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# The Effects of *Stachys lavandulifolia* Leaves Aqueous Extract on Angiogenesis in Chick Embryos Chorioalantoic Membrane and Choroids Plexus

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**Abstract**

Angiogenesis, formation of new vessels from the primary vessels is involved in physiological condition such as reproduction, wound healing and pathological like Diabetes and tumor. Tumor progression associates with persistent unregulated angiogenesis. This study aimed to investigate the effect of leaves aqueous extract of *Stachys lavandulifolia* on the angiogenesis in chorioallantoic membrane and choroids plexus of chick embryos. In this experimental study, 30 fertilized eggs of Hy-line race were randomly divided into 5 groups: control group, sham-exposed group and four experimental groups (n=6). In the second day of incubation in sterile condition, a window was opened on eggs. On 8th day, chorioallantoic membrane (CAM) was treated at sham-exposed group with normal saline and at experimental groups with *Stachys lavandulifolia* aqueous extract (25, 50, 100 mg/kg). On 12th day CAMs, the length and number of vessels were examined and

morphological disorders were studied. Data were calculated and analyzed statistically using t-test and ANOVA.
There is no significant difference in the mean number and length of vessels in the control group with the sham-exposed groups. The mean of number and length in all experimental groups shows a meaningful increase in compare with sham group (P<0/05).Morphological abnormalities were not observed in fetuses.
According to the results of this research, low doses of *Stachys lavandulifolia* aqueous extract increase angiogenesis in chorioallantoic membrane and choroids plexus, but it has antiangiogenesis effect in high doses. No significant effect on chick embryo's morphological characteristics observed.

**Keywords**: Angiogenesis, *Stachys lavandulifolia*, Chorioallantoic, choroids plexue

## Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vascular bed, plays a critical role in tissue growth and repair [1]. Angiogenesis is the complex physiological sequence of vasodilatation, degradation of basement membrane, endothelial cell migration, chemotaxis, increasing vascular permeability and eventually endothelial cell proliferation and vessel formation. The fine-tuned balance of vasculation and angiogenesis is controlled by many growth and transcription factors [2]. The molecular mechanisms underlying new blood vessel growth currently are being investigated in several laboratories. Angiogenesis or neovascularization has been implicated in various unrelated disease processes, such as retinopathy of prematurity (ROP), diabetic retinopathy, choroidal neovascularization, macular degeneration, and tumor angiogenesis. On the other hand, growth of new blood vessels is desired and beneficial in wound healing and myocardial and limb ischemia [3]. Regenerative concepts are one of the basic ideas of modern biomedical research. In regeneration, new vessel requires to deliver oxygen and survival factors to damaging tissue. This phenomena is necessary for growing, differentiating and migration the cells to repair tissue. It becomes obvious that there can never arise a regenerative course without a functioning vasculature to provide the essential cells and proteins, to ensure the oxygen and nutrient supply and to evacuate accumulating metabolic products [4]. The process of angiogenesis is controlled by chemical signals in the body. May be some natural products has essential rule in angiogenesis. Natural products of plant origin are still a major part of traditional medical systems. *Stachys lavandulifolia* is a native plant widely distributed in Iran [5]. It consists of approximately more than 300 species that are widely distributed in tropical and subtropical countries [6]. In middle ages, the European traditional medicine scientists were using *Stachys* in asthmatic, infective, rheumatic and other inflammatory disorders. Different species of *Stachys* have wound and skin inflammation, antitoxic [7], antibacterial [8], anti-nephritic [9], antiinflammatory

[10, 11] and anti-anoxia [12] effects. The basic ingredients of this genus are flavonoids, triterpenoids, steroids [13, 14]. Chick embryo chorioallantoic membrane (CAM) and choroid plexus are common experimental models for angiogenesis studies. The purpose of this research is to examine the angiogenesis properties of aqueous extract obtained from *Stachys Lavandulifolia* aerial parts on chorioalantoic membranes and choroid plexus.

## Material and methods

Materials:

The ***Stachys Lavandulifolia*** leaves (herbarium code 9420) was supplied by Islamic Azad University of Mashhad, Iran (2014). ***Stachys*** leaves were grinded. The powder was kept in a cool and dry place until extraction time.

How to prepare extract:

Aqueous extract of leaves powdered was prepared by Soxhlet apparatus method [15] and the extraction was carried on in the Laboratory of herbal researches. Most of the requisite material of this project was supplied by the sigma company.

