



**EVALUATION OF ANTI ANGIOGENIC ACTIVITY OF HYDROALCOHOLIC
EXTRACT OF RAPHANUS SATIVUS L. BY CAM ASSAY**

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ABSTRACT

Angiogenesis, the expansion of a capillary network from preexisting vasculature, is implicated in a number of pathological conditions. Now a day's stimulation or suppression of angiogenesis process is being explored as therapy. Conversely, therapeutic angiogenesis contributes to regulate various pathological conditions. This study is scientifically designed to observe the anti-angiogenic potential of hydro alcoholic extract of seeds of *Raphanus sativus* L. with Chicken Chorioallantoic Membrane (CAM) assay. *In ovo* chicken CAM assay was used to examine the anti angiogenic activity of hydro alcoholic extract of *R.sativus* seeds (HERS). After sufficient embryonic development treatment of extract was given on CAM of fertile han eggs. Quantitation of blood vessel was assessed by photo micrographic observation and analyzed for all treatments and controls. Study revealed that photomicrograph of different chick CAMs after 72hrs of treatment with control and with different concentration of HERS at a dose of 10, 20, 30 mg/ml could significantly inhibit HIF-1 α and VEGF induced angiogenesis in *in-ovo* chicken CAMs. This concurred with manual quantitation of tertiary as well as quaternary blood vessels, which indicated that HERS suppress the formation of new blood vessels as compared to control. HERS is found to produce a dose dependent anti angiogenic activity in CAM assay. This effect may be postulated due to the presence of phytochemicals like sulforaphane & sulphoraphene, gallic

acid, caffeic acid, quercetin and apigenin which is reported to have potential anti-angiogenic activity by modulating multiple signaling cascades.

Keywords: Angiogenesis, CAM Assay, *Raphanus sativus* L., sulphoraphene; VEGF

INTRODUCTION

Since ancient times, the occurrence of new blood vessel from existing one was described in both physiological and pathological conditions [1]. For the survival of cell, supply of oxygen and nutrients are necessary. This is achieved with the help of the blood vessels through circulation. Vascularization of tissues is achieved by two sequential processes: vasculogenesis and angiogenesis. **Vasculogenesis**, it is the *de novo* formation of vessels from aggregating mesodermal precursor cells. **Angiogenesis**, it is the vital process in growth and development of new blood vessels from preexisting vasculature [2]. Usually, angiogenesis is firmly balanced by various angiogenic and anti-angiogenic factors but imbalance may lead to various pathological states and therefore, the process of angiogenesis has to be tightly regulated [3, 4]. Initial steps involved in physiological angiogenesis are: Detachment of pericytes, Basement membrane (BM) degradation, endothelial cell (EC) migration, Sprouting of blood vessel.

Excessive angiogenesis is associated with increased risk of many pathological conditions viz. tumor, arthritis, psoriasis, atherosclerosis, endometriosis, diabetic

retinopathy and obesity. Conversely, insufficient angiogenesis may lead to chronic wound (with poor rate of healing), myocardial infarction, alopecia etc. Therefore, angiogenesis promoters and inhibitors have gained attention for the treatment of angiogenesis associated diseases [5, 6]. Solid cancers depend on neo-angiogenesis for growth, progression & metastasis. The academic and industrial research and development efforts have been focused on angio-therapy for effective management of cancer worldwide. There are several agents recently approved by FDA which target either the angiogenic factor and/or angiogenic factor signal cascade [7, 8].

Currently available drugs are known to have several clinical limitations like the development of resistance, and are associated with side effects like hypertension, arterial thromboembolic events, proteinuria, bowel perforation, reversible posterior leukoencephalopathy syndrome, wound complications and hemorrhage [9, 10]. Thus, identification of natural nutrients from food for controlling angiogenesis would be of greater advantage because of their low toxicity or safe with no side effects. Seeds of *Raphanus sativus* L.

have active phytochemicals like glucosinolates-precursors of isothiocyanates (sulforaphane & sulphoraphene), phenolic compounds (gallic acid, caffeic acid) & flavonoids (quercetin, apigenin) which is considered as potential angiogenesis inhibitor [11, 12, 13]. Hence, this study aimed to evaluate the angiogenesis inhibitor potentials of these herbal plant using the *in ovo* Chick Chorioallantoic Membrane (CAM) Assay. This method is suitable for examining blood vessel development in CAM, by enhancing the accessibility and easy documentation of CAM to measure angiogenesis in embryos. On the basis of above mentioned evidences, effect of hydroalcoholic extract obtained from seeds of *Raphanus sativus* L. was evaluated to determine angiogenesis inhibition during growth of chicken embryo.

