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Original Research Article

Study of antiangiogenic activity of "aqueous extract of *Nigella sativa* seeds" in chick chorioallantoic membrane (CAM) model

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ABSTRACT

Background: Angiogenesis is important for the typical physiological activities such as cure from injury, menstrual cycle and embryo growth. It is also plays a crucial role in several pathological conditions in cancer. Antiangiogenesis, e.g., inhibition of blood vessel growth, is being investigated as a way to prevent the growth of tumors and other angiogenesis-dependent diseases. The chick embryo chorioallantoic membrane (CAM) is commonly used as an experimental in vivo assay to study both angiogenesis and antiangiogenesis in response to tissues, cells or soluble factors. Given the high occurrence of cancer worldwide and the major source of the discovery of new lead molecules are medicinal plants. The objective of the present research was to study the antiangiogenic property of "aqueous extract of Nigella sativa seeds" using chick chorioallantoic membrane (CAM) assay
Methods: The chick chorioallantoic membrane (CAM) assay for screening the effect of *Nigella sativa* on anti-angiogenesis was performed according to the method given by Ribatti and co-workers.
Results: The results of present study significantly increased the antiangiogenic effect on CAM by decreasing the proliferation of capillary networks in a dose (50 to 300 µg/egg) dependent manner which is probably related to the inhibition of neovascularization.
Conclusions: It is concluded that aqueous extract of *N. sativa* seeds possesses significant antiangiogenic activity, and this is a possible rationale for its folkloric use as an anticancer agent.

Keywords: Antiangiogenic activity, Chick chorioallantoic membrane assay, *Nigella sativa*, Neovascularization

INTRODUCTION

Tumor angiogenesis is the consequence of an angiogenic imbalance in which proangiogenic factors predominate over antiangiogenic factors.[1,2] Angiogenesis is the process by which new blood vessels grow.

In the adult, except for a few physiological processes such as menses, wound healing, and placental formation, all angiogenic processes are pathologic.[3-5] By blocking the development of new blood vessels, one hopes to cut off the tumor's supply of oxygen and nutrients and, therefore, its growth and spread to other parts of the body.[6-8] The current ongoing researches are approving the plant can be useful in malignancies and at various levels and with different mechanisms.[9]

There are several important plants having anticancer action like *Atocarpus obtusus*, *Blumea balsamifera*, *Boerhaavia diffusa*, *Citrus maxima*, *Calotropis procera*, *Emblica officinalis*, *Moringa oleifera*, *Oroxylum indicum*, *Vitex negundo* and *Zingiber officinale*.[10] An effort has

been made in this study to know the antiangiogenic effect and cancer preventing effect.

Nigella sativa Linn. (Family - Ranunculaceae) is a widely used medicinal plant throughout India and popular in various indogenous system of medicine like Ayurveda, Siddha, Unani and Tibb.[11] The seeds of *Nigella sativa L*. commonly known as black seeds, have been used in traditional medicine by many Asian, Middle Eastern and Far Eastern Countries to treat headache, coughs, abdominal pain, diarrhea, asthma, rheumatism and other diseases.[12,13]

The aqueous extracts of the seeds have been shown to possess antioxidant, anti-inflammatory, anticancer, analgesic and antimicrobial activities. Based on present preliminary literature study we found that there are only few reports on anticancer activity of *Nigella sativa*.[14] Further there is a paucity of information regarding on its antiangiogenic property, thus the proposed study aims to evaluate the anti-angiogenic property of "aqueous extract of Nigella sativa seeds" in chick chorioallantoic membrane (CAM) model.

METHODS

Chemicals and reagents

pyruvic acid was purchased from SD fine-chem Ltd (Mumbai) and prednisone from Mankind Pharma Pvt Ltd (Delhi). All other chemicals and solvents were of analytical grade.

