

Evaluation of Antiangiogenic Activity *Artocarpus heterophyllus* Extract by Sponge Implantation Method in Mouse

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Received: 22.10.2019 | Revised: 25.11.2019 | Accepted: 29.11.2019

ABSTRACT

The present research work was conducted in department of Veterinary Pharmacology and Toxicology, Krantisinh Nana Patil College of Veterinary Science, Shirwal. The study was conducted in 18 male swiss albino mice divided into three groups comprising of six animals in each. All the animals were subjected to subcutaneous implantation of gel foam sponges. Group I was kept as a control (blank sponge), group II received sponge with SU5416 (25mg/kg) and group III received sponge treated with extract of *Artocarpus heterophyllus* (300 mg/kg body weight). On 14th day animals were sacrificed and gel foam sponges were collected for estimation of haemoglobin, VEGF and histopathological examination. The mean (\pm SE) haemoglobin concentration (μ g/mg weight of sponge) in mice were 1.918 ± 0.087 in untreated group, 0.365 ± 0.031 in positive control group, 0.672 ± 0.038 in *Artocarpus heterophyllus* 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 , 0.794 ± 0.148 respectively. The MVD (\pm SE) per field from processed sponges in groups I to III of mice were 14 ± 2.84 , 1 ± 0.36 , 4 ± 0.93 respectively. From the present findings it is observed that *Artocarpus heterophyllus* showed anti-angiogenic effect equals to standard angiogenic inhibitor drug. Further extensive studies are required for assessing the utility of *Artocarpus heterophyllus* in clinical set-ups.

Keywords: SU5416, *Artocarpus heterophyllus*, VEGF, MVD, Haemoglobin.

Cite this article: Abhijeet, U.P., Ghumare, B.C., Karande, V.V., Patodkar, V.R., Dhaygude, V.S., & Lambate, S.B. (2020). Evaluation of Antiangiogenic Activity *Artocarpus heterophyllus* Extract by Sponge Implantation Method in Mouse, *Ind. J. Pure App. Biosci.* 8(1), 92-96. doi: <http://dx.doi.org/10.18782/2582-2845.7819>

INTRODUCTION

Angiogenesis, the process of new blood vessel formation from pre-existing ones. It is also a hallmark of tumour progression. Angiogenesis is essential process for supply of nutrient and oxygen totumour cells. The discharge of molecules by tumour cells send signals to the neighbouring normal host tissue. This is indicated by starting of tumour angiogenesis supporting the growth of new blood vessel by activation of certain genes (Yadav et al., 2013). Angiogenesis denote vessel sprouting from blood vessels from complex network for nourishment of all tissue in the body. The cancer growth and other inflammatory diseases can be hallmarked by anomalous vessel growth. Tumour cells metastasised by vessels pathway to all over body (Potente et al., 2011).

Angiogenesis is now a days considered as an attractive therapeutic target. The indicating molecule, VEGF plays an essential role in angiogenesis and it is highly uttered in cancer (Welti et al., 2013). Vascular endothelial growth factor (VEGF) and its tyrosine kinase VEGFreceptors (VEGFRs) are the key regulators in angiogenesis. Among them, VEGF-A, the most important member of the VEGF family, binds and activates the VEGFR2, and subsequently, activates the main signalling pathway of angiogenesis (Holmes & Zachary, 2005)

A. heterophyllus has been used as traditional medicine for inflammation, malarial fever, ulcer etc. The methanolic extract of jackfruit seeds has cytotoxic activity against cancerous cell line but does not harm normal cells. Methanolic extract of *A. heterophyllus* seeds contain flavonoids, saponins and tannins. Active component in jackfruit belongs to flavonoid group showed antiangiogenic effect in cancer.

