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Original Article

The Effect of Aqueous Phase and Hydroalcoholic Extract of Stachys lavandulifolia on VEGF Gene Expression Changes and Angiogenesis of Chick Embryo Chorioallantoic Membrane

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Article Info

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Abstract

Introduction: Angiogenesis is the physiological process through which new blood vessels form from pre-existing vessels. Cardiovascular endothelial factors are involved in diseases and vascular regulation. *Stachys lavandulifolia* belongs to the *Laminacea* family and its hydroalcoholic extract is used as a traditional treatment to reduce pain and inflammation. The present study was conducted to examine the effect of aqueous phase and hydroalcoholic extract of *Stachys lavandulifolia* on the expression changes of vascular endothelial growth factor gene (VEGF) and angiogenesis of chick embryo chorioallantoic membrane.

Methods: In this study, 40 Ross fertilized eggs were randomly divided into control, sham and two experimental groups of treatment with aqueous phase and treatment with hydroalcoholic extract. On the 2nd day of incubation a window was opened on the eggs; on the 8th day a gelatin sponge of 1×4×4 diameter was put on the chorioallantoic membrane, and 75 mg/kg of each extract was added. On the 12th day, the height and weight of the embryos were measured, their blood vessel network was photographed by a stereomicroscope and the number and length of vessels around the sponges were measured via applying image J software. The VEGF Gene was sampled. Results were analyzed by Minitab software using t-test and ANOVA at a significance level of $P<0.05$.

Results: The mean of the data showed no significant difference between control and sham groups ($P>0.05$). The mean number of vessels in the experimental group 1 (aqueous phase) had a significant reduction ($P\leq0.05$), while the experimental group 2 (hydroalcoholic extract) showed no significant difference. Gene VEGF expression in the experimental groups increased compared to the sham group.

Conclusion: The aqueous phase of *Stachys lavandulifolia* has anti-angiogenic effects, which appears to work through affecting VEGF receptors.

Introduction

Angiogenesis is the formation process of new blood vessels from existing ones which is very important for normal growth and development of the body. In normal conditions, angiogenesis is the basis of multiple physiological processes such as fetal growth, the formation of the placenta and wound healing (1). Angiogenesis is a controlled process that rarely occurs in adults except in special conditions such as wound healing and menstrual cycle (2). Angiogenesis depends on a precise balance between natural body stimulators and inhibitors. In the event of imbalance in this natural state, the conditions are provided for the emergence of diseases such as corneal angiogenesis, endometriosis, obesity, psoriasis and tumor growth (1). There are many angiogenic and anti-angiogenic factors in the body, some of which directly affect endothelial cells, while others might activate other cells, such as inflammatory cells that might lead to secretion or inhibition of angiogenic factors (3). There are more anti-angiogenic factors than angiogenesis factors in normal tissues, and thus angiogenesis does not happen normally (4). Factors such as hypoxia, decreased pH, increased lactic acid, inflammatory immune responses and mutations in oncogenes and tumor suppressors which increase the concentration of angiogenic factors or decrease anti-angiogenic factors will disturb the balance and lead to angiogenesis. These factors are secreted in form of autocrine or paracrine by endothelial, inflammatory, or tumor cells (5). The most important angiogenic factors or genes involved in angiogenesis include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and angiopoietin. These are protein factors that bind to specific receptors on the surface of the cell membrane. The vessel formation onset needs two factors of b-FGF and VEGF. VEGF is one of the most important factors

