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Ethyl Acetate Fraction of *Teucrium Polium* Extract Abolishes Human Umbilical Vein Endothelial Cells (HUVEC) Tubulogenesis in Collagen Bed through Suppression of Cell Proliferation/VEGF Secretion

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ABSTRACT

Angiogenesis has essential role in growth and metastasis of tumors. Development of therapies aimed to suppress angiogenesis using medicinal plants is one of the effective approaches for prevention/treatment of cancer. The current study was performed to investigate in vitro anti-angiogenic effect of *Teucrium Polium* (TP) extract and its fractions.

The aerial part of *Teucrium Polium* was powdered and extracted with 50% ethanol. The extract was fractionated in to aqueous (AQ), n-butanol (BU), ethyl acetate (EA) and n-hexane (HE) fractions. Anti-angiogenic effect of TP was examined on human umbilical vein endothelial cells (HUVECs) in three-dimensional collagen matrix. The endothelial cells form capillary-like branches that can be visualized using phase contrast microscope and the number of tube-like structures can be quantified as a measure of in vitro angiogenesis. Furthermore, anti-proliferative and vascular endothelial growth factor(VEGF)suppressive effect of TP as important factors in the process of angiogenesis were assessed using3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)and quantitative ELISA, respectively.

Based on our findings, among the TP fractions, EA fraction showed the highest inhibitory activity on angiogenesis. This fraction with IC50: 68 μg/mL, inhibited angiogenesis at 60 μg/mL. The crude extract and other fractions of TP inhibited angiogenesis in a dose-dependent manner at doses higher concentrations than EA fraction, significantly.TP extract and EA fraction were able to inhibit proliferation of HUVEC and inhibited VEGF secretion in a dose dependent manner. The ethyl acetate fraction at 60 μg/ml inhibited VEGF secretion perfectly.

Our data indicated that ethyl acetate fraction of *Teucrium Polium* could be a potential candidate for the prevention of angiogenesis in cancer and other related disorders. However, this suggestion needs more quantitative and *in vivo* investigations for confirmation.

Keywords: Angiogenesis; Proliferation; *Teucrium Polium*; Vascular endothelial growth factor

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INTRODUCTION

Angiogenesis, a multistep process for the development of new blood capillaries from existing vessels,[1] is important in many physiological processes like embryonic development and wound repair and many pathological processes like fatigue and diabetes.[2]Angiogenesis is controlled through an accurate balance between pro-angiogenic factors, mainly vascular endothelial cell growth factor (VEGF) and anti-angiogenic factors.[3,4]These factors can initiate or inhibit angiogenesis. In addition to VEGF, conditions such as hypoxia, increased secretion of matrix metalloproteases (MMPs) and decreased pH can alter the process of angiogenesis. In angiogenesis, endothelial cells migrate out from the parental vessels, invade the extracellular matrix, proliferate and finally differentiate as a new blood vessel.[5,6] Because of the key role of excessive angiogenesis in disease progression, notably tumor growth, inhibition of new blood capillaries growth has become a target for treatment of tumor and many chronic diseases including diabetic retinopathy and rheumatoid arthritis.[7]

Several natural products from medicinal plants have anti-angiogenesis activities;[8] For example, green tea catechin,[9] *Salvia officinalis*,[10] Salvicine[11] and soybean trypsin inhibitor[12] have been isolated from natural sources and examined for their anti-angiogenesis effects. *Teucrium Polium* (TP) a member of *Teucrium* genus and belongs to the family of Laminaceae[13] has been used for over 2000 years in folk medicine. This herbal plant is found mainly in the Mediterranean area. *Teucrium Polium* have immense functions and significant power for anti-inflammatory,[14] hypoglycemic,[15] anti-spasmodic, anti-microbial,[16] anti-tumor effects[17] and is reported to have a role in memory Impairments.[18]Investigations have shown that TP contains flavonoids such as rutin and apigenin with anti-proliferative activity.[19] Anti cancer properties of TP have been shown in some studies on cancer cell lines like REYF-1.[20]

Human umbilical vein endothelial cells (HUVECs) are cells derived from the umbilical vein endothelium using proteolytic enzymes. They are used as a laboratory model for the study of the function and pathology of endothelial cells like angiogenesis. Typical characteristics of in vitro angiogenesis assay are proliferation and formation of tube-like structure by endothelial cell in cytodx-3-microcarrier beads model. In the current study, we indicated that crude extract and ethyl acetate fraction of TP have *in-vitro* anti-proliferative and anti-angiogenic functions.

