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Full Length Original Research Paper

Efficiency of *Ocimum sanctum* Linn. Leaf extract on Angiogenesis

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Abstract:
Ocimum sanctum, a holy plant is used by many traditional medical practitioners for various diseases in day to day life. This holy plant- Tulsi is used in the present investigation for study of its angiogenesis efficiency. The effect of acetone extract of *O. sanctum* leaves was studied by using chick chorioallantoic membrane (CAM) assay in ovo. The angiogenesis was studied after 48 hrs, 72 hrs and 96 hrs treatment chick embryos after day 6. The morphometry and histology was studied during this investigation. There was notable reduction in number of secondary and tertiary blood vessels along with reduction in their diameter as compared to that of in normal CAM. It is due to inhibition of angiogenic factors or due to cellular apoptosis. Angiostatic property of acetone extract of leaves support anti-cancerous ethnomedicinal property of this plant and paves the foundation to synthesize the drug against tumor.

Keywords: *Ocimum sanctum* (Tulsi), Angiogenesis, CAM, Cancer.

Introduction:

Angiogenesis is the growth of blood vessels from existing vasculature. It starts during organogenesis and continued up to death. It is normal process in adult during wound healing, growth and for action of female reproductive organs. It is highly regulated process controlled by angiogenic switches. Its imbalance leads to pathological conditions. Over proliferation of blood vessels is observed in cancer, psoriasis, arthritis, obesity, asthma and arthrosclerosis. Reduced angiogenesis leads to heart and brain ischemia, neurodegeneration and respiratory distress. Hypoxia is the main stimulant for angiogenesis. The sprouting angiogenesis is the multistep process that involves serial steps - release angiogenic factors, activation of endothelial cells (ECs), proteases are released to dissolve the basement membrane, ECs migration and proliferation, sprouting of blood vessel, dissolution of extracellular matrix, loop formation and stabilization of blood vessel by pericytes.

The complete process is controlled by the switches- angiogenic factors. Different angiogenic inducers have been identified including members of fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor (TGF), platelet derived growth factor (PDGF), tumor necrosis factor (TNF), interleukins, chemokines and angiopoietin. Angiogenic inhibitors are angiostanin, endostanin, endostanin, thrombospondin etc. The 'angiogenic switches' term implies the balance between these angiogenic and angiostatic factors. Endothelial sprouting is the basic mechanism for tumor vascularisation and controlled by these factors.

Nowadays major advances have been made in the field of angiogenesis, including elucidation of the signaling pathways of several angiogenesis

factors and discovery of several natural and synthetic angiogenesis stimulators and inhibitors leading to formulation of experimental drugs into clinical field.`

The researchers are paying more attention to complementary or alternative medicines for cancer treatment from medicinal plants due to its low cost, fewer side effects and easy availability. Tulsi is most sacred plant in India. There is no plant in the world with such universal respect, adoration and worship from people as does Tulsi. It is the plant par excellence. Different parts of plant are used in Ayurveda for prevention and cure of many illnesses and everyday ailments like common cold, headache, cough, flue, earache, fever, colic pain, sore throat, bronchitis, asthma, hepatic disease, insomnia, arthritis, digestive disorders, night blindness and influenza [1]. Anticancer activity of *O. sanctum* has been proven and cited by several investigators [2,3].

The phytochemical composition of *O. sanctum* is very complex. It contains vitamin- A, C and E, minerals- calcium, phosphorous, chromium, copper, iron, nickel and zinc [4]. The leaf oils are- eugenol, euginal, urosolic acid, cravacol, linalool and sitosterol [5]. The secondary metabolites are alkanoids, steroids, tannins, flavinoids, resins and fatty acids [6].

Tumor angiogenesis is a fast growing domain in oncology. Tumors can grow to size of approximately 1- 2 mm^3 before their metabolic demands are restricted due to diffusion limit of oxygen and nutrients. In order to grow beyond this size, blood vessels are developed in tumor from surrounding blood vessel. This process is regulated by variety of pro- and anti-angiogenic factors and prerequisite for further growth of tumor [7].

The flavinoids- orientin and vicenin from *O. sanctum* showed radioactive protective effect against radiation during cancer treatment [8] due to its antioxidant activity [9]. The essential fatty acids- linoleic and linolenic acids having anti-hypertensive, cardio protective activity [5] as well as anti-inflammatory, anti-arthritic activity [10]. The eugenol present in the leaves extract suppresses cancer by inhibiting metabolic activation of carcinogens [11].