Plant material and preparation of extracts

The study was conducted during the period April 2015 to June 2015. The seeds of *Nigella sativa* were obtained from a local supermarket in Udaipur (Rajasthan) and was authenticated by Department of Botany, Government Meera Girls College, Udaipur.

The seeds of *Nigella sativa* were dried in air, crushed to coarse powder and extracted with distilled water using Soxhlet apparatus.

The extract was dried under vacuum, stored at room temperature and protected from direct sunlight in the Department of Pharmacology, Geetanjali Medical College, Udaipur.

Preliminary Phytochemical screening

The freshly prepared aqueous extract of the *Nigella sativa* was subjected to preliminary phytochemical screening for detection of major chemical compounds.[15]

Experimental animals

Seven-week-old, specific pathogen-free Swiss albino mice weighing between (25-30 gm) procured from Animal House of Geetanjali Medical College, Udaipur. The animals were kept in standard laboratory condition and given free accesses to food and water. The protocol was approved by the Institutional Animal Ethics Committee (IAEC), GMCH, Udaipur.

Acute Toxicity Study

Acute toxicity study of aqueous extract of N. sativa (AENS) was determined in Swiss albino mice (25-30 gm) according to the OECD guidelines No.425.[16]

Experimental design:

Chicken egg Chorioallantoic Membrane (CAM) Assay

Antiangiogenic activity of crude plant extract of N. sativa was conducted on fertilized eggs by modified CAM assay method.[17]

Fertile white Leghorn chicken eggs (Gallus domesticus) were obtained from a local hatchery.

Inclusion criteria

- Three days old fertilized, medium sized healthy white leghorn chick embryos
- Without crack / Crack free embryos
- Experiment should be simple to perform and uniformly reproducible
- Technique should be able to predict the potential properties of standard drug
- It should consume minimal quantities of drugs.

Exclusion criteria

- More than three days old fertilized chicken embryos
- Large size embryos
- Diseased chick embryos
- Embryos with crack
- Double yolk embryos.

In the present investigation, a total of 42 eggs were taken and divided into six groups of 6 eggs each. The eggs were incubated at 37ºC in humified incubator for 48 hrs, placed in horizontal position and rotated several times.

The eggs were sprayed with 70% ethanol and air-dried to reduce contamination from the surface. Eggs were divided into the following groups -

- Group 1: Normal control (0.9% NaCl 5ml/kg)
- Group 2: Negative control (Pyruvic acid 300 µg/egg)
- Group 3: Positive control (Prednisone 5mg/ml)
- Group 4: AENS 50 µg/egg
- Group 5: AENS 100 µg/egg
- Group 6: AENS 200 µg/egg
- Group 7: AENS 300 µg/egg

Statistical analysis

The results were expressed as Mean±SD. Statistically significance was determined using one-way ANOVA followed by Dunnett's multiple comparison test. P values <0.05 were considered significant.

Physiochemical screening

The extract of *Nigella sativa* seeds for phytochemical study showed the presence of carbohydrates, alkaloids, amino acids, proteins, steroids, glycosides, flavonoids, tannins, saponins, terpenoids, polyphenolic compounds.

RESULTS

Table 1: The Average number of blood vessels before and after treatment of Aqueous extract of *Nigella sativa* on CAM model.

Group	Egg 1	Egg 2	Egg 3	Egg 4	Egg 5	Egg 6	Average no. of vessels	Egg 1	Egg 2	Egg 3	Egg 4	Egg 5	Egg 6	Average no. of vessels
Normal control	10	8	7	11	12	10	9.66	10	8	8	12	12	10	10
Pyruvic acid	8	12	10	9	8	10	9.5	11	15	12	13	10	14	12.5
Prednisone	12	9	8	10	9	11	9.83	3	3	2	4	2	3	2.83
AENS 50 µg	10	9	10	8	13	10	10	9	9	10	8	12	9	9.5
AENS 100 µg	10	11	9	10	11	9	10	7	8	7	8	7	7	7.33
AENS 200 µg	8	10	8	12	11	12	10.16	4	6	5	6	6	7	5.66
AENS 300 µg	13	9	9	10	9	8	9.66	4	3	3	4	3	2	3.16

Table 2: Anti-angiogenic effect of Aqueous extract N. sativa L. seeds on CAM model.