MATERIALS AND METHODS

Ethics statement

All experiments and procedures were performed in accordance with the ethical guidelines approved by Ethics Committee of Kermanshah University Of Medical Science and approval ID of this study is KUMS.REC.1394.518

Chemicals and Reagents

Cytodex microcarrier was obtained from Amersham Pharmacia Biotech of USA.RPMI-1640, fetal bovine serum (FBS), ethanol, ethyl acetate, butanol, hexane, LDH cytotoxicity assay kit were purchased from Sigma Chemical Company (Carlsbad CA) and vascular endothelial growth factor(VEGF)assay kit from R&D system. Human umbilical vein endothelial cells (HUVECs) and Gastric adenocarcinoma cells (AGS) were supplied from Cell Bank of Pasture Institute (Tehran, Iran).

Plant Material

Teucrium Polium was purchased from a local market in Yazd (Voucher number 130, Environmental Sciences Dept. Yazd University, Iran). The dried aerial parts were cleaned and ground with a blender. The powder was kept at 4°C for use in the period of experiment.

Preparation of T. Polium Extract

Aerial parts of *Teucrium Polium* plant (1 Kg) was powdered and extracted with ethanol (20 Lit, 50% v/v) and shacked in a closed container for 24 h at room temperature. The extraction was repeated three times and clear supernatant was obtained by filtering and centrifugation at 3000 g for 20 min at 4°C. The clear extract was evaporated by a rotary until dried, then re-suspended in distilled water (5% w/v) and fractionated with n-hexane (HE), ethyl acetate (EA) and n-butanol (BU) leaving residual aqueous fraction (AQ). Each fraction was evaporated to obtain HE, EA, BU and AQ fractions, respectively.

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Cell Culture

HUVECs and AGS cells were cultured in RPMI-1640 containing 10% (v/v) FBS, 100 units/mL penicillin and 100 µg/mL streptomycin in flasks at 37°C in a humidified incubator (95% air, 5%CO_2) until they reached to 80% confluent. Thereafter, all cultures were trypsinized (0.25% trypsin and 0.02% EDTA) and re-seeded into new plates or flasks.

Cytotoxicity Assay

Crude extract and different fractions were used for cytotoxic test, to determine the level of CC50.HUVECs and AGS cells were grown in 96-well microplates containing different concentration of hydroalcoholic extract and its fractions (0, 20, 40, 60, 80, 100, 150, 200, 400, 600, 800 and 1000 µg/mL). After 24 h, the medium in each well was harvested for lactate dehydrogenase (LDH) assay using a plate reader (Stat Fax 2100, Awareness Technology Inc. Palm City USA) at 490 nm (with background subtraction at 630 nm). The positive and negative controls were the wells treated with culture medium alone and culture medium containing 1% Triton-X100, respectively.

MTT Assay

HUVECs (2×10^4 cells/well) were seeded for 24 h in RPMI-1640 and 10% FBS for 24 h in 96-well plates. Thereafter, culture medium was replaced with RPMI containing 5% FBS and cells were incubated with different concentrations of TP extract and its fractions for 48 h (crude extract: 40, 60, 80, 100, 200 and 400 µg/mL. Ethyl acetate fraction 20, 40, 60, 80, 100 and 200 µg/mL. Butanol fraction: 100, 200, 500 and 1000 µg/mL). Following 48 h incubation, cell proliferation was estimated by adding 20 µL MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/ml in PBS) to each well. After 4 h, the MTT-containing medium was aspirated slightly and 150 µL DMSO was added to solubilize the formazan crystals followed by shaking for 10-15 min at a dark place. The absorbance was measured at 570 nm using a plate reader (Awareness Stat Fax 2100). The IC50 value (µg/mL), which represents the concentration of test substance that reduces the MTT by 50%, compared to the control, was calculated.

