to inhibition of MMP. In this respect, further mechanistic studies on the latter subject are in process in our laboratory.

We reported earlier that the flavonoid-rich ethyl acetate fraction of Persian Shallot show significant angiogenesis inhibitory activity on HUVEC [19]. Persian Shallot contains the highest level of total flavonoids among the *Allium* genus [45]. Furthermore, since quercetinand isoliquiritigenin (constituents of the flavonoids; [46, 47]) are found in high concentrations in Persian Shallot bulbs, it is plausible to conclude that flavonoids are potentially anti-angiogenic factors and it may be inspired that these phytochemicals contribute (in part) to appearance of the chemopreventive properties of *Allium* genus. We also demonstrated that the anti-angiogenic effect of aqueous fraction of Persian Shallot (and also quercetin; [19]) target several key events involved in the development (metastasis) of cancer, such as HUVEC proliferation, migration, tubulogenesis, and MMP expression/activity. This pleiotropic mechanism of action of shallot's aqueous extract implies that the observed anti-angiogenic properties are complex and likely arise from synergistic combinations from several flavonoids (and non-flavonoid) phytochemicals. On the other hand, flavonoid-rich extract from *Allium* species may be a strong source of anti-oxidants. It is noteworthy that anti-oxidant activity is involved in cancer prevention at the initiation stages while anti-proliferative activity (against EC/tumor cells) is targeting cancer at the promotion and progression stages, it implies that *Allium* consumption is associated with both preventive and curative benefits against several types of malignancies.

In addition to polyphenolic phytochemical derivatives, Persian Shallot as well as the other members of *Allium* species contains multiple non-flavonoid organo-sulfur constituents with potentially anti-tumor activity and thereby anti-angiogenesis/anti-cancer effects of *Allium* vegetables are also attributable to organo-sulfur compounds [48]. There are reports that the sulfur-containing constituents of *Allium* such as allicin-decomposition products (i.e, diallyl disulfide, diallyltrisulfide, and ajoene), S-allyl cysteine, and alliin not only inhibit capillary-like tube formation, migration of HUVEC and MMP activation, but also suppress experimental metastasis of cancer cells and their growth by causing cell cycle arrest and apoptosis induction. In this context, inhibition of VEGF-induced tube formation in human EC and neo-vascularization in chicken chorioallantoic membrane assay by alliin[48], suppression of HUVEC migration and tubulogenesis by diallyltrisulfide (via decreasing VEGF secretion and down-regulation of VEGF receptor-2; [49]), and inhibition of proliferation and activation of MMP-2 and 9 in EC by diallyl disulfide [50] have been reported.

So far, it is possible that observed anti-angiogenic/proliferative activity of Persian Shallot aqueous extract belong to the concerted/cumulative effects of all the aforementioned phyto-constituents. These results illustrate the need to improve current dietary recommendation by actively promoting increased consumption of *Allium* species as an essential means to prevent/reduce the incidence of cancer. Although comprehensive effects on angiogenesis need further research, our findings provide the first evidence of the direct effect of Persian Shallot extract on EC proliferation, migration, and tubulogenesis.

Conclusions

The results of this study revealed that an aqueous extract of Persian Shallot inhibits angiogenesis, in part, via the prevention of VEGF, as well as of MMP-2 and -9 production. The anti-migration, anti-angiogenic, anti-proliferative, and MMP-inhibitory activities displayed by this Persian Shallot extract - each of which could be involved in different modes of chemoprevention - should be considered as templates for the better evaluation of the "global anti-cancer potentials" of several members of the *Allium* species. Nonetheless, more quantitative investigations in animal models are needed to clarify the preventive/curative role of Persian Shallot in cancer onset/progression, *in vivo*. Further, due to the critical role of MMP in tumor invasiveness, an evaluation of MMP-9 (and MMP-1, 2, 3, and 14) activities/levels in differing human cancer cell lines in response to the Persian Shallot (as well as other different *Allium* species) extract appears worthwhile.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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