Group	Dosage	Percentage of vessels inhibition (%)						Percentage of inhibition (Mean ± SD)
		Egg 1	Egg 2	Egg 3	Egg 4	Egg 5	Egg 6	
Normal saline	0.9% NS	0	0	-14.28	-9.09	0	0	- 3.89 ± 6.26*
Pyruvic acid	300µg	-37.5	-25	-20	-44.44	-25	-40	- 31.99 ±9.91
Prednisone	5 mg/ml	75	66.66	75	60	77.77	72.72	71.19 ± 6.63***
AENS	50 µg	10	0	0	0	7.69	10	4.61 ± 5.12*
AENS	100 µg	30	27.27	22.22	20	36.36	22.22	26.34 ± 6.14**
AENS	200 µg	50	40	37.5	50	45.45	41.66	44.1 ± 5.24***
AENS	300 µg	69.23	66.66	66.66	60	66.66	75	67.37 ± 4.84***

Values are expressed as mean ± SD, (n=6). One-way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test. * P<0.05, *P<0.01, ***P<0.001 when compared with positive control group.

Antiangiogenic activity of Nigella sativa

The anti-angiogenic activity of aqueous extract of *Nigella sativa* seeds was examined using the CAM assay versus prednisone which was used as positive controls, whereas pyruvic acid was employed as negative control for the assay.

The 8th day old embryos after treatment for number of blood vessels and their reduction were examined. The analysis of blood vessel was based on the evaluation of angiogenesis by measuring the area of inhibition surrounding the applied disc.

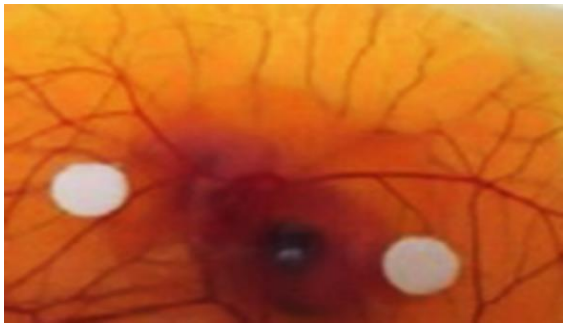

Figure 1: Effect in the control group which is treated with sterile normal saline.

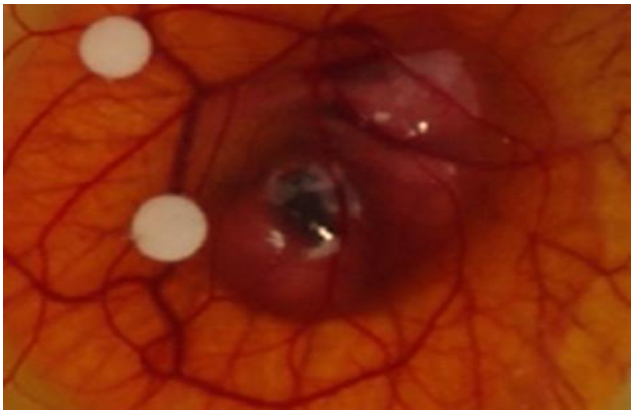

Figure 2: Angiogenesis effect of Pyruvic acid on CAM at a dose of 300 µg.

