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Keywords: *ocimum sanctum, angiogenesis, cam, cancer.*

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Influence Evaluation of *Ocimum Sanctum* Leaf Extract on Angiogenesis by using Chick Chorioallantoic Membrane (CAM) Assay

U. H. Shah [α], G. R. Gonjari [σ] & A. E. Patil [ρ]

Abstract- *Ocimum sanctum* is a sacred herb of India is also known as 'Tulsi' or 'Holy Basil'. There are several medicinal properties attributed to leaf, bark, root as well as seeds of *O. sanctum*. This holy plant is used in the present investigation to study its angiogenic efficiency. The effect of methanolic 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 of on chick CAM after day 6. The CAM was studied morphometrically and histologically. There was highly significant decrease in number of secondary and tertiary blood vessels as well as in total CAM area.

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I. Introduction

During development of vascular system two processes are involved- vasculogenesis and angiogenesis. Vasculogenesis is the process of blood vessel formation from angioblasts while neovascularisation from pre-existing blood vessel is angiogenesis. Angiogenesis is the rapid process up to organogenesis. In adults it is slow but plays very important physiological role during wound healing and reproduction. The process of angiogenesis is highly balanced and regulated by angiogenic and angiostatic factors- vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF), angiogenin, tumor necrosis factor (TNF). These factors act as switches to control angiogenesis, if imbalanced leads to pathological conditions. Excessive angiogenesis occurs in cancer, psoriasis, arthritis, diabetic blindness, asthma and atherosclerosis. Reduced angiogenesis is observed in heart and brain ischemia, neurodegenration, hypertension and respiratory distress [1]. For controlling all these pathological conditions, pro- and antiangiogenic therapies have been developed. There are various assays have been developed to study angiogenesis. The mostly used assay is chick chorioallantoic assay. It is easiest and cheapest angiogenesis assay.

In this present investigation we have used methanolic leaf extract of O. sanctum (Tulsi) to study its angigenic or angiostatic potential. *O. sanctum*, a queen of herbs is one of the holiest and health giving herb in India and subcontinent. The medicinal use of *O. sanctum* is as old as human. In ayurveda it is known as 'elixir of life' and commonly used against headaches, common cold, and soared throat. Ethanolic extract of *O. sanctum* decreases glucose level and increase glycogen in strreptozotocin induced diabetic rats [2] and having normal wound healing as well as dexamethesone depressed wound healing by fast epithelialization and wound contraction [3].

Aqueous extract of *O. sanctum* possesses radio-protective effect by reduction in rapid peroxidation in both kidney and salivary glands of rat [4]. The extract of *O. sanctum* having different pharmacological activities like- analgesic, antiulcer, antidepressant, anti-anxiety, anti-tussive, anti-thyroidic, anti-stress, anti-spasmodic, anti-pyretic and anti-plasmodial [5] . Prashar et al have reported that *O. sanctum* leaf extract having blocking effect of chemical carcinogenesis [6].

Though there were many reports explaining different ethnomedicinal properties of *O. sanctum,* its efficiency on angiogenesis has not been studied. Hence this investigation is aimed for screening the properties of methanolic leaf extract of *O. sanctum* on angiogenesis by using chick CAM assay.

II. Materials and Methods

a) *Preparation of extract*

The plant was properly identified and the leaves were collected from local area of Sangli district from Maharashtra (India). These were washed with distilled water, shed dried, mechanically powdered, strained through muslin cloth and extracted in methanol. The yield of methanol extract was 3.41%.The concentrated solution of known concentration was prepared and stored as stock solution. At the time of treatment was dissolved in dextrose with normal saline (DNS) was purchased from Mark-Bioscience Ltd, Goa (G21730031, Exp. Dec. 2015). DNS is the medicated saline used to prepare proper concentrations of the extract for treatment.

b) *Chorioallantoic membrane assay*

For screening the effect of methanolic extract of *O. sanctum* on angiogenesis chick CAM assay was

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used. The fertilized eggs of *Gallus gallus* was purchased from local farmers, sterilized by 50% alcohol and incubated in aseptic incubator at 37.5^{0}C with 70- 75% humidity. The eggs were grouped three groups- sham control, DNS control and treared. These are again sub 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 of incubation. According to mortality and cytotoxic study the dose of extract selected was 0.4 mg/ml. The window method was used for administration of desired dose [7]. The leaf extract was administrated in DNS, sealed and incubated up to day 6.