of angiogenesis (6). The vascular endothelial growth factor or VEGF gene is 14kb long, placed on chromosome 6 and consists 8 exons and 7 introns (7). VEGF plays an important role in angiogenesis. VEGF and its receptors are the key mediators of angiogenesis and are the targets for several pharmacologic agents. VEGF performs its biological action on target cells through interaction with a receptor tyrosine kinase in the plasma membrane of the cell. After binding to their ligands, these receptors become dimer and autophosphorylated and eventually lead to a cascade inside the cell. VEGF-A is the main factor in the process of angiogenesis and works via activating two VEGFR-1 and VEGFR-2 receptors (8). Medicinal plants are more popular than synthetic drugs among people due to being natural, having low risks and few complications, and being easily accessible and cheaper. Drug resistance against chemical antimicrobial drugs has led to paying more attention to and use of medicinal plants to treat diseases and infections in recent years (9). The use of medicinal plants has long been common in Iran and many other countries, and using these plants in traditional or novel pharmaceutical forms has been welcomed by the public and professionals. *Stachys lavandulifolia* is a medicinal plant with numerous therapeutic applications (10). In recent years, pharmacological studies have shown that extracts and compounds derived from plants belonging to the *Genus Stachys* have analgesic (11), anti-bacterial (9), anti-oxidant (12); anti-inflammatory (13); and anti-hypertensive properties and sedative effects (14). *Stachys lavandulifolia* is an important medicinal plant native to Iran, which is locally called tooklije, khargoosh kork kafte (15). The brewed aerial parts of the plant are used for treating gastric discomfort, insomnia, and anxiety (16). *Genus Stachys* has more than 270 species (17). The genus has 34 species in Iran, 13 of which are endemic species (18). *Stachys lavandulifolia* is a species from this family (19). The active compounds of the plant that have biological activity are phenylethanoid, terpenoid, and flavonoid (20). The study of angiogenesis process and its factors requires the use of multiple in-vivo methods. The common in-vivo models include rabbit ear, hamster cheek pouch, dorsal skin cavity, air sac, chick chorioallantoic membrane (21). Simplicity, speed and low prices of different assays using chick embryo motivated using this model in pharmacology and medical studies (22). Several studies have been displayed the effects of plant extracts on the process of angiogenesis in recent years. One example is the inhibitory effects of asafoetida on chick chorioallantoic membrane angiogenesis (23). The present study was conducted to investigate the possible effects of the aqueous phase and hydroalcoholic extract of *Stachys lavandulifolia* and VEGF gene changes on the chick chorioallantoic membrane angiogenesis. Since *Stachys lavandulifolia* is used to treat various diseases and its angiogenesis effects on consumers has not been investigated yet, the present study was designed with the aforementioned purpose.

Materials and Methods

In this in-vitro experimental study, the extract of *Stachys lavandulifolia* was prepared in the Plants Research Laboratory and the experiments were conducted in the Animal Developmental Biology Research Laboratory of the Department of Biology at Mashhad Islamic Azad University in 2015.

Stachys lavandulifolia was collected from the foothills of Hezar Masjed Mountains near Ghoochan in Iran. The plant was approved by the Herbarium Center of Faculty of Science at the University of Mashhad under the Herbarium Code 9420. The leaves of the plant were separated from the stems and after washing with tap water were dried under the shade away from the sunlight. Extraction was prepared by Soxhlet method (24). First, 22 grams of powdered *Stachys lavandulifolia* leaves was poured in extraction thimbles and placed in the device. Then 250 ml of ethanol and 250 ml of distilled water was poured into the special container. As distillation flask heated slowly, the solvent (alcohol and distilled water) heated and the *Stachys lavandulifolia* leaf extract mixed with the solvents and returned to the balloon. The extraction was prepared in 4 hours. It was condensed up to one-third of the initial volume in a vacuum distillation rotary device adjusted at 40°C. This was performed several times until the alcohol- and the water-soluble extract was obtained from the plant. The extract was put in the incubator at 45°C for 3 days and finally, 6.8470 g dry extract was obtained. Of this amount, 2.447 g was separated as the solid alcoholic extract. Then, the remaining dried extract (4.400 g) was solved in 180 ml of distilled water to prepare the aqueous phase. Next, 50 ml of n-butanol was added to the liquid in the decanter funnel and well shaken to create two phases. This was repeated twice more and then placed inside an incubator to obtain a dried extract. The lower obtained phase of the mixtures was used for the preparation of ethyl acetate fraction containing polar and intermediate components. In this stage, 50 ml of ethyl acetate was added and this was repeated for several times, too. The upper phase was separated, containing intermediate components such as phospholipids, which are dissolved in ethyl acetate. The concentrated liquid was placed inside the incubator to obtain a dried extract. After the extraction of n-butanol and ethyl acetate fractions from the distilled water and hydroalcoholic extract mixture in the decanter funnel, what was remained was the aqueous phase. After concentration, the aqueous phase was placed in an incubator to be dried, too (25).