MATERIALS AND METHODS

Plant Collection & Preparation of Extract

The dried seeds of the plant *Raphanus sativus* L. were collected from the local market of Visavadar, Junagadh, Gujarat. The fine seed powder (30g), suspended in 150ml of ethanol (85%), and agitated at 150rpm for 24hr at room temperature. The suspension was then filtered using Whatman filter to remove undissolved solids and evaporated to 1/15th of its original volume using rotary evaporator, at

40°C to remove the ethanol and to concentrate the dissolved compounds. The concentration of the total soluble compounds in the crude extract was determined by measuring the dry weight of a small aliquot of the extract dried at 65°C overnight. The dry crude extracts were stored in air-tight container at 4°C [14].

Chemical Characterization of the Extract

The extract was tested for the presence of flavonoids, alkaloids, tannins, phenols, saponins, sterols & triterpenoids, glycosides, carbohydrate etc. [15].

Chick Chorioallantoic Membrane (CAM) Assay

The fertilized chicken eggs were obtained on day zero, they were undeveloped at this point. The surface of eggs were wiped with 70% EtOH and were incubated at 37°C temperature and at 95% relative humidity for 4 days. The eggs were incubated in the horizontal position to ensure the correct positioning of the embryo (away from CAM) & manually rotated 180° at least three times a day for the duration of the test, to ensure correct development and viability of the embryo.

On day 4, eggs were wiped with 70% EtOH and a hole was made at the blunt end to puncture air sac. A window of 1 cm² was made in the shell. Care was given that no shell dust fell on the developing embryo.

Unfertilized eggs were discarded. The windows were sealed with sterile transparent paper tape and then eggs were returned to the incubator. On day 9, sterile surgical gauge with approximate inner diameter of 1.5 cm were placed on the CAMs and the eggs were returned to the incubator. On day 10, eggs were organized into groups and different concentration of extract were injected in the gauge. Eggs treated with an equal volume of DMSO served as controls. After further incubation for 72 hrs, ice cold 4% paraformaldehyde

was spread over the membrane and perfused inside such that both sides of membrane were fixed. These embryos were left at 4°C for some time. The CAMs were later excised and refixed in 4% ice cold paraformaldehyde for more than 30 min. The membrane were placed on a microscope slide, and images of control and treated CAMs were taken for comparative studies [16, 17]. HERS-hydro alcoholic extract of seeds of *Raphanus sativus* L.

Table 1: Study design

Groups(n=10)	Treatment
Normal control	0.6% DMSO
Treatment(20µL)	HERS 0.2mg/ embryo
	HERS 0.4mg/ embryo
	HERS 0.8mg/ embryo

Evaluation

The quantitation of blood vessels was carried out by counting the primary vessel and the secondary, tertiary, and quaternary branches arising from the primary vessel, manually in selected areas, and histograms were plotted. A significant decrease of number of quaternary vessels was observed with treatment groups as compared to control.

Statistical analysis

All the values were expressed as Mean \pm S.D. Statistical significance was tested using two-way ANOVA using Graph-pad prism version 8.0. Differences were

considered to be statistically significant when $P < 0.05$.

RESULTS & DISCUSSION

Plant extraction The extraction of dry powdered seeds of *Raphanus sativus* L. was carried out using ethanol and water for 24hrs. The extract was obtained in dark brown color and semisolid in nature. The percentage yield of hydro alcoholic extract of 25 gm of dried powdered seeds of *Raphanus sativus* L. was found to be 3.72% w/w.

Phytochemical screening

Preliminary phytochemical screening of *Raphanus sativus* L. confirmed presence of phyto-constituents such as carbohydrates,

alkaloids, glycosides, saponins, saponins, tannins, flavonoids, phenolic compounds except sterols and triterpenoids.

Estimation of anti-angiogenic activity by quantitation of blood vessels

The anti-angiogenic activity of seeds of *Raphanus sativus* L. was evaluated by CAM assay. The quantitation of blood vessels was carried out in chick embryo by counting different branches of blood vessels, manually in selected area of corioallantoic membrane of developing embryo. Comparison of tertiary and quaternary blood vessels of developing embryo at Day 12 (72hrs after the treatment has been given at 9th day) in HERS treated embryos with control treated embryo was carried out (Table 2). Various concentration of HERS resulted in significant decrease in number of tertiary and quaternary blood vessels as compared to control as evident in Fig. 3. This effect was observed in dose dependent manner suggesting a strong anti angiogenic potential of hydro alcoholic extract of seeds of *Raphanus sativus* L. This effect may be postulated because of the presence of phytochemicals like sulforaphane & sulphoraphane, gallic acid, caffeic acid, quercetin and apigenin which have potential anti-angiogenic activity.