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

i *Evaluation of CAM angiogenesis*

The morphometric evaluation was made as described by Meloknian [8]. The CAM area was calculated-

Area= (1/2 A) X (1/2 B) X π

Where A is longest length, B is longest width and π= 3.14

Morphometric study of number of secondary and tertiary blood vessels were counted manually on computer image by counting branching points [9]. Histological evaluation of CAM was made by processing of CAM for paraffin embedding and sectioning. The sections were cut at 5 μm thickness with the help of rotator microtome.

c) Statistical analysis

The data was expressed in mean ± SE. The statistical analysis between the groups was made by one way ANOVA. The value of $p< 0.05$, $p< 0.01$ and $p<0.001$ were considered as significant.

III. Results and Discussions

In this present investigation we have studied effect of acetone methanol extract of O. sanctum leaf by using CAM assay. The chick CAM assay is ideal model to study angiogenesis [10]. After treatment at different developmental stages the chick embryos were evaluated morphometrically and histologically at 144 hrs of development.

Table 2 : Morphometric evaluation of chick CAM after treatment of *Ocimum sanctum* leaf extract

Treatment (hrs)	Groups	Number of blood vessels		CAM area(sq.cm)
		Secondary	Tertiary	
48 (0.4mg/ml)	Normal	11 ±0.489	113±3.162	26.50±2.4
	Sham control	10±0.632	110±3.149	26.00±2.46
	DNS control	11±0.439	117±3.915	28.00±1.65
	Leaf extract treated	9±0.436	98±1.183	22.93±1.73
72 (0.4mg/ml)	Normal	11±0.510	115±2.9	25.98±2.5
	Sham control	10±0.461	108±1.994	25.50±2.56
	DNS control	12±0.616	120±3.055	29.50±2.38
	Leaf extract treated	9±0.439	88±1.825	20.48±1.73
96 (0.4mg/ml)	Normal	12±0.50	115±2.89	26.50±2.01
	Sham control	9±0.754	108±3.438	24.00±2.5
	DNS control	12±0.461	120±2.983	30.50±2.66
	Leaf extract treated	8±0.336	77±2.983	18.32±2.016

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

a) Morphometric evaluation

The morphometric evaluation was made by considering branching points to differentiate primary, secondary and tertiary blood vessels. There was very slight decrease in angiogenesis in sham operated embryos and very marginal increase in DNS controlled embryos as compare to that of normal. The significant decrease in number of primary and secondary blood vessels was observed at 48 hrs treatments- 15.84% and 14.9% respectively. More significant decrease in number of blood vessels was observed at 72 hrs of treatment (34.3% and 43.3%respectively) was reported. Total area of CAM was decreased 18.10% at 48 hrs treatment, 30.57% at 72 hrs treatment while 39.93% at 96 hrs treatment of methanolic extract of *O. sanctum* (Plate I, Table 2 and Fig. 1-3).

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b) Histological evaluation (Plate II)

For histological evaluation T. S. of CAM was studied as described in materials and methods. Histologically CAM consisted of mesodermal cells intervening between ectodermal and endodermal layers. There is presence of capillary plexus near the ectoderm. Formation of capillary plexus was studied by Melkonian et al [13]. The undifferentiated mesenchymal cells are forming capillary plexus. The blood vessel associated with capillary plexus is considered as part of capillary plexus. Normal CAM is with numerous mesodermal blood vessels with capillary plexus by day 6. The considerable inhibition of blood vessels associated with capillary plexus in extract treated embryos by day 6. The thickness of CAM was also decreased in treated CAM. Sham controlled CAM was with slight decrease in blood vessels and capillary plexus while in DNS controlled CAM was with slight increase in number of capillary plexus with main blood vessel.