Though ethnomedicinal properties *O. sanctum* was screened in detail, its role in angiogenesis was not studied. During this investigation we have used acetone extract of *O. sanctum* to study its anti-cancerous effect on angiogenesis by using chick CAM assay *in ovo*. There are several assays to study angiogenesis – matrigel plug, corneal angiogenesis, hind limb ischemic, aortic ring etc. We have used chick CAM assay as it is readily accessible, eggs are inexpensive and results can be quantified rapidly with minimum equipments allowing large scale screening.

MATERIAL AND METHODS:

1. Preparation of Ocimum sanctum leaves extract:

Properly identified leaves of *O. sanctum* were collected from local area of Sangli district, Maharashtra, India. These were washed and cleaned in distilled water. The leaves are shade dried, powdered mechanically and strained through muslin cloth. Twenty grams of powder was extracted in acetone. The extract was concentrated by evaporation using high speed vacuum evaporated (Buchi type). The yield of extract was 1.85%. The powder of extract was dissolved in acetone to make stock solution of known concentration. At the time of treatment it was dissolved in dextrose with normal saline (DNS) was purchased from Mark- Bioscience Ltd, Goa (G21730031, Exp. Dec. 2015). DNS is the

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medicated saline used to prepare proper concentration of acetone extract.

2. Chorioallantoic Membrane (CAM) Assay (in ovo):

CAM assay was used for screening the effect of acetone extract of *O. sanctum* leaves on angiogenesis was performed by window method. Fertilized eggs of *Gallus gallus* were purchased from local farmers. These were properly sterilized and incubated in aseptic incubator adjusted at 37.5°C with 70-75% relative humidity. The eggs were divided into four groups as- normal, sham controlled, DNS controlled and acetone extract treated. The treatment was given at 48 hrs, 72 hrs and 96 hrs of incubation. The development is continued up to 144 hrs of incubation. After day 6 the CAM was evaluated.

The dose was selected on the basis of mortality, abnormality and toxicity study. After completion of scheduled time the eggs were treated according to Table 1.The window method was used for administration of desired dose [12].

The windows were prepared by removing shell at broad end in aseptic condition. 0.5 mg/ml acetone extract of *O. sanctum* leaves was spread on CAM in DNS. The window was sealed with medicated tape in experimental group. One group of eggs was incubated as normal. The embryos of operated control group were sham operated and other group was with administration of 1 ml DNS as a control. All eggs were incubated for 144 hrs.

3. Evaluation of CAM angiogenesis:

The CAM evaluation was made by measuring CAM area with some modifications, which was described by [13]. The CAM area was calculated-

Area = (1/2 A) x (1/2B) x π, where A-longest length, B- longest width and π = 3.14.

For morphometric study number of secondary and tertiary blood vessels was counted manually on computer, taking into consideration the bifurcation points.

The CAM was studied morphometrically as well as histologically. For histological preparation the CAM was fixed in calcium acetate formalin (CAF) fixative. After paraffin embedding sections were cut at 5µm thickness with the help of rotatory microtome.

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

Groups	Exposure to treatment in hrs.			Treatment in hrs
	48	72	96	
I	-	-	√	48
II	-	√	-	72
III	√	-	-	96

4. Statistical Analysis:

The data was expressed in Mean ± SE. The statistical significance between groups was analyzed by using one-way ANOVA. The values of $p < 0.05$, $p < 0.1$ and $p < 0.001$ were considered as significant.

RESULTS AND DISCUSSION:

In the present investigation we have studied the efficiency of acetone extract of *O. sanctum* leaves on angiogenesis. For this study chick CAM assay has been used. Cancer biologists, developmental biologists and ophthalmologists have described chick CAM as a model for studying development [14], cancer behavior [15], properties of biomaterial [16], angiogenesis [17] and photodynamic therapy [18].

After treatment at three stages, the developing embryos were observed and studied morphometrically and histologically at 144 hrs of incubation.

1. Morphometry:

During the present investigation the morphometric study was done as described by [13].