Oktavia and associates (2016) reported the antiangiogenic potential of jackfruit seeds using CAM assay. Thiruselvi and Durairaj (2018) demonstrated that the hydroethanolic seed extract of *A. heterophyllus* strongly inhibited the angiogenesis *in vivo* and *in vitro* experiments and evaluate the arrest of

angiogenesis by blocking VEGF signalling pathway. Similarly Zuraidah and Mimi (2014) also reported that the extract of *A. heterophyllus* seed prevented growth of proliferating cells in cancer cell lines.

MATERIALS AND METHODS

Plant material

A. heterophyllus seeds were botanically authenticated by plant taxonomist from Department of Botany, Shripatrao Kadam Science College, Shirwal Dist Satara. A specimen was preserved at 4°C. The dried seeds of plant were mechanically powdered and extracted with 96% ethanol and evaporated with rotary evaporator to render the extract alcohol free. The extract was kept in a refrigerator at 4°C till further use.

Animals

Male swiss albino mice weighing 18-25 g. were recruited in the study 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 manage mental conditions (24±2°C, 12-h light:10-h dark cycle) with pelleted food and water *ad libitum* as per standards of the CPCSEA.

Mouse sponge implantation method

Mice (18) were divided into three groups comprising six animals in each and designated as Group I (untreated), Group II (SU5416) and Group III (*A. heterophyllus*). 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 foams were cut (5mm×5mm×5 mm) and hydrated in sterile PBS in the Petri dish. 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 in a mouse sponge was squeezed aseptically and then dipped (5 min) in 0.5 ml of the extract prepared in Eppendorf tubes (9 mg in 0.5 ml) so that all the extract gets absorbed in the foam sponge. Dose of SU5416 was 25 mg /kg body weight of animal (0.75

mg in 0.5 ml). 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 minutes to allow solidification of agarose. Excess agarose was removed using sterile blade and the implants were kept ready for further use.

Mice were anaesthetized with ketamine (100 mg /kg) and xylazine (10 mg/kg) combination. An incision of size 0.5 cm was made along the midline at caudal back area and one sponge was inserted into each subcutaneous pocket created laterally and incision was closed aseptically. The animals were allowed to recuperate for 14 days. On the 14th day, animals were sacrificed and the gel foam sponges were harvested. The gel foam sponges were processed for estimation of mean vessel density (MVD), mean haemoglobin concentration and VEGF estimation by ELISA.

Haemoglobin determination by cyanmethemoglobin method

Hemoglobin estimation was performed as per method of Drabkin and Austin (1935). Gel foam implants were 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 (eppendrof) for six minutes, and the supernatants were filtered through a 0.22 µm filter. Hemoglobin (Hb) in the samples was quantified calorimetrically at 540 nm using a spectrophotometer

VEGF estimation by ELISA

High-sensitivity VEGF ELISA kits were obtained from Biospecs pvt ltd and used for determination of VEGF from the sponges. 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×g in a cooling micro centrifuge (eppendorf). The cytokines in the 50 µl of supernatant from each

implant were measured by using Immunoassay ELISA kits for murine VEGF as per the manufacturer's protocol.

Histopathology

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

RESULTS AND DISCUSSION

Angiogenesis plays crucial role in both physiological and pathological conditions. Pivotal balance between multiple pro-angiogenic and antiangiogenic factors required for proper angiogenesis and a shift in this balance can lead to pro- or anti-angiogenic effects, angiogenesis can be instigated by a reaction to inflammation, hypoxia, and other conditions. It is moderated by angiomodulatory factors that are liberated by tumours and other diseased cells. The development of pathological condition can be arrived due to variance in these factors. Ribatti et al. (1997) reported the advantages of using the gelatin sponge compare to other in vivo method. Hasan et al. (2004) studied the sponge implant assay and reported that it could be used to study tumour and inflammatory angiogenesis. The development of vasoactive regulatory systems and pharmacological reactivity of neovasculature had also been investigated in the sponge models.