In this study, one-day-old Ross fertilized eggs were used as *in-vitro* models that were procured from Mashhad Agriculture-Jahad poultry company. A total of 40 eggs (excluding losses) were randomly distributed into 4 experimental groups including the control group (kept in normal conditions), the sham group (treated with normal saline), the experimental group 1 (treated with aqueous phase), and experimental group 2 (treated with a hydroalcoholic extract of *Stachys lavandulifolia).* The extracts were injected with a dose of 75 mg/kg. After sterilizing, the eggs were placed in a research incubator (Made in Iran) at 38°C and relative humidity of 65% and were rotated twice a day. On the second day of incubation, a window was opened on the eggs in sterile conditions under a laminar hood using sterile forceps inside the autoclave. First, a hole was made in

the wider end of the eggs. Then, the small-scale part of the shell was removed slowly from the longitudinal part of the egg. After removing the shell, the window site was covered by sterile plates and paraffin and the eggs were transferred to the incubator (26). On the 8th day of incubation, after placing a gelatin sponge (egg albumin and agar solution in normal saline with equal proportions, with dimensions of 1×4×4 mm), 10 µl of normal saline was added to the sponge in the sham group; the experimental group 1 received 10 µl of aqueous phase extract at a dose of 75 mg/kg; the experimental group 2 received hydroalcoholic extract at a dose of 75 mg/kg; and then the windows were closed by sterile plates and paraffin. The eggs were returned to the incubator and kept there until the 12th day of incubation. All the studied groups were photographed on the 12th day of incubation by research stereomicroscope (Zeiss, Germany) at the site of the sponge in the chorioallantoic membrane with a magnification of X40. The pictures were studied using Image J software in a 15-inch monitor. A part of the chorioallantoic membrane of each sample was isolated using scissors for RNA extraction and transferred to a sterilized porcelain mortar, frozen by liquid nitrogen, and pulverized. The resulting powder was transferred into 1.5 micro-tubes and then the process continued with respect to the RNA extraction protocol included in the kit (Zist Asia, Iran). The micro-tubes contents were centrifuged according to the extraction protocol and Total RNA was extracted at the end. It was stored in a freezer at -20°C. Embryos in the eggs were removed and their weight and height were measured by digital scales and caliper. Data was analyzed using Minitab software. The T-test was used for determining the significance of independent groups, and ANOVA was used to compare groups at a significance level of $P<0.05$. The results are presented in Mean±SD form. The Total RNA underwent cDNA synthesis by a special kit (Zist Asia, Iran) and then the samples were stored in the freezer. Primer sequences were determined for VEGF gene expression (Table 1). Finally, the samples and the added primers were examined by the RT-PCR method to determine VEGF gene expression changes and the resulting data was analyzed (Figure 6).