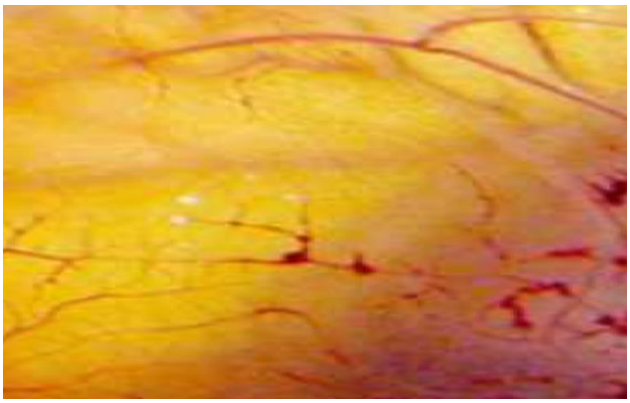

Figure 3: Antiangiogenesis effect of Prednisone 5 mg/ml on CAM.

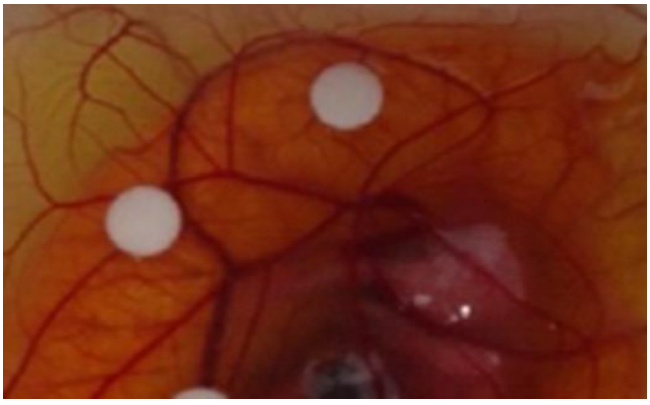

Figure 4: Antiangiogenesis effect of AENS on CAM at a dose of 50 µg/egg.

The average number of vessels in aqueous extract of Nigella sativa 50, 100, 200 and 300 µg were 9.5, 7.33, 5.66 and 3.16 where as in pyruvic acid and prednisone treated CAM was 12.5 and 2.83 respectively (Table 1).

Prednisone and aqueous extracts of N. sativa (300 µg) showed the higher antiangiogenic activities 71.17±6.63 and 67.37±4.84 respectively. The *Nigella sativa* inhibition percentage is shown in the Table 2.

A marked rise in average number of blood vessels was observed in pyruvic acid treated group as compare to normal control (normal saline).

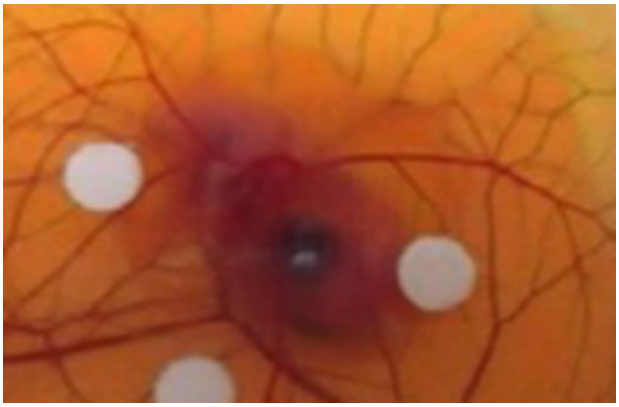

Figure 5: Antiangiogenesis effect of AENS on CAM at a dose of 100 µg/egg.

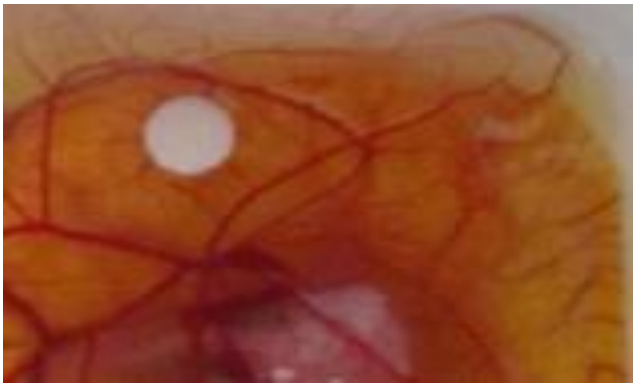

Figure 6: Antiangiogenesis effect of AENS on CAM at a dose of 200 µg/egg.