Proliferation, migration, and tubular formation are essential characteristics of endothelial cells for the generation of new blood vessels but this phytochemicals alter various biochemical pathways which directly affect physiological angiogenesis.

Sulphoraphane (SFN) exerts antiangiogenic terms of tumor angiogenesis, Sulphoraphane (SFN) has been reported to inhibit NF- κ B-regulated VEGF expression in human prostate cancer cells; and hypoxia-induced HIF-1 α and VEGF expression in human colon cancer cells [18, 19, 20, 21, 22]. CA is a phenolic compound, reported to suppress tumor angiogenesis by blocking STAT3-mediated VEGF expression. In addition, it is able to inhibit NF- κ B activation and inhibit iNOS expression [7]. GA inhibits in vitro angiogenesis induced by OVCAR-3 in ovarian cancer cells [23]. Quercetin is shown to inhibit angiogenesis by targeting VEGFR-2 regulated AKT/mTOR/P70S6K signaling pathways [24, 25, 26, 27]. Apigenin, flavonoid has been revealed to inhibit tumor angiogenesis through decreasing HIF-1 α and VEGF expression [28, 29, 30, 31]. This mentioned phytochemicals are directly or indirectly responsible for strong anti angiogenic activity of seeds of *Raphanus sativus* L.



Figure 1: Normal pattern of blood vessels [17]

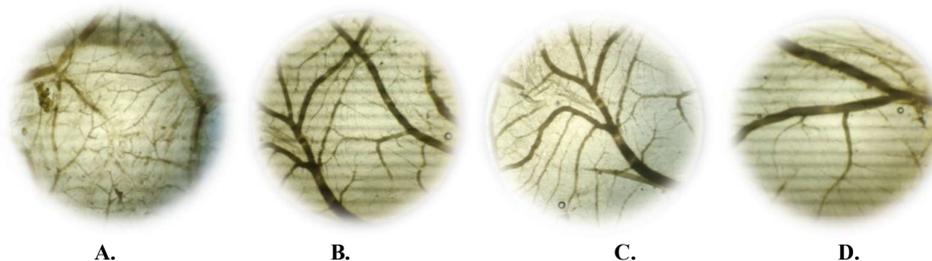


Figure 2: Photomicrograph of different chick embryo chorioallantoic membranes (CAM) after 72hrs of treatment with A-control DMSO, B-0.2mg of HERS, C-0.4mg of HERS, D-0.8mg of HERS

Table 2: Effect of HERS at various concentrations on angiogenesis at 12th day of incubation in fertilized chicken embryo

GROUPS	PRIMARY	SECONDARY	TERTIARY	QUATERNARY
CONTROL	2.142±0.69	9.142±2.73	25.142±5.84	47.428±11.83
HERS(0.2mg)	1.5±0.577	8.333±4.131	22.666±3.54	33±8.049*
HERS(0.4mg)	2±1	8.2±4.494	18.6±4.037	18±6.519*
HERS(0.8mg)	1.5±0.577	5±2.581	6±0.816 [#]	4.75±1.892*

All values are expressed as mean± SD for each group

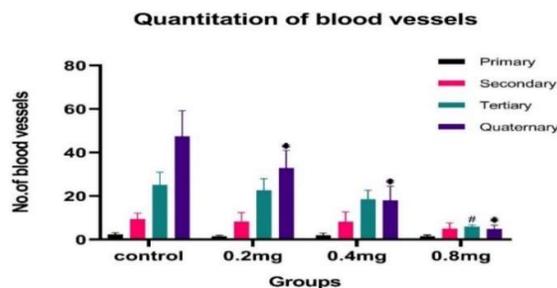


Figure 3: Effect of HERS at various concentrations on angiogenesis at 12th day of incubation in fertilized chicken embryo

All values are expressed as mean± SD for each group.

*indicates significant difference from control quaternary at p<0.05

[#]indicates significant difference from control tertiary at p<0.05

CONCLUSION

The present investigation revealed that hydroalcoholic extracts prepared from the seeds of *Raphanus sativus* L. shows anti-angiogenic activity which is associated

with presence of various anti-angiogenic phytochemicals which may shows significant reduction in the levels of vascular endothelial growth factor (VEGF), PDGF, FGF, angiotensin expression and/or

stimulation of expression of anti-angiogenic factors like, Thrombospondin, Endostatin and Angiostatin.

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