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ASSESSMENT OF AQUEOUS LEAF EXTRACT *OF FICUS RACEMOSA* L. FOR VASCULOGENESIS AND ANGIOGENESIS USING *IN VIVO* MODEL OF CHICK CAM ASSAY

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ABSTRACT

The influence of aqueous extract of *Ficus racemosa L.* leaf was evaluated, using *in vivo* model of CAM assay with window method, to study the pro/antiangiogenic and vasculogenic properties. The doses were administered in HBSS at 48, 72 and 96 hrs in twelve groups (each of the incubation interval has 4 groups viz., normal, sham operated, HBSS control and experimental and each group contained 6 eggs in development). The observations include photographic data and quantitative analysis of primary, secondary and tertiary vessels, that were observed after 144 hrs for each of the incubation interval groups. The results indicate strong antiangiogenic effects of the aqueous extract directly proportional to the hours at which administration was carried out.

KEYWORDS: *Ficus racemosa L.*, leaf extract, Vasculogenesis, angiogenesis, CAM assay.

INTRODUCTION

Angiogenesis is a progressive multistep physiological process by which new blood vessels are formed from pre-existing ones. New blood vessels form by sprouting of existing blood vessels and the process is also termed as neovascularization. This process is integral part of both, normal developmental process and many pathologies ranging from tumor formation, metastasis to retinopathies. The process is essential in tissue repair, fetal development and female reproductive cycle too. Angiogenesis is controlled by many growth factors and cytokines as well.[1] Any drug/substance /phytochemical influencing neovascularization will be of importance to target the isolation of the effective substances that are pro/anti

angiogenic. Our laboratory is testing extracts of many plants and plant products on vasculogenesis and angiogenesis[2,3] so that pro and anti-angiogenic chemicals can be isolated and analyzed further for modern therapeutic use.

Ficus racemosa L is used in many traditional systems of medicines such as Ayurveda, Unani, Siddha, Homeopathy etc. to treat many disorders.[4,5,6,7] Due to the importance of therapeutic values, the aqueous extracts of its bark and leaves have been tested for their toxic effects.[8]

The mortality data[8] showed that 0.5mg/egg is effective dose without any developmental deformity in chick embryos. Therefore, in the present work, the influence of the same dose of aqueous extract of the leaves on vasculogenesis and angiogenesis was studied using window method for chick Chorioallantoic Membrane(CAM) assay.

MATERIAL AND METHODS

1. Preparation of aqueous Extract of Leaves

Leaves of *Ficus racemosa*. were obtained from campus of Shivaji University, Kolhapur 416410, Maharashtra state, India. Cleaned and shed dried leaves were powdered and strained through muslin cloth. The known amount of powder was mixed with known amount of sterilized water to get aqueous extract. The extracts were then dried at low temperatures. The yield of aqueous extract of leaves was 8.1%. Known amount of such extract was dissolved in Hanks Balanced Salt Solution (HBSS-HIMEDIA, India) to prepare the stock solutions so that suitable concentrations can be used for the applications. The minimum influencing dose (0.5 mg/egg) which showed no associated embryonic toxicity and abnormality, but that was with average 20% mortality was selected from toxicity studies data.[8] Results of 0.5mg/egg dose that are studied on CAM vasculature are presented here.

2. Experimental protocol

Fertilized eggs of *Gallus gallus* were obtained from Quality poultry products, Malgaon, Tal. Miraj, Dist. Sangli (416416), MS, India. Selected eggs of similar size and weight were used. Surfaces of the eggs were disinfected with 70% alcohol. They were divided in twelve groups each containing 6 eggs. The groups were: normal (at 48, 72 and 96 hrs), sham operated (at 48, 72 and 96 hrs), HBSS control (at 48, 72 and 96 hrs) and experimental (dose administered at 48, 72 and 96 hrs). Thus, each of the incubation interval had 4 groups of eggs. Vasculogenesis and angiogenesis of CAM being the interest, the hours of administration of extract were selected as 48hrs, 72hrs and 96hrs. Aseptic status of the incubator was

maintained and the eggs were incubated at 37.5°C temperature where relative humidity was maintained at 70-75%. The treatment doses were administered at above stated hours and development was continued up to 144hrs. i.e. on completion of CAM development and capillary networking. (Table1).