At 48 hrs., 72 hrs and 96 hrs there was very slight decrease in vasculature in sham operated embryos. At the same time DNS controlled embryos showed marginal increase in neo-vasculature. The number of secondary blood vessels showed marginal decrease at 48 hrs and 72 hrs of treatment as compared to controlled embryos, but significant decrease was noticed after 96 hrs of treatment. More significant decrease in number of tertiary blood vessels was observed after 48 hrs of treatment, while significant decrease in its number was recorded after 72 hrs of treatment. Highly significant decrease in number of blood vessels was observed after 96 hrs of treatment (Table 2 and Fig. 1-3). More significant decrease in CAM area was observed after 48 and 72 hrs of incubation, while highly significant decrease was reported after 96 hrs of treatment. During the present investigation, normal, sham controlled and after treatment, all embryos showed the same branching pattern of angiogenesis. There were 25.5%, 38.46% and 45% decrease in number of secondary blood vessels after 48hrs, 72 hrs and 96hrs of treatment respectively. The tertiary blood vessels were decreased by 10%, 12.60% and 21.90% after 48 hrs, 72 hrs and 96 hrs of treatment respectively. The percent inhibition of CAM area was 11.37%, 10.61% and 12.34% after 48 hrs, 72 hrs and 96 hrs of treatment respectively (Plate-I).

Table 2: Acetone extract of *Ocimum sanctum* and profile of number of blood vessels and area of chick CAM

Treatment (hrs)	Groups	No. of blood vessels		CAM area (sq.cm)
		Secondary	Tertiary	
48 (0.5mg/ml)	Normal	11±0.003	156±.565	23.8±0.12
	Sham control	10±0.046	149±0.336	21.38±0.376
	DNS control	12±0.748	158±0.783	23.31±0.274
	Leaf extract treated	7±0.282	142±0.730^{bry}	20.66±0.306^{cz}
72 (0.5mg/ml)	Normal	12±0.894	156±0.496	23.28±0.190
	Sham control	10±0.783	140±0.565	22.38±0.110
	DNS control	13±0.800	159±0.730	27.0±0.222
	Leaf extract treated	8±0.454^{cqy}	139±0.894^{cy}	20.87±0.263^{cr}
96 (0.5mg/ml)	Normal	12±0.469	155±0.658	23.60±0.087
	Sham control	11±0.658	135±0.522	22.60±0.105
	DNS control	12±0.594	161±0.594	23.50±0.034
	Leaf extract treated	6±0.398^{cz}	126±0.611^{cz}	20.00±0.402^{cpx}

(Results expressed as mean ± S.E. of 6 embryos. 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).

2. Histology:

For histological study T. S. of CAM was studied after H-E staining as described in material and methods. Histological sections of normal CAM have shown that the capillary plexus formed below ectoderm by migration of mesodermal blood vessels to ectoderm. Some of the mesodermal blood vessels attach to ectoderm near the capillary plexus. There is continuity of blood vessel endothelial cells as shown in plate II.

T. S. of normal CAM showed more capillary plexus as compared to that in sham controlled CAM.

Slight increase in number of blood vessels along with capillary plexus was observed in DNS controlled CAM. After the treatment with acetone leaf extract thickness of CAM was decreased. The treated CAM in histological sections showed decreased capillary plexus.

Formation of capillary plexus was observed in developing embryo in serial sections during day 5 to day 6. CAM consisted of ectodermal and endodermal layers with intervening mesoderm that contained blood vessels. Vessels are considered part of capillary plexus if they are immediately adjacent to the ectoderm. The number of plexus vessels increased by day 6 [13]. The same findings were observed in the normal chick embryo CAM sections, during this investigation. According to Melkonian [13], suramin and cytochalasin, the angiostatic substances decrease the thickness of CAM as well as inhibit angiogenesis. Treated CAM at day 6 showed less number of capillary plexus near the ectoderm. The present investigation also showed same findings after treatment of *O. sanctum* extract (Plate III).

This angiostatic property of *O. sanctum* extract may be used to cure cancer. Cancer continues worldwide killer despite of great advances made in modern medicines during the past decades. Nowadays radiotherapy and chemotherapy are two important treatments for malignancy, but having ample of side effects. Andreas Moritz [19] in his book `cancer is not a disease' has quoted experienced oncologists professor, Dr. Jones who said "my studies have proven conclusively that cancer patient who refuse chemotherapy and radiation, actually live up to four times longer than treated cases, including untreated breast cases."