Method:

We used 30 fertilized ross eggs held in an incubator with 38°C temperature and 65% moisture. In day 2 of incubation, windows were opened for eggs under sterile condition [16] and eggs were divided into 5 random groups including:
1) Control that were held in normal condition.
2) Sham-exposed that was treated with sterile serum.
3) Treated with aqueous extract of ***Stachys Lavandulifolia*** ( 25mgkg $^{-1}$)
4) Treated with aqueous extract of ***Stachys Lavandulifolia*** ( 50mgkg $^{-1}$)
5) Treated with aqueous extract of ***Stachys Lavandulifolia*** ( 100mgkg $^{-1}$).
In day 8, a gelatin sponge with 1 × 4 × 4 diameter was placed on chorioalantoic membrane (CAM) and soaked with 10 microliter of extract solution in members of groups 3, 4 and 5. Chorioalantoic membrane were examined daily and photographed (by photo-stereomicroscope, Zeiss, Germany) at day 12 in 0.65 × 10 × 4 magnification.
Data were analyzed for the number and length of angiogenic blood vessels in chorioalantoic membrane by software programs and t-test.

**Sampling:**

Under the pentobarbital anesthesia the brain of embryos was rapidly removed and fixed in 10% paraformaldehyde. For histological evaluation samples were placed in same fixative overnight and were embedded in paraffin. Serial cross –sections were cut and stained with hematoxylin and eosin.

**Stereological methods**:

The volume of choroids plexus were measured with Cavalieri method and the length density of capillary by counting the number of capillary particles as described by [17].

**Statistical analysis:**

Student's t test was used for comparison when only two groups were analyzed and a one-way ANOVA followed by a Scheffe' f test when more than two groups were analyzed. Statistical significance was chosen as $p<0.05$. All results are reported as mean ± SEM.

## Results

**Number and Length of CAM vessel**

The result of examining angiogenesis effect of ***Stachys Lavandulifolia*** aqueous extract are presented (Fig.1) as counting number and Length of vessel in chorioalantoic membrane and choroids plexus in tables 1 and 2. The results do not show significant difference between control and sham groups ($p<0.1$) but there is a significant difference between sham and Experimental 50 in number of vessels (Fig.2).

Table1. Comparison between average number (N) and length (L) of vessels in experimental groups (±SE) in chorioalantoic membrane

| Groups | Control | Sham | Exp. 25 | Exp. 50 | Exp. 100 |
|---|---|---|---|---|---|
| Number of vessels | 21.5 ± 2 | 19.3 ± 1 | 22.5 ± 2 | 28.5 ± 2 | 20.17 ± 3 |
| Length of vessels | 3.8 ± 0.3 | 3.6 ± 0.2 | 4.1 ± 0.5 | 4.5 ±0.5 | 4 ± 0.4 |

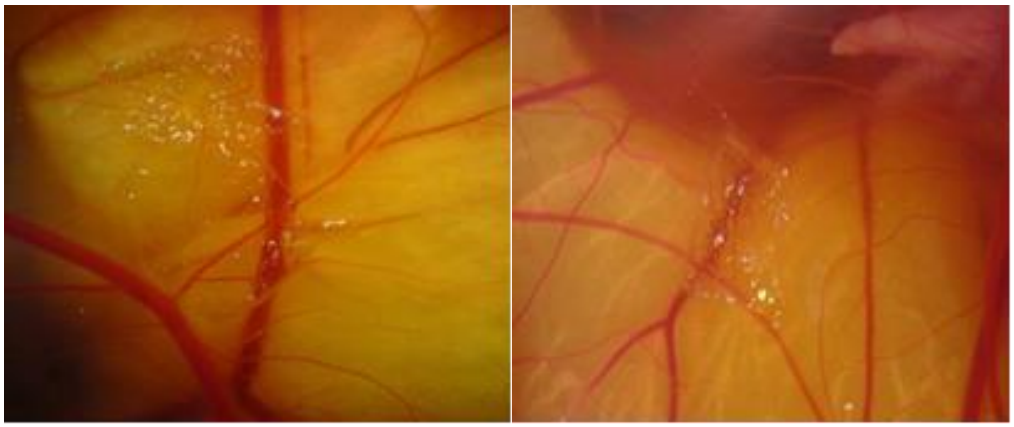

Fig.1: Chorioalantoic membrane. Left: Exp.50. Right: Control

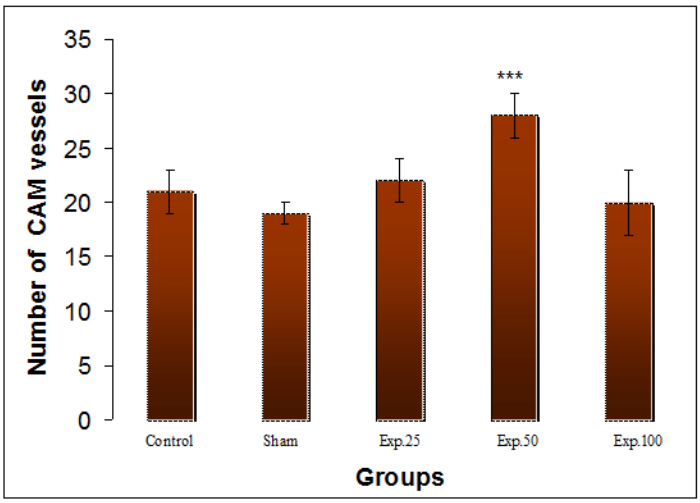


Fig.2: Compression between Number of CAM vessels in sham and experimental groups with aqueous extract in 3 different dosages (25, 50, 100 mg kg-1)