In vitro Angiogenesis Model

To assess the effect of TP extract and its fractions on tubulogenesis of HUVEC, the *cytodex-3-microcarrier beads were* hydrated and swollen *in PBS* and sterilized by autoclaving. HUVECs were mixed with microcarrier beads in RPMI-1640 containing 10% FBS. The mixture was shaken briefly every 20 min for 8 h, then transferred to a 24-well culture plate and incubated for 12-16 h at 37^0C and 5% CO2. After attachment of cells to the beads, the mixture was cultured in collagen matrix, RPMI-1640 with 10% FBS and different concentration of TP extract and fractions.(Crude extract: 60, 200 and 400 µg/mL. Ethyl acetate fraction 20, 40, 60 and 80 µg/mL. Butanol fraction: 100, 500 and 1000 µg/mL. Aqueous fraction: 100, 500 and 1000 µg/mL) added. After 48 h, the results of sprouting were analyzed microscopically and compared against controls.

Analysis of VEGF Level Using ELISA Method

The AGS cell line was cultured in 25 cm^3 flasks. In logarithmic phase, the cells were trypsinized and cultured in RPMI medium and 10% FBS at 24-wells plates. In serum free medium, the AGS cells were treated with different concentration of ethyl acetate fraction (60 and 100 µg/mL) and hydroalcoholic extract (100, 200, 400 and 600 µg/mL) of *Teucrium Polium.* After 24 h, supernatants of wells were harvested and tested for VEGF concentration using a quantitative ELISA kit (R&D systems).

Statistical Analysis

The comparison between the control and tests was performed by ANOVA. Data are the mean±SE of three independent experiments, vs control. *p*-values≤0.001 were considered statistically significant.

RESULTS

Extraction and Fractionation of *Teucrium Polium* (TP)

The aerial parts of TP (1 Kg) was extracted with ethanol 50% and dried to give hydroalcoholic extract (250 g).After fractionation of hydroalcoholic extract, the yield of fractions were n-hexane (HE: 1.86%), ethyl acetate (EA: 9.80%), n-butanol (BU: 11.42%) and aqueous (AQ: 42.63%). Because of very low solubility of hexane fraction in cell compatible solvents, this fraction was not further tested.

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Effect of Crude Extract and Fractions on Viability of HUVECs and AGS

The results of viability test (LDH assay) on the endothelial cells and AGS cells indicated that HUVEC cells had viability more than 50% at 640 µg/ml of hydroalcoholic extract. The EA fraction was 40% toxic for HUVEC at 640 µg/ml and toxicity of BU and AQ fractions for HUVECs were 29% and 17% respectively at this dose (640 µg/mL). For AGS cells, CC50 of hydroalcoholic extract, EA, BU and AQ fractions were estimated 890, 635, 615 and 786 µg/mL, respectively (Table 1A, B).

Anti-proliferative Effect of TP Extract and Fractions on HUVECs

As the findings indicated, ethyl acetate fraction at 68 µg/mL inhibited 50% proliferation (IC50) of HUVECs as compared to untreated wells, however CC50 of this fraction for HUVECs was estimated 700 µg/mL. On the other hand, IC50 of hydroalcoholic extract and BU fraction for HUVECs were 250 and 680 µg/mL, respectively (Table 1 C).

Anti-Tubulogenesis Effect of TP Extract and Fractions (In vitro Angiogenesis Model)

We used three-dimensional culture of HUVECs as an in vitro model of angiogenesis to assess the effect of hydroalcoholic extract and its fractions of TP. After 48 h incubation of HUVECs with crude extract and its fractions, untreated control wells showed several tube-like structures projected from the beads. Among the fractions, the EA fraction showed the highest inhibitory activity on angiogenesis (Figure 1A). EA fraction at 60 µg/mL inhibited angiogenesis perfectly. In comparison, hydroalcoholic extract (Figure 1B), BU (Figure 1C) and AQ fractions (Figure 1D) inhibited tubulogenesis at 200,500 and 1000 µg/ml, respectively. All the fractions and crude extract inhibited angiogenesis in a dose-dependent manner at doses lower than their CC50 concentrations.

