OCIMUM SANCTUM INHIBITS VASCULAR ENDOTHELIAL GROWTH FACTOR AND ANGIOGENESIS IN CHICK CHORIOALLANTOIC MEMBRANE


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ABSTRACT

Ocimum sanctum (Linn.) is the sacred herb. In India it is known as ‘Tulsi’ or ‘Holy Basil’. Every part of the plant is used in ethanomedicines to cure several pathological conditions. In this present investigation angiogenic efficiency of aqueous leaf extract of O. sanctum was studied by using chick chorioallantoic (CAM) assay. The CAMs were studied morphometrically, histologically and immunohistochemically. There was significant decrease in secondary and tertiary blood vessels at early hrs of treatment. The angiogenesis was inhibited more significantly at 72 and 96 hrs treatments. Decreased immunoperoxidase staining for VEGF indicated inhibition of VEGF synthesis by the extract. The antiangiogenic property of aqueous leaf extract of O. sanctum supports anticancerous ethanomedicinal property of this plant and paved the way to synthesize the new drug against tumor.

KEYWORDS: Angiogenesis, Ocimum sanctum, CAM.

INTRODUCTION

During embryonic development the initial development of blood vessels in intraembryonic region takes by the process of vasculogenesis. The haemangioblasts give rise to primary blood vessels. Angiogenesis is the process of neovascularization from pre-existing microvasculature. It may be of sprouting and intussusceptive type. The intussusceptive type of angiogenesis is accompanying sprouting type after 8-10 days of embryonic development in chick. Angiogenesis plays an important role in various physiological and pathological
conditions. It is not only observed during fetal development but also in tissue repair after surgery or trauma. It is essential for wound healing and during menstrual cycle (Pluda, 1997). In adult the process of angiogenesis is quiescent and balanced by various angiogenesis switches- pro- and anti-angiogenic factors. Angiogenesis is initiated by vasodilatation and increasing permeability of primary blood vessel. Due to destabilization the endothelial cells (ECs) migrate to form new vessels by angiogenesis, which are stabilized by pericytes. The important angiogenic factors are vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin and platelet derived growth factor (PDGF). The Angiostatic factors are angiostatin, endostatin, thrombospondin etc. The steady state of angiogenesis becomes disrupted under some pathological conditions. Excessive angiogenesis is associated with cancer, psoriasis, arthritis and atherosclerosis (Folkman et al., 1992). The myocardial ischemia, scleroderma and ulcers are associated with insufficient angiogenesis. As these pathological conditions are associated with angiogenesis, there is unending quest to study and apply angiogenesis modulators for therapeutic angiogenesis. Nowadays many angiostatic drugs are used against cancer while angiogenic drugs are used for wound healing in diabetic patients (Folkman et al., 1991). Various drugs used against angiogenesis related diseases are having its own side effects, lack of specificity and affecting normal tissue along with diseased. Hence scientists are in search of natural products to treat the diseases. In this present investigation we have tried to unveil the angiogenesis potential of aqueous leaf extract of *O. sanctum* by using chorioallantoic membrane (CAM) assay.

*O. sanctum* has been used since many years in Ayurveda for its different healing properties. The sacred Tulsi is renowned for its religious and spiritual sanctity. It is used traditionally in India to cure bronchitis, malaria and arthritis. It is having anticancer, antidiabetic, antifungal, analgesic and antispasmodic activity (Ali and Dixit, 2012).

**MATERIAL AND METHODS**

**Preparation of extract**

The plant was properly identified and the leaves were collected from Sangli district, Maharashtra (India). These were washed with distilled water, shed dried, mechanically powdered, strained through muslin cloth and extracted in distilled water. The yield of aqueous extract was 12.80%. The concentrated solution of known concentration was prepared and stored as stock solution. At the time of treatment the desired concentration was prepared.
in dextrose with normal saline (DNS) purchased from Mark-Bioscience Ltd., Goa (G21730031, Exp. Dec. 2015).