The length of vessels in experimental groups (25, 50) was increased but this increase in Experimental 50 was significant ($p<0.001$) (Fig.3). In experimental group 100, data was shown a remarkable decrease.

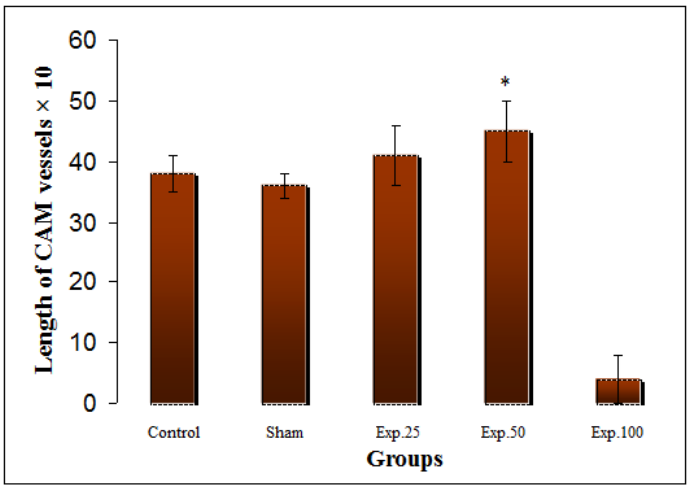


Fig.3: Compression between length of CAM vessels in Sham and experimental groups with aqueous extract in 3 different dosages (25, 50, 100 mg kg-1)

**Volume of choroid plexus**

There is no significant increase in volume of choroid plexus between control and sham groups ($p>0.05$)(Table.2). The volume of choroid plexus in all experimental groups was increased but this increase in Experimental 50 was meaningful ($p<0.001$) (Fig.4).

Table 2. Comparing volume of choroids plexus and length of vessels in Sham and experimental groups

| **Groups** | **Control** | **Sham** | **Exp. 25** | **Exp. 50** | **Exp. 100** |
|---|---|---|---|---|---|
| Choroids plexus volume | o.346 ± 37 | o.284± 45 | 0.367± 40 | o.515 ± 38 | 0.390± 44 |
| Length of choroid plexus vessels | 0.012 ± 0.5 | 0.008 ± 0.2 | 0.02 ± 0.3 | 0.049±0.4 | 0.019± 0.1 |

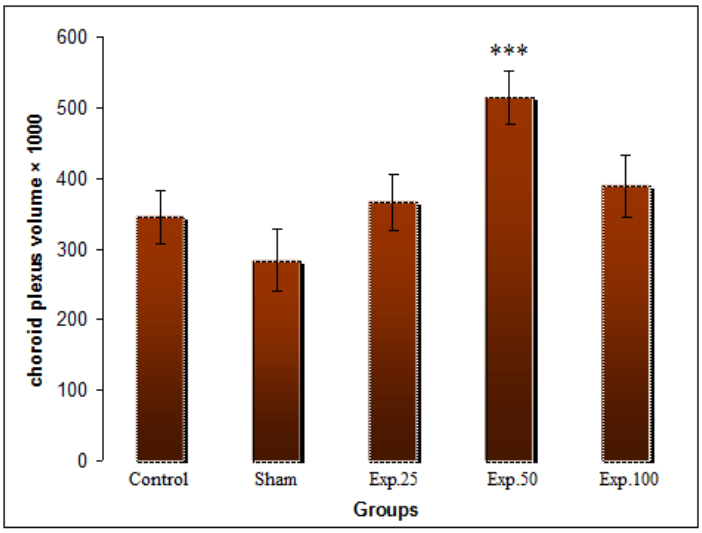


FIG. 4. Comparing volume of choroids plexus in the Sham and Experimental groups

**Length density of capillary in choroid plexus**

As expected, the length of choroid plexus capillary in all experimental groups has increased. This increases in experimental 50 was significant ($p<0.001$) (Fig.5).