VEGF Secretion

Vascular endothelial growth factor (VEGF) is the most important growth factor to induce endothelial cells migration and tubulogenesis. Therefore, we examined the inhibitory effect of EA fraction and crude extract of TP on the VEGF secretion by AGS, a gastric adenocarcinoma cell line, to produce considerable level of VEGF. Based on the results, secretion of VEGF was reduced in culture supernatants of hydroalcoholic and EA fraction-treated cells in a dose dependent manner. VEGF concentration in control well was 1300 pg/mL and after adding the EA fraction at 60 µg/mL, level of VEGF reduced to 500 pg/ml. For hydroalcoholic extract at 100 and 400 µg/mL, the VEGF levels were 1000 and 300 pg/mL, respectively (Figures 2 and 3 respectively).

Table 1. (A)Survival-inhibitory effects of *Teucrium Polium* on human umbilical vein endothelial cells (HUVECs) and (B) gastric adenocarcinoma (AGS) cell line. (C) Growth inhibitory effect of *Teucrium Polium* on HUVECs. Cells were treated with the various concentrations of *TP* extract and fractions for 24 h (lactate dehydrogenase assay) and 48 hours MTT assay

	Sample	Toxicity (%) at 640 µg/mL
A	Crude extract	35 %
	Ethyl acetate fraction	40 %
	Butanol fraction	29 %
	Aqueous fraction	17 %
B	Sample	Toxicity (%) at 640 µg/mL
	Crude extract	46 %
	Ethyl acetate fraction	50 %
	Butanol fraction	53 %
	Aqueous fraction	40 %
C	Sample	50% growth inhibition (IC50)
	Crude extract	250 µg/mL
	Ethyl acetate fraction	68 µg/mL
	Butanol fraction	680 µg/mL

MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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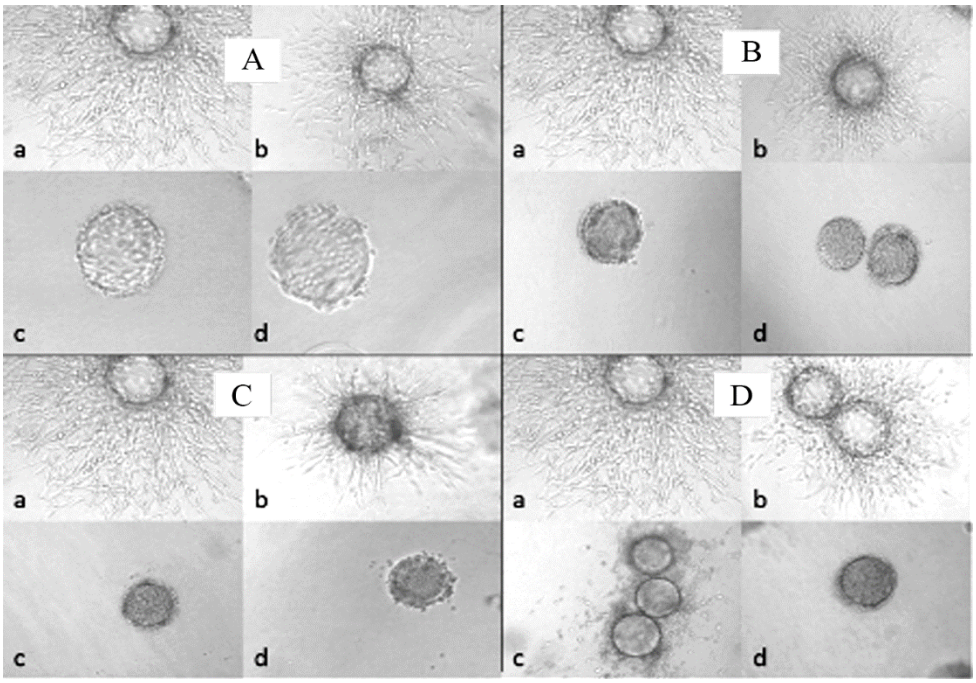

