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Evaluation of antiangiogenic activity *C. papaya* ethanolic extract by sponge implantation method in mouse model

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Abstract
Cancer is a major cause of death worldwide, and angiogenesis is critical in cancer progression. The development of new blood vessels and the nutrition of tumor cells are heavily dependent on angiogenesis. Thus, angiogenesis is an important process that occurs both during health and disease. *Carica papaya* has been used in traditional medicine to treat many ailments like immunomodulation, anti-inflammatory analgesics etc. The present study was undertaken to evaluate the effect of the ethanolic extract of *C. papaya* on angiogenesis in a mouse model. The sponge implantation method investigated the antiangiogenic potential, wherein significant inhibition of blood vessel formation, hemoglobin concentration and VEGF concentration were recorded. Animals (30) were divided into five groups where group I was kept as untreated, group II was treated with SU5416 and group III was treated with ethanolic extract of C. papaya. The results were compared with positive control SU5416 and the untreated group. By using the Cyanmethemoglobin method the mean (±SE) hemoglobin concentrations (µg/mg weight of sponge) in group I to III of mice were 1.868 ±0.07, 0.391±0.03 and 1.069±0.09, respectively. The mean (±SE) VEGF concentration (pg/mg weight of sponge) in groups I to III of mice were 2.442±0.20, 0.481±0.06 and 1.607±0.13, respectively. The MVD (±SE) per field from processed sponges in groups I to III of mice were 12.4 ±2.1, 1.2 ±0.29 and 10.8 ±1.01, respectively. The results of the present study suggested that the ethanolic extract of *Carica papaya* leaves possesses antiangiogenic activity.

Keywords: Angiogenesis, cancer, SU5416, VEGF

Introduction
Angiogenesis is a complex mechanism in which there is a growth of new blood vessels from the pre-existing ones. Angiogenesis is an essential phenomenon for the growth and survival of tumors. Tumor angiogenesis is the proliferation of blood vessels penetrating the cancerous growth for the supply of oxygen and nutrients.

The process of angiogenesis is a requisite for metastasis (Yadav *et al*., 2013) [11]. The signaling molecule vascular endothelial growth factor (VEGF) plays a central role in angiogenesis and is frequently expressed in cancers (Welti, 2013) [10]. VEGF receptors were first identified in endothelial cells (VEGF-A). VEGF is a major pro-angiogenic factor. Hypoxic cells produce VEGF and up-regulate VEGF receptors on preexisting endothelial cells (EC). The functions of VEGF receptors are to induce endothelial cell proliferation, promote endothelial cell survival and also to increase the migration and invasion of endothelial cells, which is required in the process of angiogenesis. VEGF interacts with its tyrosine kinase receptors and transmits signals to various downstream proteins (Byrne *et al*., 2005) [1].

Carica papaya fruit, which belongs to the family of Caricaceae grown in different areas of the world, is well recognized as a potential medicinal fruit possessing unique food values and biological potentials. Medicinal uses of *C. papaya* fruits have been reported, such as leaves and smoke for cancer, diabetes, asthma relief and poultice for nervous pains, pulp for preventing rheumatism and urine acidity and flowers for jaundice and hypertension. (Islam *et al*., 2019) [5]. Therefore, The multiple uses of *C. papaya* in traditional medicine encouraged many researchers to isolate its active components, including of Quercetin, Ascorbic acid, Riboflavin and lycopene, A large number of *in vitro* and *in vivo* studies have been conducted on laboratory animals in order to investigate pharmacological properties of *C. papaya*, like immunostimulant, anti-inflammatory, antihypertensive, antimicrobial, antiparasitic, antioxidant as well as anticancer activities (Tayal *et al*., 2019) [9].

Fauziya *et al*. (2013) [4] conducted *in-vitro* activity of papaya, where the study showed good efficacy on many cancerous cell lines and also physiochemical properties possessed anticancer activities. Papaya is rich in the enzyme papain which is effective against cancer. Nguyen *et al*. (2015) [12] investigated the *in vitro* cytotoxicity of aqueous and ethanolic extracts of *Carica papaya* leaves on the human oral squamous cell carcinoma (SCC25) cell line the principal compounds identified were flavonoids or flavonoid glycosides, particularly compounds from the kaempferol and quercetin families, of which several have previously been reported to possess anticancer activities. These results confirm that papaya leaf is a potential source of anticancer compounds.

Materials and Methods

Plant material

C. papaya leaves were purchased from the local herbalist in Pune. The leaves were botanically authenticated by a specialist in plant taxonomy from the biology department of the S K Kadam College of Science, Shirwal, Dist. Satara (MS). A specimen has been preserved at 4 °C. The leaves were identified, cleaned, dried, mechanically powdered and extracted with 96% ethanol with Soxhlet apparatus 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 the necessary approval from the 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 five groups comprising ten animals in each group and designated as Group I (untreated), Group II (SU5416 @ 25 mg/kg), Group III (*C. papaya* @ 300 mg/kg), Group IV (*E. officinalis*@ 300 mg/kg) and Group V (*C. papaya* + *E. officinalis* @ 300 mg/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) [6]. Absorbable gel foam was cut (5 mm×5 mm×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 of, gel foam in a mouse sponge was removed from the petri dish, 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 a water bath. Agarose-treated implants were transferred aseptically to a 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 the 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 to estimate mean vessel density (MVD), 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 were removed on the 14th day. The sponges were homogenized in 2 ml of Drabkin's reagent for 20 minutes on ice. The samples were spun at 12,000 rpm in a cooling microcentrifuge (Eppendorf) 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 kits were used for determinations. Mouse VEGF ELISA kits were procured from Biospes to estimate VEGF by the ELISA method. Standards were analyzed 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 microcentrifuge (Eppendorf). The cytokines in the 50 µl of supernatant from each implant were measured using Immunoassay ELISA kits for murine VEGF per the manufacturer's protocol.