Table 1. The primers sequence of the specific gene (VEGF) and housekeeping gene for real-time (RT-PCR):

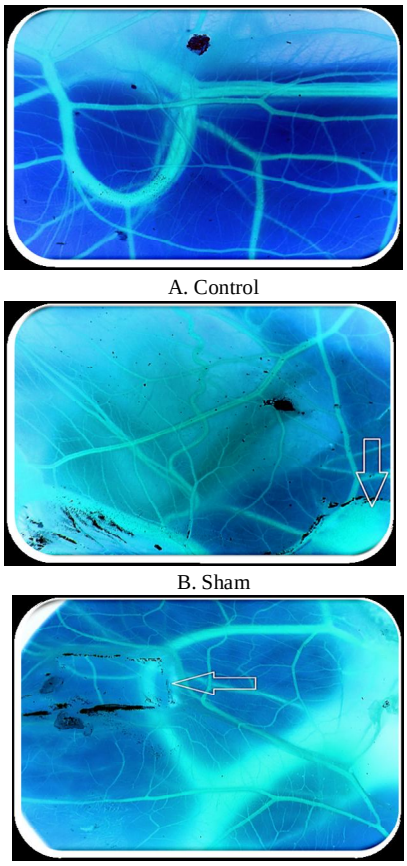
VEGF-A								
Forward: 5'	CAA	TTG	AGA	CCC	TGG	TGG	AC 3'	20 Open
Reverse: 5'	TCT	CAT	CAG	AGG	CAC	ACA	GG 3'	20 Open
G6PDH								
Forward:5'	CGG	GAA	CCA	AAT	GCA	CTT	CGT 3'	21 Open
Reverse: 5'	CGC	TGC	CGT	AGA	GGT	ATG	GGA 3'	21 Open

Results

Expanded blood vessels were observed on the surface of chorioallantoic membrane on the 12th day of incubation (Image 1). The results were obtained from the comparison in terms of mean and standard deviation (MEAN ± SD). The comparison of the mean length of vessels in the control group (20.750±4.83) showed no significant difference with the sham group (18.00±6.30). The comparison of the number of vessels in the control group (10.750±4.68) and the sham group (12.375±3.06) showed no significant difference. The comparison of the mean length of vessels in the experimental group 1 (aqueous phase), (20.875±3.52) with the sham group showed no significant difference (Figure. 1). The comparison of the mean length of vessels in the experimental group 2 (hydroalcoholic extract), (24.250±6.56) with the sham group showed no significant difference, either (Figure 1).

The comparison of the mean number of vessels in the control group (10.750±4.68) showed no significant difference with the sham group (12.375±1.08) (Figure 2). The comparison of the mean number of vessels in the experimental group 1 (aqueous phase), (9.500±2.39) with the sham group (12.375±3.06) showed a significant decrease (Figure 1). The comparison of the mean number of vessels in the experimental group 2 (hydroalcoholic extract), (9.375±3.43) with the sham group (12.375±3.06) showed a significant decrease (Figure 2).

The comparison of the mean weight of embryos in the control group (2.8750±0.64) showed no significant



A. Control

B. Sham

C. Experimental 1 (aqueous phase)

Image 1. Stereomicroscope images of vessels in the chorioallantoic membrane surface in the control sample with a magnification of 40 X

difference with the sham group (2.6250±0.263). The comparison of the mean weight of embryos in the experimental group 1 (aqueous phase), (2.3750±0.74) with the sham group showed no significant difference (Figure 3). The comparison of the mean weight of embryos in the experimental group 2 (hydroalcoholic extract), (2.7500±0.70) with the sham group showed no significant difference (Figure 3).

The comparison of the mean height of embryos in the control group (33.250±1.83) showed no significant difference with the sham group (34.000±1.69). The comparison of the mean height of embryos in the experimental group 1 (aqueous phase), (30.625±3.62) with the sham group showed a significant decrease (P<0.05) (Figure 4). The comparison of the mean height of embryos in the experimental group 2 (hydroalcoholic extract), (32.000±4.21) with the sham group showed no significant difference (Figure 4).