Figure 1.Inhibitory effect of *Teucrium Polium* extract and fractions on in-vitro endothelial cell tube formation in collagen matrix. Spontaneous formation of capillary-like structures by human umbilical vein endothelial cells (HUVEC) on dextran-coated cytodex-3 microcarriers was used to assess anti-angiogenic potential. (A).Effect of ethyl acetate fraction of TP on tubulogenesis of HUVEC: control (a), 20 (b), 60 (c) and 80 µg/ml (d). (B) Effect of crude extract: control (a), 60(b), 200(c) and 400 µg/mL (d). (C) Effect of butanol fraction: control (a), 100(b), 500(c) and 1000 µg/mL (d). (D) Effect of aqueous fraction: control (a), 100 (b), 500(c) and 1000 µg/mL(d). The tube-like structures were observed under a microscope and photographed with a digital camera. Photomicrographs shown are representative example of three independent experiments. 10×magnification

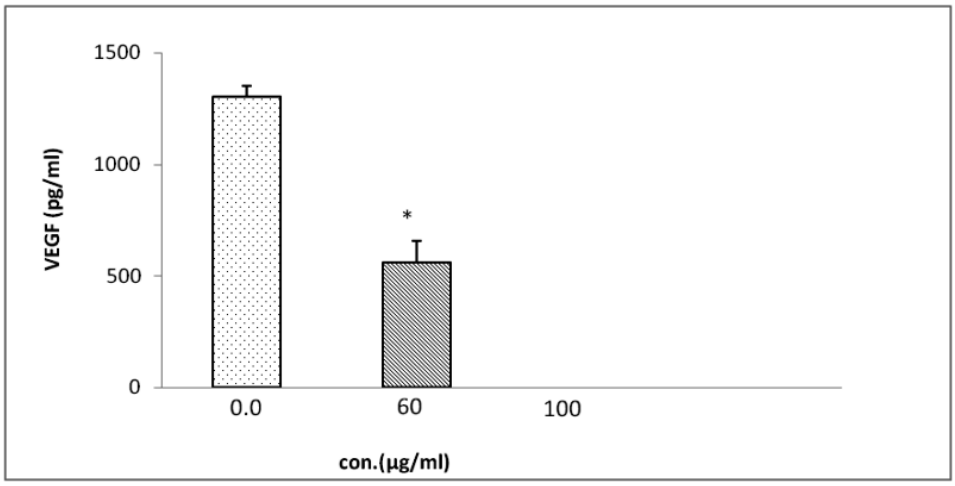

Figure 2.Effect of ethyl acetate (EA) fraction of *Teucrium Polium* on vascular endothelial growth factor (VEGF) secretion by gastric adenocarcinoma) cell line (AGS). Inhibitory effect induced by ethyl acetate fraction on VEGF, was assessed with quantitative ELISA, 24 hours after incubation. EA fraction decreased VEGF secretion from cultured endothelial cells dose dependently (60 µg/mL). Concentration (0.0) is as a control. Data are the mean±SE of three independent experiments, *p<0.001, vs control.**