On scheduled period of incubations (viz., 48, 72 and 96 hrs of incubation) the windows were prepared in egg shells[9] of respective group of eggs under aseptic conditions and extracts of leaves of *F. racemosa*. were spread on the embryonic plates in the final volume of 0.5 ml HBSS. The required concentration was adjusted in the final volume of 0.5 ml, with appropriate pH, to spread on the embryonic plate of each egg of all groups. One group of embryos was maintained as normal group. Embryos of operative control were sham operated for window preparation and embryos of HBSS control received plain 0.5ml HBSS alone. The HBSS and all the treatment doses were brought to 37°C before administration. The windows made for administration were sealed with sterilized adhesive tapes and the eggs were immediately transferred to the incubator to continue further incubation hours adjusting the experimental time slot until completion of 144hrs. On 144 hrs of incubation, the shells were removed and the embryos were observed and used for analysis. Total distribution of blood vessels on CAM were taken for measurements.

Experimental data analysis

The experimental schedule is presented in Table I.

Quantification of growth of embryo

The embryo and CAM both were assessed for their growth by taking embryo weight and CAM weight. A fine balance (Shimadzu BL-220H make) was used for the weight measurements. Weights of embryo and CAM after different treatments are given in tableII.

Quantification of vasculogenesis and angiogenesis

Angiogenesis was quantified by using morphometry and microscopic observations. For CAM analysis, computerized image analysis system was used. Number of primary vitelline vessels (largest) was measured. Number of secondary vitelline vessels (branches of primary vitelline vessels) were measured (initiation of branching point to capillary network branching). Tertiary vitelline vessels at peripheral zone emerging from main secondary vitelline vessels were measured. The observed alterations are presented in Table III, and Figs II(a & b).

Statistical analysis

The quantitative data is expressed as Mean ± S.D. and the statistical significance between groups was analyzed by using student's t test. The values of p,0.05, p,0.01 and p,0.001 were considered as significant.

RESULT AND DISCUSSION

Evaluation of growth parameters of Embryo and CAM

The data of embryo weight and CAM weight is presented in table 2. The differences in the weight of embryo and CAM for normal group of eggs, for sham operative group of eggs and HBSS treated group of eggs (at all hours of administration), were not statistically significant. Upon administration of the plant extract at 48 hrs, the embryo weight was found to be reduced by 30% whereas weight of CAM showed only marginal decrease with respect to HBSS treated ones. When the doses were administered at 72 hrs of incubation, the alterations in the weight of embryo as well as CAM was only marginal. Same is the case upon administration of extract at 96 hrs of incubation. The factors responsible to bring upon a drastic reduction in the weight of embryo at 48 hrs of treatment seems to become adaptive and remain unaffected at later hours. This indicates that 48 hrs period is more sensitive for embryo growth and is influenced by extract so also by HBSS. HBSS promoted CAM growth which is also true for HBSS plus extract treatment. This indicates that reduction on growth of embryo is sole effect of the components of aqueous extract.

Macroscopic evaluation of CAM

Macroscopic evaluation of CAM after different treatments is represented in Figure I. Macroscopic evaluation of Normal and Sham control of CAM at 144 hrs of development shows normal angiogenesis with dendritic pattern of blood vessel formation.[10] Operative control did not affect either the growth of embryo and CAM or the blood vessel formation on the CAM at 144 hrs of development. HBSS when administered at early hours (48 hrs), showed hardly any difference in the pattern formation of blood vessels at 144 hrs of development. On the other hand, HBSS when administered at 72 hrs and 96 hrs of development has shown remarkable enhancement in the blood vessels. Although the pattern of blood vessel formation remained same as dendritic, HBSS had induced tortuosity in many smaller blood vessels. It also caused sprouting of smaller blood vessels and thus the number of tertiary blood vessels had increased.

As far as primary vessels are concerned, there is no influence of any treatment (HBSS or Extract) on its number or pattern. In 48 hrs experimental group secondary blood vessel's

number was hardly affected but tertiary vessels were increased marginally (1.16 folds) due to administration of HBSS, whereas upon the administration of leaf extract of *F. racemosa*, 35% decrease in the number of tertiary vessels was observed. In 72 hrs experimental group, number of secondary vessels was hardly influenced but tertiary vessels were increased in number by 1.59 folds due to administration of HBSS. This increased number was brought down by 65% due to administration of aqueous extract of *F. racemosa*, indicating the most sensitive stage influenced by the extract. In 96 hrs of experimental group, HBSS increased the secondary vessels marginally and tertiary vessels were increased significantly (1.59 folds) as in 72 hrs experimental group. Tertiary vessels were significantly reduced (73%) by the leaf extract. (Table 3, Figure II).