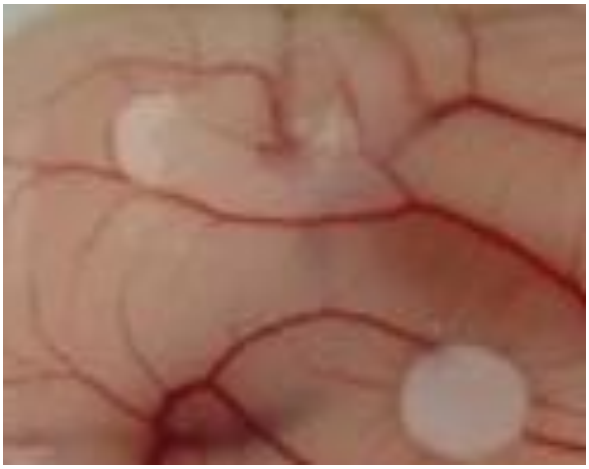

Figure 7: Antiangiogenesis effect of AENS on CAM at a dose of 300 µg/egg.

Whereas, treatment with AENS (50, 100, 200 and 400 µg/egg) caused significant reduction in the average number of blood vessels in a dose dependent manner. The

antiangiogenic effect of aqueous extract at was found similar effective to the reference standard, prednisone (Table 2). The observations were shown in the Figure 1-7.

DISCUSSION

Angiogenesis, the formation of new blood vessels from the existing vascular bed, is one of the hallmarks of cancer, playing an essential role in tumor growth, invasion and metastasis. Because of genetically instability and difference of tumor blood vessels to normal vessels, they are potential targets in therapy for all types of cancer. In many other pathological conditions, including diabetic retinopathy, inflammation, hemangiomas, arthritis, psoriasis and atherosclerosis, cancer seems to be driven by persistent upregulated angiogenesis.[20,21] Angiogenesis is a strictly controlled process in normal human body and regulated by a variety of endogenous angiogenic and angiostatic factors.[22]

The investigation is based on the need for newer antiangiogenic agents from natural source with potent activity to substitute chemical therapeutics. Realizing the fact this study was carried out to evaluate the antiangiogenic activity of *Nigella sativa* seeds extract using CAM model in this direction.

Using chick CAM model, the new pharmacological effects of *Nigella sativa* have been confirmed by the proven inhibition of angiogenesis. Authors showed that *Nigella sativa* seed extract had significant antiangiogenic activity at all doses of treatment studied (Table 2); where these extracts of *Nigella sativa* reduce neovascularization of the CAM as well as distortion of existing vasculature (Figure 1-7).

Folkman et al have reported, by blocking the development of new blood vessels, one hopes to cut off the tumor's supply of oxygen and nutrients and, therefore, its growth and spread to other parts of the body.[6-8] This may be due to the induction of apoptosis by phytochemical present in these plants.[23]

The results of the present study suggest that aqueous extract of *Nigella sativa* seeds exhibits strong antiangiogenic action in concentration dependent manner and also may have the potential to be a useful inhibitor of tumors and other angiogenesis-dependent diseases. It is due to the presence of active principles such as flavonoids and tritrepenoids may responsible for this activity. Hence, *Nigella sativa* can be used as a potent antiangiogenic agent. Nevertheless, further studies needed to substantiate these findings.

CONCLUSION

It is concluded that aqueous extract of *Nigella sativa* seeds possesses significant antiangiogenic activity, and this is a possible rationale for its folkloric use as an anticancer agent. This property of *Nigella sativa* extract might have been executed either by preventing signaling of angiogenic agents from epithelial cells or by induction of apoptosis, preventing promotional events in the CAM tissue through free radical scavenging mechanism. Further research on mechanism of action of *Nigella sativa* has to be worked out in future studies.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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