

Evaluation of Antiangiogenic Activity of *Nigella sativa* Extract by Sponge Implantation Method in Mouse Model

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ABSTRACT

Angiogenesis has crucial role in malignant tumor growth. *Nigella sativa* has been used in traditional medicine to treat many ailments like immunomodulation, anti-inflammatory analgesic etc. The present study was undertaken in order to evaluate the effect of ethanolic extract of *N. sativa* on angiogenesis in mouse model. The antiangiogenic potential was investigated by sponge implantation method, wherein significant inhibition of blood vessel formation, haemoglobin concentration and VEGF concentration were recorded. Animals (18) were divided in three groups where group I was kept as untreated, group II treated with SU5416 and group III was treated with ethanolic extract of *N. sativa*. The results were compared with positive control SU5416 and untreated group. By using Cyanmethemoglobin method the mean (\pm SE) haemoglobin concentrations (μ g/mg weight of sponge) in mice were 1.918 ± 0.087 in untreated group, 0.365 ± 0.031 in positive control group, 0.425 ± 0.037 in *N. sativa* extract treated group. The mean (\pm SE) VEGF concentration (pg/mg weight of sponge) in groups I to III of mice were 2.369 ± 0.198 , 0.562 ± 0.074 , 0.675 ± 0.209 respectively. The MVD (\pm SE) per field from processed sponges in groups I to III of mice were 14 ± 2.84 , 1 ± 0.36 , 3.5 ± 0.764 respectively. The results of the present study suggested that the ethanolic extract of *Nigella sativa* seeds possesses antiangiogenic activity.

Keywords: Angiogenesis, *Nigella sativa*, SU5416, Tumor, VEGF

INTRODUCTION

Angiogenesis is a complex mechanism in which there is growth of new blood vessels from the pre-existing ones. Angiogenesis is an essential phenomenon for the growth and

survival of tumor. Tumor angiogenesis is the proliferation of blood vessels penetrating the cancerous growth for the supply of oxygen and nutrients.

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The process of angiogenesis is a requisite for metastasis (Yadav et al., 2015). The signalling molecule vascular endothelial growth factor (VEGF) plays a central role in angiogenesis and is frequently expressed in cancers (Welti, 2013). VEGF receptors were first identified in endothelial cells (VEGF -A). VEGF is major pro-angiogenic factor. Hypoxic cells produce VEGF and upregulate VEGF receptors on pre-existing endothelial cells (EC). The functions of VEGF receptors are to induce endothelial cells proliferation, promote endothelial cells survival and also to increase the migration and invasion of endothelial cells, which is required in the process of angiogenesis. VEGF interact with its tyrosine kinase receptors and transmits signals to various downstream proteins (Byrne et al., 2005).

The multiple uses of *N. sativa* in traditional medicine encouraged many researchers to isolate its active components, including thymoquinone, thymohydroquinone, dithymoquinone, thymol, carvacrol, nigellimine-N-oxide, nigellicine, nigellidine and alpha-hederin. The bioactive compound of *N. sativa* thymoquinone (TQ) has been used for medicinal purpose for more than 2000 years. A large number of in vitro and in vivo studies have been conducted on laboratory animals in order to investigate pharmacological properties of *N. sativa*, like immunostimulation, anti-inflammatory, antihypertensive, antimicrobial, antiparasitic, antioxidant as well as anticancer activities (Randhawa & Alghamdi, 2002). Acute and chronic toxicity studies on laboratory animals have reported that *N. sativa*, its oil and thymoquinone, the most widely studied active principle, are quite safe, particularly when given orally. It was reported that thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and ERK signalling pathways (Padhey S. et al., 2008) Thymoquinone also showed immunomodulatory properties. Thus, thymoquinone could be used as a potential drug candidate for cancer therapy (Yi et al., 2008).

MATERIALS AND METHODS

Plant material:

N. sativa seeds were purchased from the local herbalist in Mumbai. The seeds were botanically authenticated by a specialist of plant taxonomy from biology department of Botanical survey of India institute. A specimen has been preserved at 4°C. The seeds were identified, cleaned, dried, mechanically powdered and extracted with 96% ethanol with rotary evaporator to render the extract alcohol free. The extract was kept in a refrigerator at 4°C.