According to Melkonian number of capillary plexuses was decreased in CAM treated with cytochalasin D and suramin which inhibit angiogenesis. The same findings were observed in the present investigation. There was decrease in CAM thickness indicates that metanolic extract of *O. sanctum* inhibit angiogenesis.

This antiangiogenic property of *O. sanctum* can be used for antiangiogenic therapy against cancer. No tumor can develop without angiogenesis to meet increasing demand of food and oxygen. Due to severe side effects and non targeting killing of neoplastic cells by chemotherapy, scientists are in search of alternative medicines. For safer and effective approach of antiangiogenic drugs, the herbalists are trying to search such drugs from herbs. Morphin was the first drug isolated from plant, Papaver somniferum in 1805. According to Manikandan et al [14] there is differential sensitivities of gastric carcinoma and normal stomach tissue to growth control and apoptosis induction by ethanolic o. sanctum leaf extract. Administration of ethanolic leaf extract selectively induces apoptosis in MNNG- treated rats but not in normal rats. The *O. sanctum* contains phytochemicals like- eugenol, ursolic acid, carvacrol, apigenin, luteolin and carvacrol. Eugenol, ursolic acid and cavacrol have been repoeted to inhibit cell proliferation in vitro [15]. Eugenol, ursolic acid and apigenin were found to induce apoptosis by influencing Bcl-2/ax ratio and cytochrome C mediated caspase 3 activation [16]. Present investigation supports the above findings. This extract is having combination of different phytoconstitutents which acts together to reduce sprouting angiogenesis. This antiangiogenic property of *O. sanctum* was also reported of acetone leaf extract [9].

Hence antiangiogenic property of *O. sanctum* may be aimed to halt new blood vessel growth to treat disease like cancer, diabetic blindness, rheumatoid arthritis etc. This therapy will starve the tumor for oxygen and nutrients.

IV. Conclusion

O. sanctum is used traditionally in our country and called 'Elixir of Life'. However the leaf extract have to be screened by ethno pharmacological research. As it is promising plant for anticancer therapy, extensive investigations on the metabolism, pharmcodynamic interactions of individual metabolism as well as molecular mechanism has to be studied.

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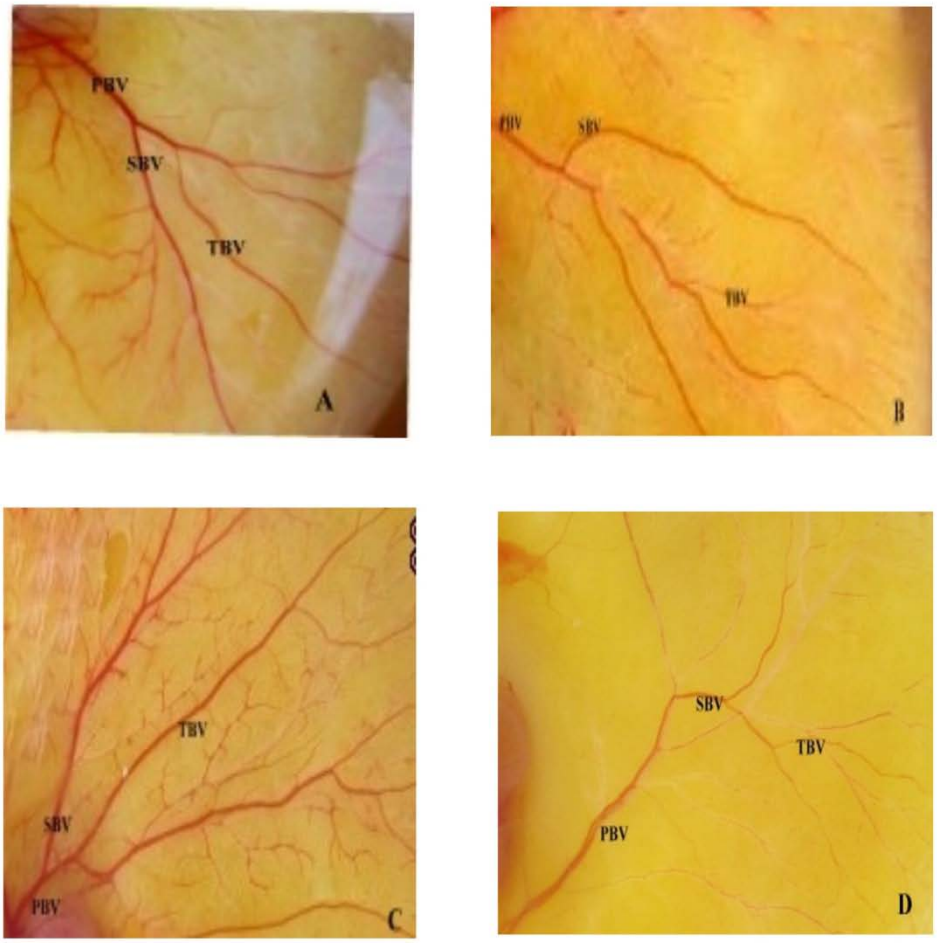
Plate I