One of the important photochemical of *O. sanctum* is eugenol induce apoptosis via the mitochondrial pathway by modulating the Bcl-2 family proteins, Apaf-1, cyto-C and caspases, inhibiting invasion as well as angiogenesis as evidenced by changes in activities and expression of VEGF and VEGF-1. Eurosolic acid and oleanlic acid possess anticancer activity. There is reduction in tumor size and increase life span of mice having Sarcoma-180 solid tumors [20]. However individual active compounds are less potent than the total herbal extract from which they are isolated [21]. According to Manikandan *et al* [22] eugenol is an attractive candidate for preventing tumor progression. Along with eugenol, eurosolic acid and oleanlic acid must be taken into consideration.

CONCLUSION:

The scientists with innovative foresight are looking forward to have an alternative or complementary natural medicine with devoid of deleterious side effects caused to cancer patients during treatment. Scientific studies proved that *O. sanctum* could be effective in treating several cancers. The challenge is to develop drug with suitable pharmacokinetics and toxicity profiles to test this hypothesis in clinical trials. It is necessary to define molecular basis and pathways of angiostatic activity of *O. sanctum* leaf extract in a more integrated manner so as that the excitement of the science can be converted into development of an efficient and safe therapies.

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Fig.1
Acetone extract of *Ocimum sanctum* and profile of number of blood vessels in chick CAM

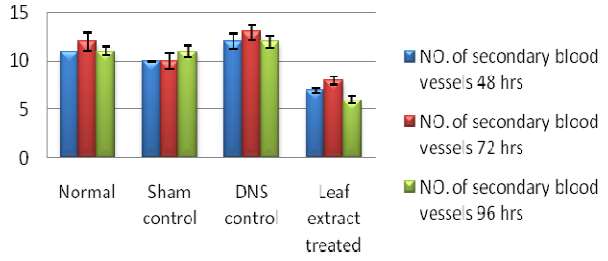


Fig.2
Acetone extract of *Ocimum sanctum* and profile of number of blood vessels in chick CAM

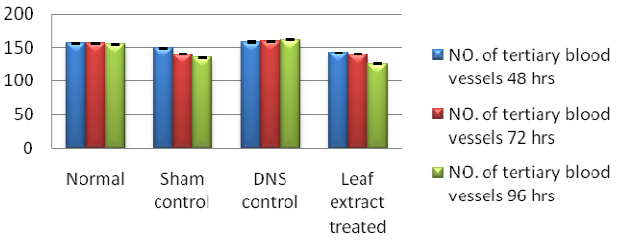


Fig. 3
Acetone extract of *Ocimum sanctum* and profile of CAM area in chick

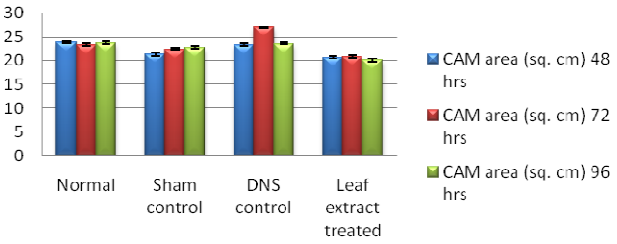
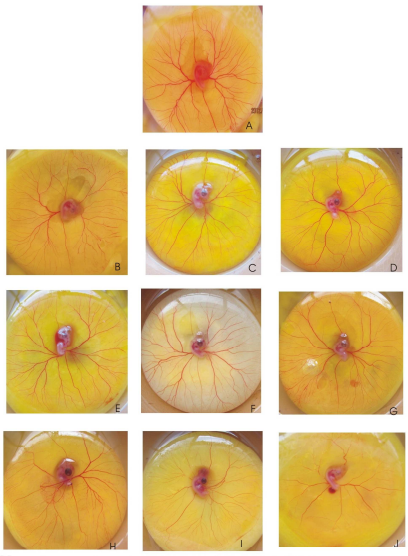
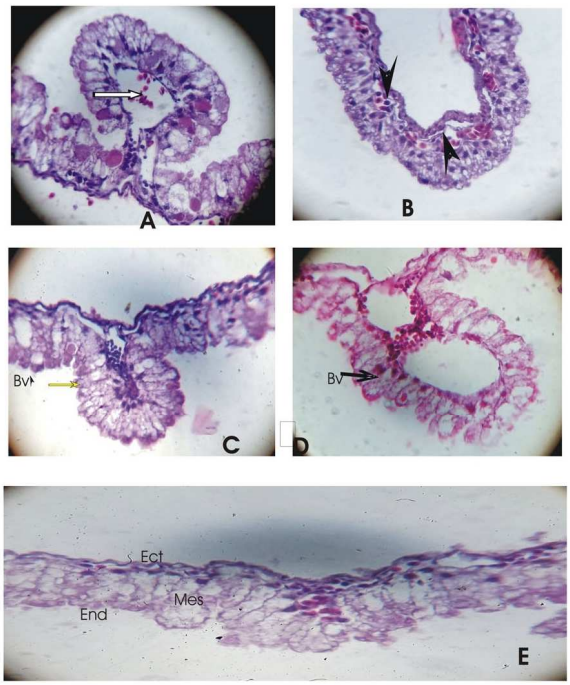