Histopathology

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

Results and Discussion

Table 1: The Mean (±SE) concentration of hemoglobin (µg/mg wt. of sponge), Mean (±SE) concentration of VEGF (pg/mg wt.) of sponge, and mean vascular density (MVD) per microscopic

Group No.	Treatment group	Mean (±SE) Hb concentration (µg/mg wt. of sponge)	Mean (±SE) VEGF concentration (pg/mg wt. of sponge)	MVD (per microscopic field of sponge)
I	Negative Control	1.868 ±0.07^{c}	2.442±0.20^{c}	12.4±2.1^{c}
II	Positive Control	0.391±0.03^{a}	0.481±0.06^{a}	1.2±0.29^{a}
III	*Carica papaya*	1.069±0.09^{b}	1.607±0.13^{b}	10.8±1.01^{b}

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

Table 1: The Mean (±SE) concentration of hemoglobin (µg/mg wt. of sponge), Mean (±SE) concentration of VEGF (pg/mg wt.) of sponge, and mean vascular density (MVD) per microscopic? field from processed sponges of different groups of mice for hemoglobin determination, the Mean (±SE) hemoglobin concentration estimated (µg/mg weight of sponge) in groups I to III were 1.868 ±0.07, 0.391±0.03 and 1.069±0.09, respectively. (Table 1). The mean hemoglobin concentration in group I was significantly higher than SU5416 and the test group. The mean hemoglobin concentration of the sponges from group I (control) was significantly highest than in group II (SU5416) and III (*C. papaya*). Thus, treatment with blank sponges in group I indicated normal progression of angiogenesis. The mean Hb concentration (µg/mg wt. of sponge) from group II was found to be the lowest than group I and III, and the later two groups also differed significantly. The group treated with Papaya extract showed a significantly low concentration of Hb than group I which shows that it is able to reduce hemoglobin concentration.

Duru *et al*. (2012) [3] studied the toxic effect of *Carica papaya* on body weight, hematology and some biochemical parameters in mice. Decrease in Hb levels reported in their study. Ojo *et al*. (2018) [8] evaluated the protective potentials of aqueous extract of *C. papaya* roots on arsenic-induced biochemical and genotoxic effects in Wistar rats. The study revealed that there were a decrease in catalase, glutathione peroxidase, superoxide dismutase, plasma hematological profile and also a decrease in Hb concentration. The reports of these scientists support the current findings.

Groups I, II and III revealed mean (±SE) VEGF concentration (pg/mg weight of sponge) as 2.442±0.20, 0.481±0.06 and 1.607±0.13, respectively. Group I (Blank) had the highest mean VEGF concentration showing statistical differences from the other groups. The mean VEGF value of group II (SU5416) was the lowest among the other groups. Group III (*C. papaya*) showed lower VEGF concentrations than control Group I. Thus *C. papaya* was able to reduce VEGF concentration to a certain level Tayal *et al*. (2019) [9] investigated the anti-angiogenic properties of the *Carica papaya* leaf. Docking behavior of known bioactive compounds of the leaf as ligands with angiogenic receptors VEGFR1 and VEGFR2 was used to assess the antiangiogenic activity of papaya leaf, and leaf aqueous extract was used for implantation in CAM egg yolk angiogenesis model based on docking results (*in vivo*) which inhibit angiogenic receptors VEGFR1 and VEGFR2.

The MVD (±SE) per field from processed sponges in groups I to III of mice were 12.4 ±2.1, 1.2 ±0.29 and 10.8 ±1.01, respectively. The mean (±SE) MVD concentration from gel foam sponges treated with papaya extract (Group III) was 10.8 ±1.01b. This group varied significantly ($p \leq 0.05$) from group I (Blank), II (SU5416).

Tayal *et al*. (2019) [9] studied an Anti-angiogenic study of leaf extract on a CAM model where CAM assay showed the inhibitory effect of the *Carica papaya* leaf with respect to its reduction in length, size and junctions of blood capillaries compared to untreated egg yolk. And ascorbic Acid, Quercetin, Riboflavin and, Lycopene (leaf compounds) can attenuate angiogenesis in pathological conditions. Munir *et al.* (2022) [7] studied the therapeutic potential of *Carica papaya* Leaf against thrombocytopenia. in mice models. Their study showed that *C. papaya* leaf reduced blood capillaries' size, length and junctions.

Conclusions

1. Sponge implantation method in mice is a good model for studying angiogenesis and related research.
2. *C. papaya* has great potential to inhibit angiogenesis.
3. Further phytochemical investigation of these plants needs to find out a more precise mechanism of action.

Fig 1: Carica papaya leaves

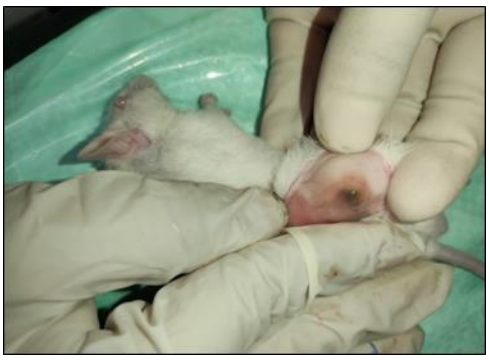

Fig 2: Implanted sponge after 14th day

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