Morphometric evaluation of chick CAM for angiogenesis

A- Piece of normal CAM
B- piece of sham control CAM
C- Piece of DNS control CAM
D- Piece of *O. sanctum* leaf extract treated CAM
PBV- primary blood vessel
SBV- secondary blood vessel
TBV- tertiary blood vessel

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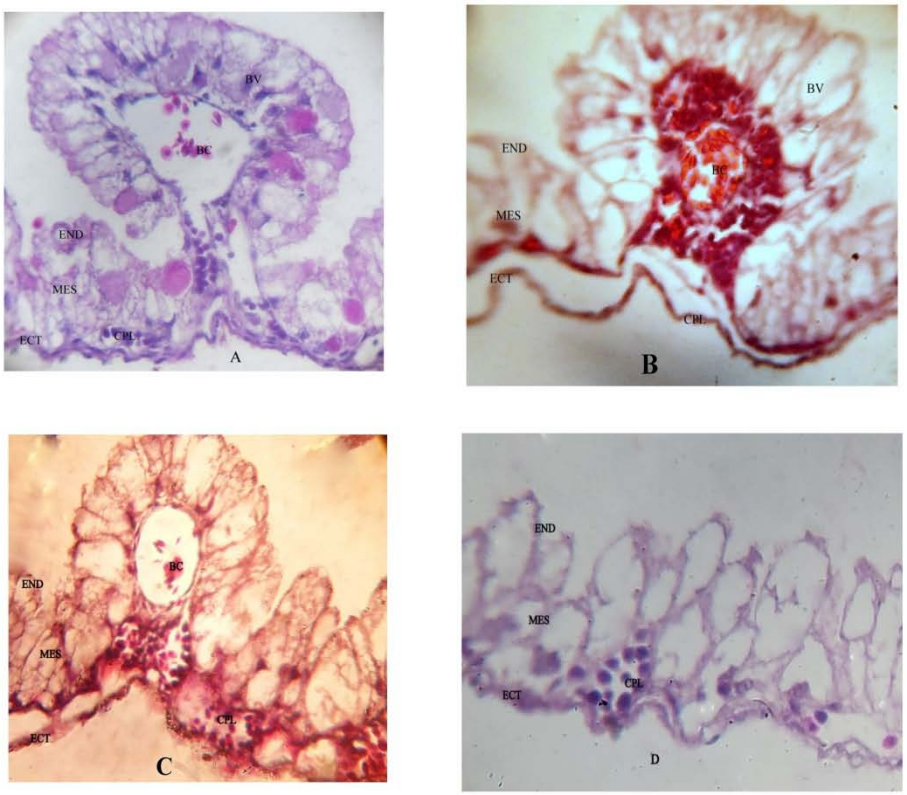
PlateII



Histological evaluation chick CAM for angiogenesis

T. S. of chick CAM
A-Normal CAM
B-Sham control CAM
C- DNS control CAM
D- *O.sanctum* leaf extract treated CAM
BV- blood vessel
CPL- capillary plexus
ECT-ectoderm
END- endoderm
MES- mesoderm

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Fig. 1
Effect of *O. sanctum* leaf extract on number of blood vessels in chick CAM