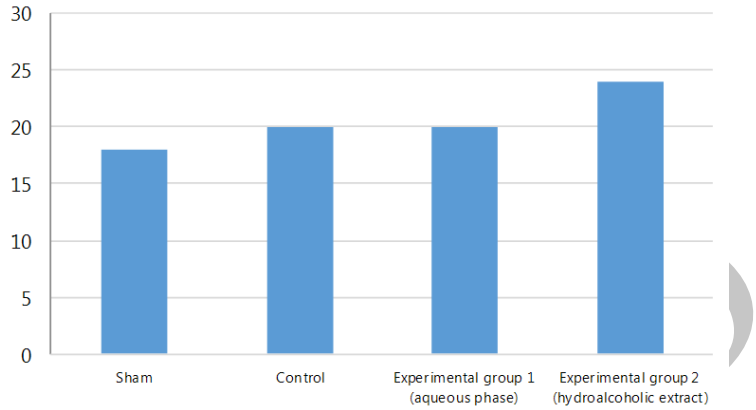


Figure 1. Comparison of the mean length of blood vessels in the experimental groups
There was no significant difference between any of the groups.

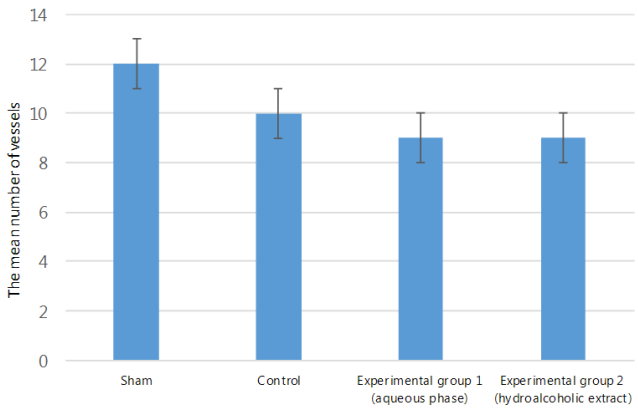


Figure 2. Comparison of the mean number of blood vessels in the experimental groups
The experimental group 1 (aqueous phase) showed a significant decrease (P<0.05).

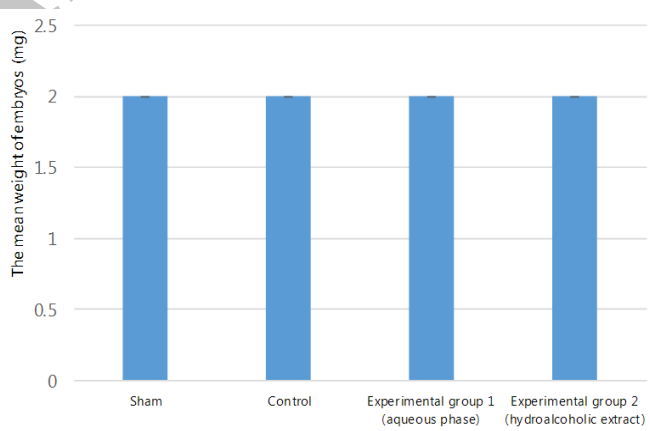


Figure 3. Comparison of the mean weight of embryos in the experimental groups
There was no significant difference between any of the groups

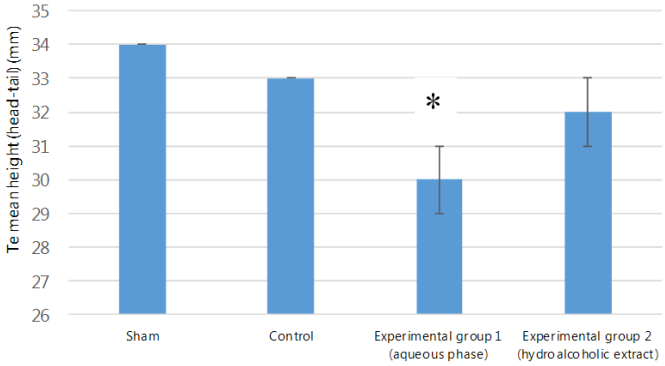


Figure 4. Comparison of the mean height of embryos in the experimental groups
* Only the experimental group 1 (aqueous phase) showed significant differences