The results indicate that *F. racemosa* leaf extract is a potent antiangiogenic at every stage of embryo development (in sprouting phase), hardly affecting the embryo growth, primary vessels, partially affecting the secondary vessels and significantly reducing the tertiary vessels.

The differences in chemical composition of leaf and/or blood vessel growth responding factors are synchronously leading to antiangiogenic effects. Its chemical composition is lupeol, β-sitesterol, stigmasterol and calcium oxalate in abundance.[4] Leaf components are sterols, triterpenoids, alkaloids, tannins and flavonoids. A new tetracyclic triterpeneglauanol acetate and racemosic acid are also isolated from leaves.[11] The leaf extracts, are known to have antibacterial[12] and antifungal[13] activities as well.

Presence of endogenous FGF-2 protein has been demonstrated in chick CAM extracts. FGF-2 mRNA is expressed in the CAM, and that the cellular source of this angiogenic factor changes during development.[10] At day 5, chorionic epithelial cells express high levels of FGF-2 mRNA. Endogenous chick FGF-2 may affect proliferation, migration, redistribution and invasive behavior of endothelial cells responsible for the development of the vasculature.[14] The FGF-2 released by chorionic epithelial cells may induce an angiogenic response in undifferentiated vessels in the CAM mesoderm by stimulating endothelial cell proliferation, movement and protease production.[15]

The experimental design of the present study includes administration of extracts at early hours of development (48 hrs, 72 hrs). This period marks the beginning of organogenesis and cellular differentiation. Therefore, it seems that the phytochemicals introduced onto CAM by

administration of extracts could be interfering the process of m-RNA formation, its expression into FGF-2 protein and sequestration of the protein itself. It could also interfere with the signal receptors associated with the FGF-2 protein. Such interference could lead to inhibition of the proliferative activity of the sprouting blood vessels, manifested as antiangiogenic/angiostatic effect. The very significant reduction in the density of the tertiary blood vessels (35% at 48 hrs, 65% at 72 hrs and 73% at 96 hrs) strongly suggests the interference in the FGF-2 protein induced angiogenic response. Further isolation of the components that may lead to antiangiogenic effects is in progress.

Table I: Exposure schedule of extracts of *F. racemosa* at different developmental stages of chick embryo in hrs.

Group	Developmental stage in hrs	Corresponding HH stage	Time of exposure to treatment of doses in hrs			Final observation at hrs
			48	72	96	
I	48	12	√	---	---	144
II	72	20	---	√	--	144
III	96	24	---	---	√	144

Table II: Effect of extracts of *F. racemosa*. at various hours of incubation on growth parameters of chick embryo.

Initiation of treatment in hrs	Groups	Emb wt	CAM wt
48hrs	normal	0.94±0.08	0.71±0.13
	SHAM Control	0.91±0.09	0.66 ± 0.09
	HBSS	0.82± 0.09	0.89+0.08
	Aqueous extract	0.56±0.04	0.82±0.09
72 hrs	normal	0.94±0.08	0.67±0.13
	SHAM Control	0.91±0.09	0.66±0.09
	HBSS	0.83±0.03	0.75±0.02
	Aqueous extract	0.86±0.04	0.73±0.03
96 hrs	normal	0.94±0.08	0.67±0.13
	SHAM Control	0.91±0.09	0.66±0.09
	HBSS	0.84±0.07	0.81±0.08
	Aqueous extract	0.72 ± 0.1	0.82±0.08

weights are expressed in grams

Table III: Alteration in the secondary and tertiary blood vessels upon exposure to extracts of *Ficus racemosa*. at various hours of incubation evaluated by CAM assay.

Initiation of treatment in hrs	Groups	No of secondary blood vessels	No. of tertiary blood vessels
48hrs	normal	19±1.6	137±7
	SHAM Control	18±1.2	139±7
	HBSS	21.14±0.69	160±8
	Aqueous extract	18.71±0.5	104.57±7
72 hrs	normal	19±1.6	137±7
	SHAM Control	18±1.2	139±7
	HBSS	22.57±0.78	222.43±7
	Aqueous extract	18.85±0.69	76.72±9
96 hrs	normal	19±1.6	137±7
	SHAM Control	18±1.2	139±7
	HBSS	22.72±0.75	221±5
	Aqueous extract	12.71±0.75	60.0±5