Animals:

Male swiss albino mice weighing 25-30 gm were used in this experiment after getting necessary approval from IAEC of the institute. The mice were maintained in Central Laboratory Animal House, Krantisinh Nana Patil College of Veterinary science, Shirwal under standard managemental conditions (24±2°C, 12-h light:10-h dark cycle) with pelleted food and water ad libitum as per standard guidelines for animal ethics.

Mouse sponge implantation method:

Mice were divided in three groups comprising six animals in each group and designated as Group I (untreated), Group II (SU5416 @ 25mg/kg) and Group III (*N. sativa*@ 300mg/kg). The in vivo angiogenesis assay involving subcutaneous implantation of gel foam sponges in mice was performed according to the method of McCarty et al. (2002). Absorbable gel foam was cut (5mm×5mm×5 mm) and hydrated in sterile PBS in the petridish. It was exposed to U. V. light for 15 minutes in order to make them sterile and sponges were allowed to get soaked overnight. About half an hour prior to implantation of gel foam in a mouse sponge was removed from the petridish, squeezed aseptically and was then dipped (5 min) in 0.5 ml of the extract prepared in eppendorf tubes so that all the extract gets absorbed in the foam. Subsequently these foams were dipped in sterile 0.4% agarose maintained at 39°C in water bath. Agarose treated implants were transferred aseptically to sterile petri dish and kept for 10-15 min for allowing solidification

of agarose. Excess agarose was removed using blade and the implants were now ready for use.

Mice were anaesthetized with ketamine and xylazine combination. An incision was made (0.5 cm) along the midline at caudal back area and one sponge was inserted into each subcutaneous pocket created laterally. The animals were allowed to recuperate for 14 days. On the 14th day, animals were sacrificed and the gel foam sponge was harvested. The gel foam sponges were processed for estimation of mean vessel density (MVD), mean hemoglobin concentration and VEGF estimation by ELISA.

Haemoglobin Determination by cyanmethemoglobin method:

Hemoglobin estimation was performed as per the method reported by Drabkin and Austin. For hemoglobin estimation, gel foam implants removed on 14th day. The sponges were homogenized in 2 ml Drabkin's reagent, for 20 minutes on ice. The samples were spun at 12,000 rpm in a cooling micro centrifuge (eppendorf) for six minutes, and the supernatants were filtered through a 0.22 μ m filter. Hemoglobin (Hb) in the samples was quantified calorimetrically at 540 nm using a spectrophotometer

VEGF estimation by ELISA:

High-sensitivity kits were used for determinations. For estimation of VEGF by ELISA method mouse VEGF ELISA kits were procured from Biospes. Standards were analysed in duplicate. The sponge implants were removed on day 14th post implantation and manually homogenized in 1.0 ml sodium phosphate buffered saline (PBS) with pH 7.4 containing 0.05% Tween 20. The homogenized implants were centrifuged at 4 °C for 10 min at 10,000 \times g in a cooling micro centrifuge (eppendorf). The cytokines in the 50 μ l of supernatant from each implant were measured by using Immunoassay ELISA kits for murine VEGF as per the manufacturer's protocol.

Histopathology:

The gels were fixed in formalin and sectioned (< 4_m), stained with H&E. The number of vessels were counted in 15 consecutive fields using a 20 \times objective and the mean MVD was calculated.

RESULTS AND DISCUSSION

Angiogenesis and proangiogenic factors are logical objects for pharmacological manipulation, proving the vital role they play in cancer formation, growth, and proliferation, using a number of distinct mechanisms.

For hemoglobin determination the Mean (\pm SE) hemoglobin concentration was estimated (μ g/mg weight of sponge) in group I, II and III were 1.918 ± 0.087 , 0.365 ± 0.031 , 0.425 ± 0.037 respectively. Mean hemoglobin concentration in group I was significantly high than SU5416 and test group.