**Table 1: Treatment schedule at different developmental stages of the chick embryo**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Exposure to treatment (hrs)</th>
<th>Treatment (hrs)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>III</td>
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**Chorioallantoic membrane**

For screening of aqueous leaf extract of *O. sanctum* on angiogenesis chick CAM assay was used. The fertilized eggs of Gallus gallus was purchased from local farmers sterilized by 50% ethyl alcohol and incubated in aseptic incubator at 37.5°C with 70-75% humidity. The eggs were grouped into three- 48, 72 and 96 hrs for treatment according to schedule in Table 1. Some eggs were incubated for normal development. All eggs were observed after 144 hrs incubation. According to mortality and cytotoxic study the dose of extract selected was 0.4mg/ml. The window method was used for administration of desired dose (Korn and Kramer, 2009).

**Evaluation of CAM**

The normal, sham control, DNS control and treated CAMs were evaluated morphometrically, histologically and immunohistochemically for angiogenic study. The morphometric study was carried out as described by Melkonian et al. (2002). For quantification of secondary and tertiary blood vessels branching points were taken into consideration.

The histological evaluation was made by processing of CAMs for paraffin embedding and sectioning by microtome (5µ thickness). The images were documented for further evaluation.

Immunohistochemical evaluation was done by using VEGF antibodies. The primary antibodies used are anti-VEGF polyclonal antibodies reactive against chicken. The dilution was made 1:200 in PBS at pH 7.0.

The secondary antibodies used from Jackson Immuno Research, USA. These are peroxidase-conjugated AffiniPure Goat Anti-rabbit IgG. The proper dilution was prepared in PBS at pH 7.0. The peroxidase was derived from horse radish root. After the immunohistostaining, the sections were examined and documented using photographic images.
Statistical analysis
The data was expressed in mean ± SD of 6 embryos in each experimental group. The comparative t-test was applied. The ‘p’ values were calculated with the help of XLSTAT 7.5 program. The probability ‘p’ value was calculated for given number of degree of freedom is compared with Fisher’s table (Fisher and Yates, 1938). The ‘p’ values are significant according to following conventions-
‘p’< 0.05- The difference is said to be almost significant.
‘p’< 0.01- The difference is said to be significant.
‘p’< 0.001- The difference is said to be highly significant
‘p’> 0.05- The difference is said to be insignificant.

RESULTS AND DISCUSSION
The aqueous crude leaf extract of O. sanctum was biochemically analyzed for qualitative evaluation of main groups of phytoconstituents. In the leaf extract alkaloids, flavonoids, tannin and terpenoids are present.

During angiogenesis in chick CAM, the primary blood vessels sprout out to form secondary blood vessels, which in turn give tertiary blood vessels. The influence of aqueous leaf extract of O. sanctum was studied morphometrically, histologically and immunohistochemically.

Morphometric evaluation
The normal, control and treated CAM were evaluated at the end of ED6 to quantify the number of secondary and tertiary blood vessels (Table 2). The number of secondary and tertiary blood vessels in normal CAMs was 11 and 115 respectively. The operative control CAMs were with 8-10 and 107-108 numbers and DNS control chick embryos were with 11-13 and 120-122 numbers of secondary and tertiary blood vessels respectively. In sham control CAM there was slight decrease and in DNS control there was slight increase in number of blood vessels but is not significant.