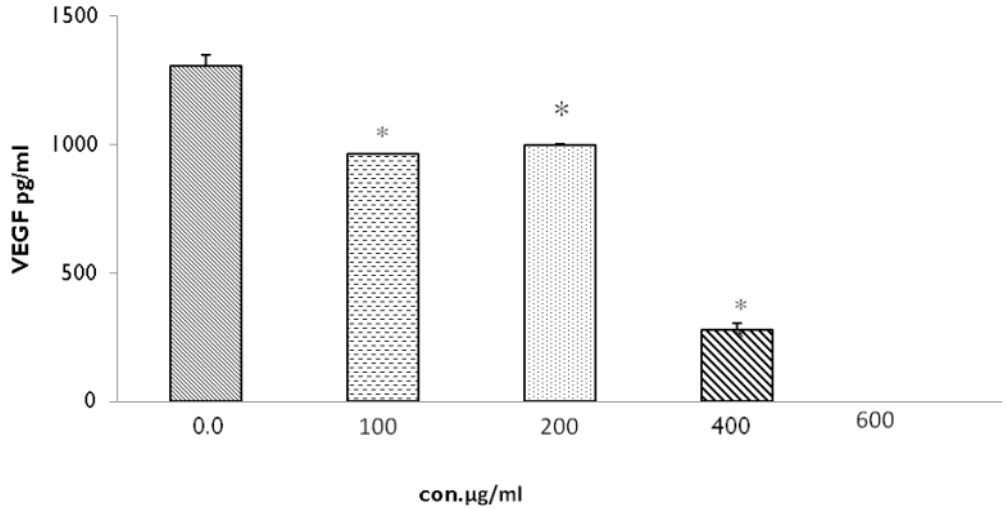

Figure 3. Effect of crude extract of *Teucrium Polium* on vascular endothelial growth factor (VEGF)secretion by gastric adenocarcinoma cell line (AGS) cell line. Inhibitory effect induced by crude extract on VEGF, was assessed with quantitative ELISA, 24 hours after incubation. Crude extract decreased VEGF secretion from cultured endothelial cells dose dependently (100-400 µg/mL).Concentration (0.0) is as a control. Data are the mean±SE of three independent experiments, *p<0.001, vs control.**

DISCUSSION

In this study, the effect of *Teucrium Polium* (TP) extract and its fractions on angiogenesis and proliferation of HUVECs was studied. The extract and its fractions especially EA fraction reduced the growth of HUVECs and production/secretion of VEGF level in AGS cell line in a dose dependent manner. On the basis of our results, it is apparent that EA fraction of *Teucrium Polium* exerts the most potent anti-proliferative activity on HUVECs. In addition, the fraction inhibited VEGF secretion from AGS cells at 60 µg/mL significantly. Kandouz et al[20] showed that TP extract inhibits cell proliferation and invasion of human prostate cancer cells via restoration of the E-cadherin/catenin complex. Furthermore, Eskandary et al[21] reported that methanolic fraction of TP has anti-proliferative effect on REYF-1 cell line, their results indicated that IC50 for methanolic fraction is 95 µg/mL in comparison to 1400 µg/mL for aqueous fraction. In comparison, we showed that IC50 of ethyl acetate fraction is 68 µg/mL and IC50 of aqueous fraction is more than 1000 µg/mL. Based on our results and the mentioned reports, it is evident that semi-polar fractions of TP like methanolic and ethyl acetate fractions contain constituents that could inhibit proliferation of target cell lines. To date, different compounds have been isolated from TP of that the main groups are terpenoids, flavonoids and iridoid. Ethyl acetate fraction of TP have the highest antioxidant capacity, total phenolic and flavonoids.[22] These constituents could be responsible for anti-proliferative and anti-tubulogenesis effects of ethyl acetate fraction. Some responsible components in ethyl acetate fraction are able to influence a variety of cell functions including cell proliferation and formation of tube-like structures. However, Menchini et al[23] attributed the anti-proliferative effect of TP on Caco2 and C32 cell lines to other constituents like sesquiterpenes (caryophyllene).

According to our data and other studies, TP has shown anti-angiogenesis activity.Sheikhbahaei[24] clearly showed that combination therapy of *T. Polium* and tranilast could significantly increase anti-angiogenic properties of these agents in an *in vitro* model of angiogenesis using HUVEC cells through anti-proliferative effect. Here in, we showed that ethyl acetate fraction of TP extract has the strongest effect on angiogenesis. The exact mechanism and responsible component of ethyl acetate fraction is not clear; however, some components like flavonoids including gluteolin, rutin and eupatorin[25,26] that are extracted preferentially in ethyl acetate fraction have potentials to inhibit proliferation and angiogenesis.

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VEGF as the most critical modulator of angiogenesis, is mainly secreted by cancer cells. This growth factor induces tubulogenesis in the tumor mass. Thus, inhibiting the VEGF secretion could inhibit tumor progression.[27] Based on our results, inhibition of HUVEC proliferation and angiogenesis of EA fraction and hydroalcoholic extract of TP may be due to the inhibition of VEGF expression/secretion.

Our results apparently addressed *in vitro* anti-angiogenic effect of *T.Polium* extract and especially its ethyl acetate fraction, and suggested the anti-angiogenesis effect of TP along with the its anti-VEGF effect. Finally, it was concluded that EA fraction of *Teucrium Polium* contains low toxic component(s) with high potential to inhibit proliferation of endothelial cells and angiogenesis in tumors. However, identification and molecular mechanisms of responsible component(s) needs more quantitative investigations an *in vivo* studies could highlight the potential of TP for therapy of angiogenesis related disorders.

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