Mean hemoglobin concentration observed in *N. sativa* treated group was lower than the group I and slightly higher than positive control group. Ethanolic extract of *N. sativa* had showed comparable activity like Group II which can be result of antiangiogenic potential. Rajani et al. (2006) studied by comparison of control (vehicle) group with treatment group using sponge implantation method and reported that the gels from vehicle control group appeared to be normally vascular with blood vessels growing towards the gel foam sponge.

Yi et al. (2014) observed the bioactive compound of *N. sativa* thymoquinone (TQ) completely abolished angiogenesis in vivo in matrigel plug assay, where the plug has lowest Hb content. It also had inhibited micro vessel growth in vitro in aortic ring assay. Similar results were obtained in the present study showing that the *N. sativa* extract is having antiangiogenic potential.

The mean (\pm SE) VEGF concentration (pg/mg weight of sponge) in groups I, II and III of mice were 2.369 ± 0.198 , 0.562 ± 0.074 and 0.675 ± 0.209 respectively. *N. sativa* extract treated group showed lower VEGF concentration than group I (untreated) but no significant differences were observed when compared with group II. Which showed that

group III had inhibitory effect on stimulating VEGF proving its antiangiogenic potential.

Yi et al. (2008) reported that the active component of *Nigella sativa*, thymoquinone showed inhibitory effects on VEGFR2 specific inhibition assay, suggesting that thymoquinone act by suppressing VEGF-induced ERK activation. Paramsivam and associates reported antiangiogenic effect of thymoquinone, as bioactive compound of *N. sativa* on zebrafish angiogenesis model. Which showed thymoquinone inhibited growth of intersegmental vessel in zebrafish by RBC staining assay. It also downregulates the expression of VEGF-A mRNA (Paramsivam et al., 2012). In present study it was also observed that *N. sativa* downregulates VEGF concentration.

The MVD (\pm SE) per field from processed sponges in groups I, II and III of mice were 14 ± 2.84 , 1 ± 0.36 and 3.5 ± 0.764 respectively. After examinations of the histopathological slides of the skin tissues it was observed that the most of the sponge material placed in the subcutaneous tissues were get phagocytized. There was significant difference between group I and II in vessel density. *N. sativa* treated group showed non-significant differences with positive control but vessel density was much lesser than that of untreated group.



Fig. 1: *Nigella sativa* seeds

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Histopathological examination of liver tissue showed no changes when treated with *N. sativa* extract (Fathy & Nikaido, 2017). Peng et al., (2013) examined that treatment with thymoquinone a bioactive compound of *Nigella sativa* marked tumor destruction, reduced micro vessel density in tissue histology. It showed inhibition of angiogenesis in mouse osteosarcoma model. Hence from the present study following conclusions can be drawn:

1. For Anti-angiogenic study, subcutaneous implantation of gel foam in mouse is one of the most suitable model.
2. Extracts of *Nigella sativa* plant showed higher potential against angiogenesis as compared to untreated group.
3. Sponge implantation method offers some distinct advantages like biocompatibility, absence of inflammatory angiogenesis, depot preparation of pro-angiogenic molecule and test sample and feasibility of long-term studies. Most importantly, the grafts can be implanted in rodents of normal immune status. Future research regarding the role of critical mediators in altering tumor microenvironment concerned in tumor angiogenesis may result in novel therapeutic applications.

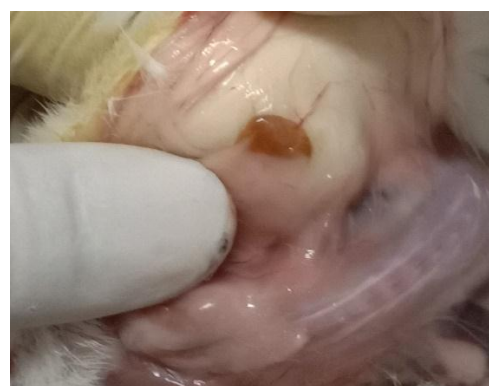


Fig. 2: Implanted sponge after 14th day

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