Table 2. The results of embryo mortality in 4 studied groups

	The number of live embryos	The number of dead embryos	The viability percentage
Control	10	0	100%
Sham	10	0	100%
Experimental 1 (aqueous phase)	6	4	60%
Experimental 2 (hydroalcoholic extract)	8	2	80%

Interpretation of VEGF gene expression:

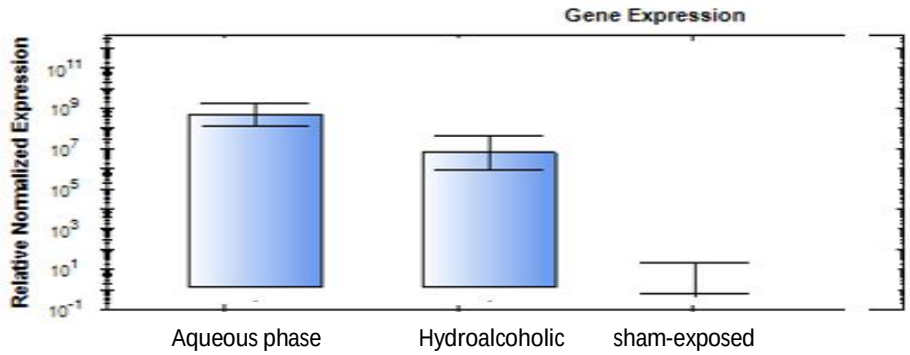


Figure 6. VEGF gene expression in different groups by RT-PCR or real-time methods by a thermocycler device

According to the interpretation of VEGF gene expression, *Stachys lavandulifolia* extracts stimulated VEGF gene expression in the experimental groups (aqueous phase and hydroalcoholic extract).

Discussion

According to the results, the hydroalcoholic extract and aqueous phase of *Stachys lavandulifolia* affected the angiogenesis of chick embryo chorioallantoic membrane. The hydroalcoholic extract and aqueous phase of *Stachys lavandulifolia* reduced the number of vessels in the chorioallantoic membrane (Figure 2). It can be concluded that both of them inhibited angiogenesis, while both increased the length of the vessels (Figure 1), thus they were involved in cell proliferation, as the VEGF gene expression also increased in the aqueous phase and the hydroalcoholic extract of *Stachys lavandulifolia*. This gene is more involved in cell proliferation. This study revealed that both the aqueous phase and the hydroalcoholic extract of *Stachys lavandulifolia* decreased the height of the embryos (Figure 4). The morphological observations of embryos in the group treated with the hydroalcoholic extract of *Stachys lavandulifolia* indicated one single-eye defective embryo because of the active compounds present in the hydroalcoholic extract. The teratogenic effects of this extract in high doses have been previously proven in various researches (27). Therefore, the results of this study in relation to the teratogenic effects of high doses of the extract are probably in line with the results of other scientists. Since one way of treating cancer is inhibiting angiogenesis, therefore *Stachys lavandulifolia* can probably have its health impacts through inhibition of tumor angiogenesis. Our results suggested that the aqueous phase and hydroalcoholic extract reduced the number of vessels, hence the fraction and the extract have anti-angiogenesis effects and can be used to treat a variety of tumors. In the study of Safai, the amount of apigenin compounds was compared in aqueous phase and hydroalcoholic extract of *Stachys lavandulifolia* and the results showed that the amount of apigenin was higher in the hydroalcoholic extract (28), thereby it can be claimed that the presence of this estrogenic compound in the *Stachys lavandulifolia* extract might be one of the causes of the abnormality observed in this study. Jafarzadeh et al. investigated the effect of *Stachys lavandulifolia* on abortion in mice. They showed that the plant caused abortion at high doses and led to fetal growth disorders in the mice (29). Their results were in line with the results of this study with the single-eyed