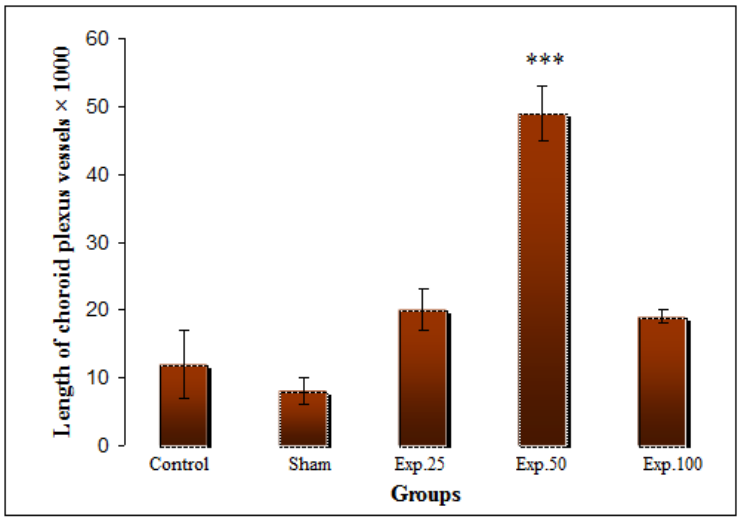


FIG.5. Comparing the C.P total length capillaries in Sham and Experimental groups

## Discussion

Therapeutic angiogenesis is a proven clinical strategy for promoting faster wound healing in diabetic foot ulcers and other wounds with delayed regeneration [18]. Regeneration means origination in stark contrast to substitution. This process aims not to replace or to reconstruct but to restore the physical integrity of cells, tissues and organs by means of the organisms' own repair mechanisms. The fundament of regeneration is the tissue's potential to grow, to differentiate and therefore to continually bridge permanently emerging damages. The process of angiogenesis is controlled by chemical signals in the body. These signals can stimulate both the repair of damaged blood vessels and the formation of new blood vessels. Other chemical signals, called angiogenesis inhibitors, interfere with blood vessel formation. Normally, the stimulating and inhibiting effects of these chemical signals are balanced so that blood vessels form only when and where they are needed [19].

As data show the number of vessel and length in chorioalantoic membrane Increased in experimental groups in compare with sham group (Fig.1,2). This data show a kind of angiogenesis. This increase in number of vessel or length in experimental groups that receive low doses (25, 50) of extract is remarkable. But may be with use of high doses (100) the rate of angiogeneses is decreased.

Studies show that aerial parts of *Stachys* contain flavonoids, phenylethanoid glycosides, and diterpenes (13) quinines, Iridoids [20], Germacrene-D, beta pinene, alpha pinene, myrcene and beta phellandrene have been reported to be the main compounds of the essential oil *S. lavandulifolia* [21]. It is also likely that the extract may have components with different anti- and pro-angiogenesis effects. As established Cornel iridoid glycoside promotes neurogenesis and angiogenesis and improves neurological function after focal cerebral ischemia in rats [22]. Maybe the effect of angiogenesis that we see in this research due to effect of iridoid glycoside. This glycoside increase expression of vascular endothelial growth factor (VEGF) and its receptor Flk-1(18). Angiogenesis requires the binding of signaling molecules, such as vascular endothelial growth factor (VEGF), to receptors on the surface of normal endothelial cells. When VEGF and other endothelial growth factors bind to their receptors on endothelial cells, signals within these cells are initiated that promote the growth and survival of new blood vessels [23]. The effects of VEGF as a promoter not only of angiogenesis but also of bone regeneration have been reported in a femur fracture model in mice and in a rabbit radial segmental defect; improved ossification and callus maturation where observed [24]. As data show in experimental groups with high doses of extract angiogenesis was decreased. Flavonoids are one of the most components in this plant. Flavonoids could inhibit angiogenesis under high doses use [25]. Probably this extract has anti angiogenesis effect in high doses as many of researches indicate.

Choroids plexus is another vascular system that be change in response to some exogenous material. Understanding the factors that control brain angiogenesis is critical for determining developmental aspects of the blood–brain barrier (BBB)

as well as cerebrovascular changes after injury. Several factors have been shown to be angiogenic *in vivo*, but only vascular endothelial growth factor (VEGF) is a secreted mitogen that is specific for the vascular endothelium [26].

We followed our research in choroids plexus for angiogenesis effect of extract. Data show the volume of choroids plexus and length of capillaries in all experimental groups were increase but just in experimental 50 was significant (Fig.3,4). This data are parallel with CAM data. As resulted suggest *Stachys* extract has angiogenesis effect in low doses but could has anti angiogenesis effect in high does. This hypotheses need to more researches. The results presented in this study should be taken as base for further investigation in *Stachys* extract.

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