In the present study the mean (±SE) haemoglobin concentration estimated (µg/mg weight of sponge) in group I, II and III were 1.918 ± 0.087 , 0.365 ± 0.031 , 0.672 ± 0.038 respectively. Mean haemoglobin concentration in group I was significantly higher than SU5416 and group III (treated with *A. Heterophyllus* extract), which showed angiogenesis in group I. Group III showed significantly low concentration of haemoglobin than group I. Group III which was treated with ethanolic extract of *A. heterophyllus* had showed comparable activity as in Group II which can be result of antiangiogenic potential of the this plant.

Oktavia et al., (2016) observed *A. heterophyllus* activity in CAM assay method where microscopic observation and quantification of new blood vessels revealed inhibition of angiogenesis with increased dose. Histopathological examination revealed two different blood vessel types in CAM, thick walled blood vessel with dense erythrocyte and thin walled vessel with fewer erythrocyte. This support the present finding of haemoglobin estimates.

The mean (\pm SE) VEGF concentrations (pg/mg weight of sponge) in groups I, II and III of mice were 2.369 ± 0.198 , 0.562 ± 0.074 and 0.794 ± 0.148 respectively. *A. Heterophyllus* extract treated group showed lower concentration of VEGF than group I (untreated) but no significant difference was observed when compared with group II. Which showed that group III had inhibitory effect on stimulating VEGF proving its antiangiogenic potential.

Thiruselvi and Durairaj (2018) studied hydroethanolic extract of *A. Heterophyllus* significantly inhibited (VEGF)-mediated angiogenesis in the HUVECs in culture dose-dependently. Further, the new blood vessel formation was observed to be inhibited by the extract in Matrigel plug model in C57BL/6 mice. However, the effect was enhanced in higher concentration demonstrating the in vivo antiangiogenic activity of the extract also inhibited nearly 100 % of HEP2 cells up to 1:4 dilution of the crude extract and started decreasing with increase in dilution. Rajendran and Ramakrishnan (2008) investigated the methanolic extract of *Artocarpus heterophyllus* used to study an anticancer activity. Zuraidah and Mimi (2014) demonstrated that the extract *A. heterophyllus* seed prevented growth of proliferating cells in cancer cell lines. Oktavia et al., (2016) concluded that the extract treatment of *Artocarpus heterophyllus* inhibit the formation of new blood vessels in CAM

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 4 ± 0.93 respectively. After examinations of the

histopathological slides of the skin tissues it was observed that the most of the sponge materials placed in the subcutaneous tissues were phagocytized. The significant differences were observed between group I and II in vessel density. *A. heterophyllus* treated group III showed non-significant difference with positive control but vessel density was much lesser than that of untreated group.

Thiruselvi and Durairaj, (2018) demonstrated that the hydroethanolic extract of jackfruit significantly inhibited vascular endothelial growth factor (VEGF)-mediated angiogenesis in the HUVECs. Complete inhibition of tube formation was observed at higher doses. Effect of *A. heterophyllus* on angiogenesis further studied in vivo in Matrigel plug angiogenesis assay showed altered neovasularization. H and E staining revealed significant blood vessel formation in untreated group than the treated. There was more than 50% inhibition of blood vessel in mice treated with higher concentration was observed. Similarly, Oktavia et al., (2016) also reported that macroscopic observation and quantification of new blood vessels and revealed that all treatments Jackfruit seed inhibited the VEGF induced formation of new blood vessels in CAM. This support present study of antiangiogenic activity of *A. heterophyllus* in mouse.

Hence from the present study following conclusions can be drawn:

1. Mouse is the suitable *invivo* model to study anti-angiogenic potential of herbal compounds.
2. Extracts of *Artocarpus heterophyllus* plant showed higher potential against angiogenesis as compared to untreated group. Further extensive studies are required for assessing the utility of these remedies in clinical set-ups.

Acknowledgement

Authors are thankful to the Associate Dean, KNPCVS, Shirwal for availing the research facilities.

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