chick embryo observed in the experimental group 2 (hydroalcoholic extract) which was probably due to the above-mentioned compound in the hydroalcoholic extract of *Stachys lavandulifolia*. Nasri et al. investigated the analgesic and anti-inflammatory effects of *Stachys lavandulifolia* hydroalcoholic extract in mice and concluded that the phytochemical study of the extracts identified two iridoid glycosides, one flavonoid glycoside, and one phenylethanol glycoside. These glycosides exist specifically in the aqueous phase. The flavonoids and iridoid compounds in the extract are responsible for its antioxidant, anti-inflammatory and analgesic properties, and the reason for its traditional use. Also, the significant antioxidant activity of the extract confirms the presence of glycosylated phenolic compounds (30). In the present study, the examination of the angiogenesis process found a relationship between glycosylated phenolic compounds and angiogenesis. The presence of flavonoid compounds processed in the hydroalcoholic extract of the plant is effective in anti-inflammatory and regenerative processes by increasing cell proliferation and reducing the activity of free oxygen. The results of this study are in line with the opinions of other scientists as the VEGF gene expression, the number and length of vessels in the hydroalcoholic extract experimental group was higher compared to the other groups. Nitric oxide (NO) derived from endothelial cells originally promote angiogenesis as endothelium relaxing factors, and play an important role in vascular reconstruction, maintaining and integrity (31). It can be concluded that nitric oxide stimulates angiogenesis and increases angiogenesis in the groups treated with hydroalcoholic extract compared to other treatment groups. Curcumin is a phenolic compound extracted from turmeric that has anti-angiogenesis activities (32). Phenolic compounds reduced angiogenesis in the aqueous phase group. Therefore, the angiogenesis results in the hydroalcoholic extract in this study were probably due to the presence of nitric oxide, and the anti-angiogenesis results observed in the aqueous phase group was related to the activity of its phenolic compounds (Figure 2).

Rafieian et al. examined the toxicity of *Stachys lavandulifolia* extract on rat liver and concluded that probably at least one of the factors that caused a toxic effect of *Stachys lavandulifolia* was the terpenoid compounds (12). Since Germacrene and Alpha-Pinene are available compounds in *Stachys lavandulifolia* with a terpene structure, and according to other researches, it can be concluded that according to Table 2, the cause of increased rate of fetal death in the experimental group 1 (aqueous phase) compared to the experimental group 2 (hydroalcoholic extract) was due to these compounds.

Nabavizadeh et al. studied the protecting effect of this plant against gastric ulcers. The results showed that *Stachys lavandulifolia* extract reduced the mucosal damage caused by alcohol. That might be due to the effect of nitric oxide release (33). The present study showed that the hydroalcoholic extract has angiogenesis properties due to the presence of nitric oxide. Nabavizadeh also indicated that angiogenesis healed alcohol-induced wound.

Analgesic and anti-inflammatory effects in some species of *Stachys lavandulifolia* led to the healing properties similar to or stronger than non-steroidal anti-inflammatory drugs, which highlights the possibility of their therapeutic use (11, 34). The results of a study suggested that extracts of the *Stachys lavandulifolia* had proper effects on both acute and chronic pain, and reduced pain compared to morphine (35). The hydroalcoholic extract of *Stachys lavandulifolia* has anti-inflammatory and analgesic effects and might be a good alternative to anti-inflammatory and analgesic drugs. The flavonoid and iridoid compounds and saponins in *Stachys lavandulifolia* extract resulted in the use of this herb in traditional medicine as an analgesic and anti-inflammatory drug (13).

Conclusion

Data analysis indicated that the aqueous extracts of *Stachys lavandulifolia* inhibited angiogenesis in chick embryo chorioallantoic membrane by reducing the number of blood vessels. However, the different results of increased length of vessels and decreased number of vessels in using the extract at a dose of 75 mg/kg highlight the need for further studies about this extract. Also, due to the increased expression of VEGF gene, the reason of decreased angiogenesis in the aqueous phase on the expression of other genes such as VEGF receptors can be further explored.

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