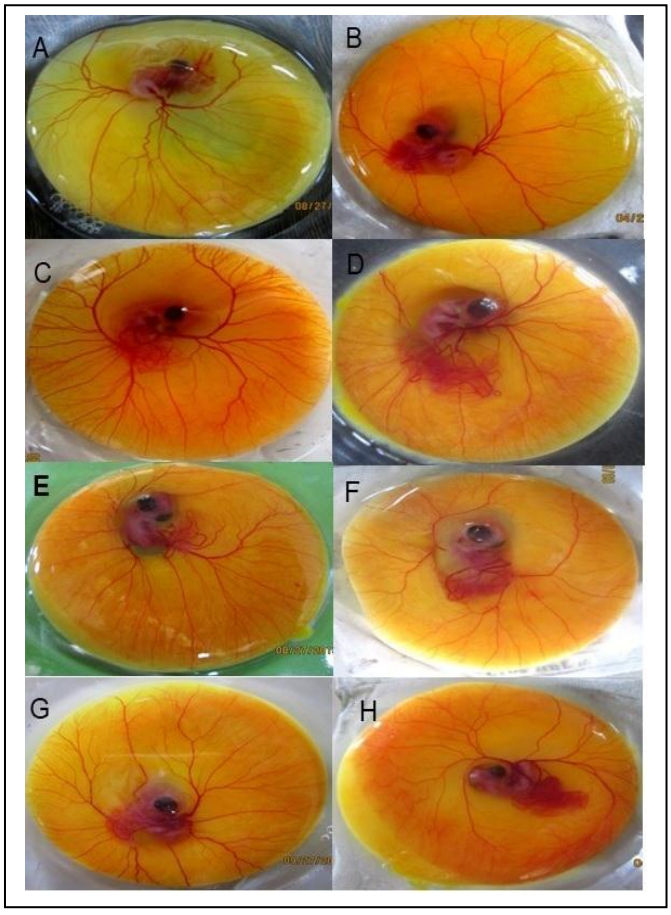

Figure I: Images showing comparison of vascular development of different treatment groups of eggs. The groups are normal (untreated, A), Sham operated (B), HBSS treated (C at 48 hrs; E at 72 hrs and G at 96 hrs) and extract treated (D at 48 hrs, F at 72 hrs and H at 96 hrs).

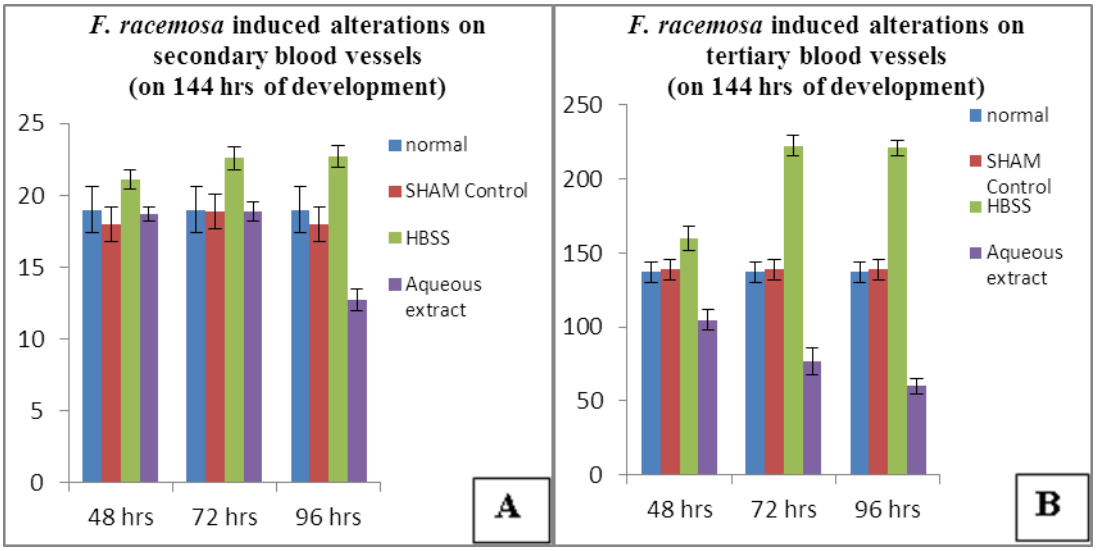

Figure II A&B: The alterations in the secondary (A) and tertiary (B)blood vessels at 144 hrs of development upon initiation of different experimental conditions at 48, 72 and 96 hrs.

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