The aqueous leaf extract treated CAMs were with reduced numbers of blood vessels. The percent decrease in secondary blood vessels are 28.37, 28.38 and 35.46 at 48, 72 and 96 hrs treatment. The numbers of tertiary blood vessels are reduced to 98, 92 and 81 at 48, 72 and 96 hrs treatment [(Fig. 1 and 2) (Plate 1)].
Plate I

Effect of *Ocimum sanctum* leaf extract on angiogenesis in chick CAM:
A: normal
B: sham control
C: DNS control
D: 48 hrs treated
E: 72 hrs treated
F: 96 hrs treated
Table No.2 Effect of *O. sanctum* aqueous leaf extract on number of blood vessels in chick CAM

<table>
<thead>
<tr>
<th>Treatment (hrs)</th>
<th>Groups</th>
<th>Number of blood vessels</th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>Secondary</td>
<td>Tertiary</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Normal</td>
<td>11 ± 0.49</td>
<td>115 ± 3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham control</td>
<td>10 ± 0.63</td>
<td>108 ± 1.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNS control</td>
<td>11 ± 0.44</td>
<td>122 ± 3.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>9 ± 0.23</td>
<td>98 ± 1.22</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>Normal</td>
<td>11 ± 0.49</td>
<td>115 ± 3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham control</td>
<td>9 ± 0.23</td>
<td>107 ± 1.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNS control</td>
<td>13 ± 0.54</td>
<td>120 ± 3.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>8 ± 0.34</td>
<td>92 ± 1.61</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>Normal</td>
<td>11 ± 0.49</td>
<td>115 ± 3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham control</td>
<td>8 ± 0.28</td>
<td>108 ± 1.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DNS control</td>
<td>13 ± 0.54</td>
<td>122 ± 3.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>8 ± 0.28</td>
<td>81 ± 2.49</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± S.E. of 6 embryo

P-values- a < 0.05, b < 0.01, c < 0.001 vs. Normal embryos.

p < 0.05, q < 0.01, r < 0.001 vs. Sham control embryos.

x < 0.05, y < 0.01, z < 0.001 vs. DNS control embryos.
Histological evaluation
The normal CAM is with outer ectoderm, inner endoderm and middle mesoderm. All these are formed from extraembryonic chorion and allantois. The mesoderm is enriched with blood vessels open to ectoderm by capillary plexus near the shell membrane for the gas exchange. In sham control and in DNS control CAMs, the numbers of capillary plexus are slightly decreased and increased respectively. That decrease and increase is insignificant.

The treated CAMs are with less numbers of capillary plexus and increased thickness of the mesoderm. The ectoderm is found to be separated from mesoderm due to increased thickness of the mesoderm (Plate 2).

Immunohistochemical evaluation
The normal, DNS control and treated CAM sections were treated with immunoperoxidase staining for VEGF. The DAB staining was used with the help of endogenous peroxidase activity as described in Material and Methods.

- In normal CAM, the VEGF staining was observed at ectodermal and endodermal region (Plate II, D). Though the staining was diffused, it was reported at cellular boundaries in the mesodermal cells.
- The staining intensity was slightly increased in DNS treated CAM (Plate II, E).
- The CAM treated with crude aqueous leaf extract of O. sanctum showed significant decrease in staining intensity (Plate II, F).

During development, the blood vessels in the embryo are formed by the process of vasculogenesis. It is the process of differentiation of endothelial cells (ECs) from mesoderm and their coalescence into primary blood vessels (Risau, 1997). Hence the phytoconstituents present in the leaf extract of O. sanctum are having angiostatic effect.

VEGF is the angiogenic key factor in the process of angiogenesis. The immunoperoxidase staining was decreased significantly after treatment of aqueous leaf extract of O. sanctum. The VEGF is the potent diffusible molecule which plays an important role in the regulation of physiological and pathological growth of blood vessels (Plouet et al., 1989). The main phytoconstituents in the O. sanctum are eugenol, ursolic acid, linol, linolic acid, vacenin and ocimarin (Fulda, 2008). The antiangiogenic property of O. sanctum was also shown by Shah et al. (2014 and 2015). Single antiangiogenic agent in the drug is with limited efficiency in
preventing the development of blood vessels. Natural drugs contain a range of complex organic chemicals that may have synergistic activity.

As the leaf extract is having antiangiogenic property it can be used to cure the cancer like diseases. Though we have unveiled its antiangiogenic property, pre-clinical trials are required to decide the desired dose.

REFERENCES