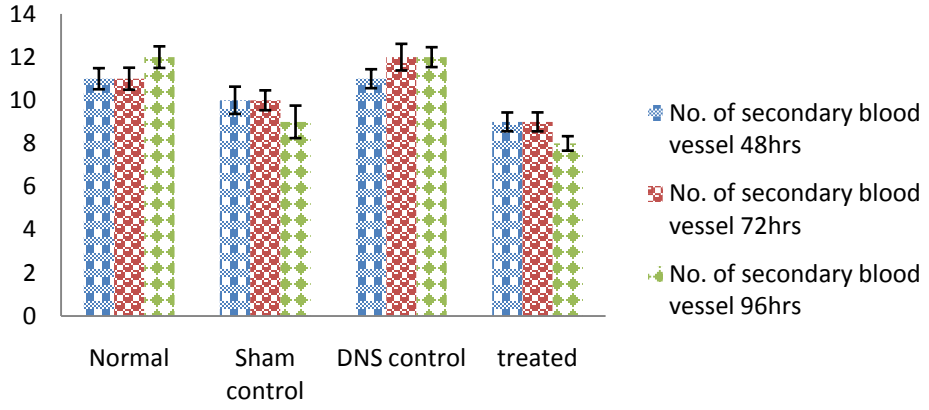


Fig. 2
Effect of *O. sanctum* leaf extract on number of blood vessels in chick CAM

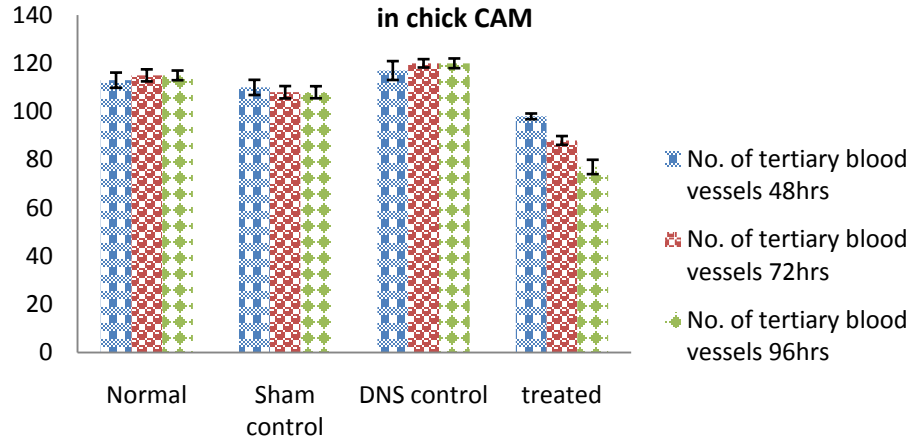
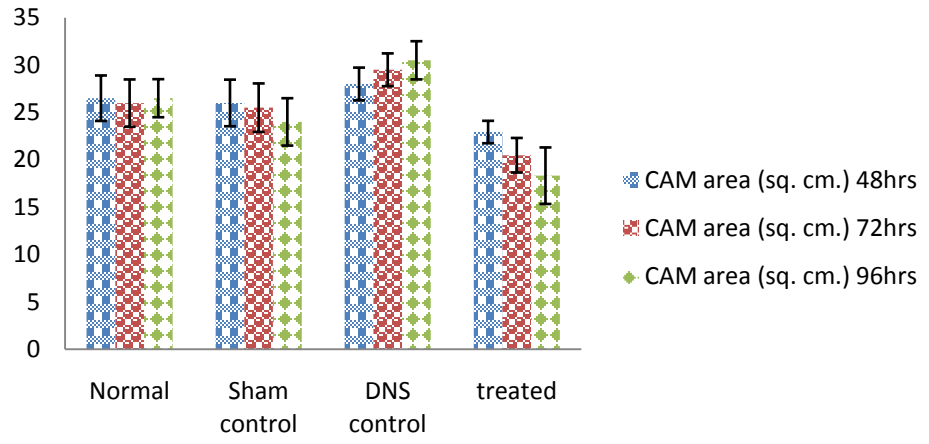


Fig. 3
Effect of *O. sanctum* leaf extract on area of chick CAM



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