Plate- I
Angiostatic effect of acetone extract of *O. Sanctum* leaves on chick CAM



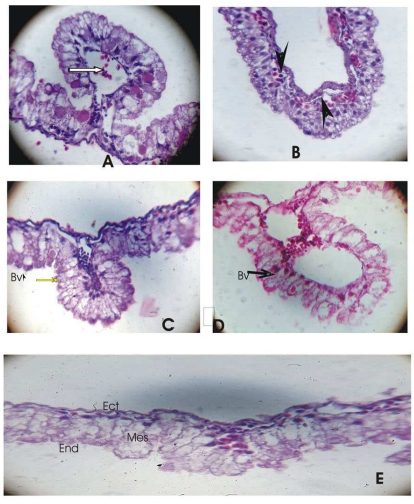
Vasculogenesis and angiogenesis in chick CAM
A- Normal CAM
B to D- Sham controlled CAM (after 48hrs, 72 hrs and 96 hrs)
E to G- DNS controlled CAM (after 48hrs, 72 hrs and 96 hrs)
H to J- Acetone extract of *O. Sanctum* leaves treated CAM (after48hrs, 72 hrs and 96 hrs)

T. S. Of chick CAM showing angiostatic effect of *O. Sanctum* leaves extract



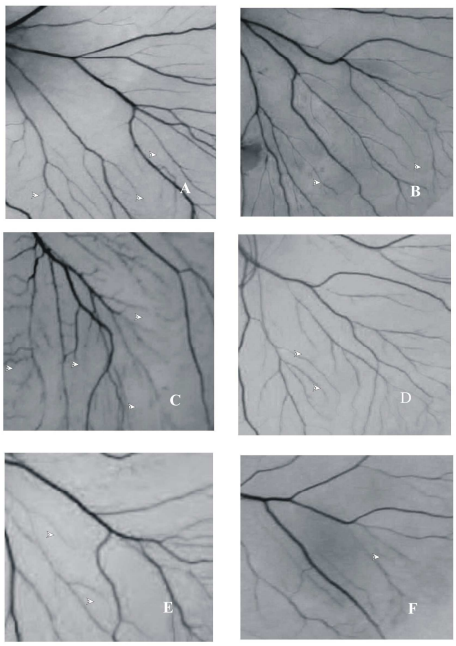
T. S. Of chick CAM(400X)
A- Normal CAM showing blood vessel and blood cells
B- Normal CAM to show capillary plexus.
C- Sham controlled CAM (after 96hrs treatment)
D- DNS controlled CAM (after 96 hrs treatment)
E- Treated with acetone extract of *O. Sanctum* leaves (after 96 hrs of treatment)
Arrow- Blood cells
Arrow head- capillary plexus
Ect- ectoderm
Mes- mesoderm
End- endoderm

T. S. Of chick CAM showing angiostatic effect of *O. Sanctum* leaves extract



T. S. Of chick CAM(400X)
A- Normal CAM showing blood vessel and blood cells
B- Normal CAM to show capillary plexus.
C- Sham controlled CAM (after 96hrs treatment)
D- DNS controlled CAM (after 96 hrs treatment)
E- Treated with acetone extract of *O. Sanctum* leaves (after 96 hrs of treatment)
Arrow- Blood cells
Arrow head- capillary plexus
Ect- ectoderm
Mes- mesoderm
End- endoderm

Plate III
Inhibition of capillary plexus due to *O. Sanctum* leaf extract



Piece of chick CAM showing capillary plexus
A- Normal
B- Sham controlled
C- DNS controlled
D to F- Treated (48, 72, 96 hrs.)
Arrow head showing capillary plexus.

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