Effect of Leaf Extract of Morus Albaon Angiogenesis Bychorio Allontoic Membrane Assay Cam in Chick

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Abstract

Chick embryo study reflects valuable information in future development of vertebrates; it is used as valuable tool to study angiogenesis. Angiogenesis is a natural, fundamental process in cancer, ischemic diseases and other inflammatory disorders. Various plant extract affect formation of blood vessels and related effects on CAM (SuaibLuqman, 2012**).**In the present investigation effects of leaf extract of Morus alba on angiogenesis were studied. Fertilized eggs of Gallus gallusdomesticus were incubated at 37^0 C and 72-75% related humidity. After 48 hrs, 72 hrs and 96 hrs embryo CAM were exposed and injected with 70 µg /ml leaf extract of M. alba. Eggs were further incubated up to 144 hrs and CAM were studied.A significant inhibitory effect of leaf extract of Morus alba were observed on the number and area of primary , secondary and tertiary vitelline veins of 48hrs, 72 hrs CAM than 96 hrs CAM incubation as compared with control.

KEYWORDS: Angiogenesis, Morus alba, CAM assay, leaf extract.

INTRODUCTION

Gallus gallusdomesticus and their eggs have been used extensively as research models throughout the history of biology(p). Chick embryo study reflects valuable information in further development; it is used as valuable tool to study angiogenesis. Propagation of new blood vessels from pre-existing vessels is nothing but angiogenesis. Angiogenesis is a natural, fundamental process observed in cancer, ischemic diseases and other inflammatory disorders. Various plant extract affect formation of blood vessels and related effects on CAM (q**)**.

Angiogenesis, process of generating new blood vessels from pre-existing ones is a fundamental step in variety of physiological and pathological conditions including wound healing, embryonic development, chronic inflammation, tumor progression and metastasis(d). The angiogenic process is controlled by a variety of activators and inhibitors . Activators of blood vessel formation include Fibroblast growth factors (FGF); vascular endothelial growth factors (VEGF) and Angiopoietin-1.while inhibitors of angiogenesis are angiostatin, endostatinand thrombospondin compounds.

Morus alba(Sanskit : Tuta) known as mulberry. Mulberry plant is native of china. It grows well on a wide variety of soils, but prefers a moist, well drained soil with lots of sun light. Various extracts of mulberry is considered to be antibacterial, astringent, diaphoretic, hypoglycemic, odontolgic and ophthalmic. Its leaves are usually used in treatment of colds, sore throats, flu, eye infections(s).

Chorioallontoic Membrane (CAM) of chick embryo lies beneath the egg shell and perform function as gas exchanger and waste elimination(n). It is one well established and most commonly used in vivo model for evaluation of angiogenesis.

MATERIAL AND METHODS

Extraction of mulberry leaves

The properly identified leaves of Morus albawere collected from the local gardens of Satara, Maharashtra, India. Leaves were shed dried for a week and powdered mechanically and strained through muslin cloth. Leaf extract were prepared by Soxhlet method in 70% alcohol. The alcohol extract was dissolved in Hanks Balanced Salt Solution (HBSS-HIMEDIA India).

ChorioAllontoic Membrane Assay (CAM)

Fertilized eggs of Gallusgallusdomesticus were obtained from the government hatchery (Assistant commissioner of animal husbandry, Central hatchery GodoliSatara). The eggs were cleaned and disinfected with 70% alcohol and divide into 3 groups viz. normal, treated and control. The eggs were incubated in an aseptic condition in vertical position at optimum 37˚C Temperature and 72-75% Relative Humidity.

The incubation were carried out to obtain embryos of 48 hrs, 72 hrs, and 96 hrs development. After completion of incubation of 48 hrs, 72 hrs, and 96 hrs the embryo's CAM were exposed to 70 µg/ml leaf alcohol extract as described in Table No. 1 by window method (j). The embryos were further incubated up to 144 hrs. and CAM was studied.

Table No. 1: Shows Exposure schedule of Leaf extract to different developmental stages of chick embryo in different hrs.

Groups according to developmental stages	Groups according to time of exposure to the treatment			Final development in hrs.
	A	B	C	
	48	72	96	
I (Normal) CAM	-	-	-	144 hrs
II(HBSS as control) CAM	✓	✓	✓	
III Treated CAM	✓	✓	✓	

The doses were selected on the basis of mortality and toxicity study. After the incubation time the eggs were treated according to Table No. 1.70 µg / ml alcohol extract of M.alba were spread on CAM in HBSS solution on treated group of eggs. One group of eggs were incubated as normal and control group were with administration of 1 ml HBSS as a control. All eggs were in cubated for 144 hrs.

The CAM evaluation were made by measuring CAM area with some modifications, which was described by Melkomianet al. (2002)For morphometric study number of secondary andtertiary blood vessels were counted with the help of computer, by considering place of bifurcation points

OBSERVATIONS AND RESULTS

In our study, phytochemical screening of Morus alba leaves showed, presence of alkaloids, flavonoids, tannins, cardiac glycosides, carbohydrate, proteins and steroids. It has been observed that influence of extract of mulberry leaves showed inhibitory effects on blood vessels such as primary, secondary and tertiary vitelline veins as compared to normal and control Chorio Allontoic Membrane of chick embryo after 144 Hrs. of incubation (See Plate No.1.

Plate No. 1- Development of CAM of Normal, Control & Treated chick embryo

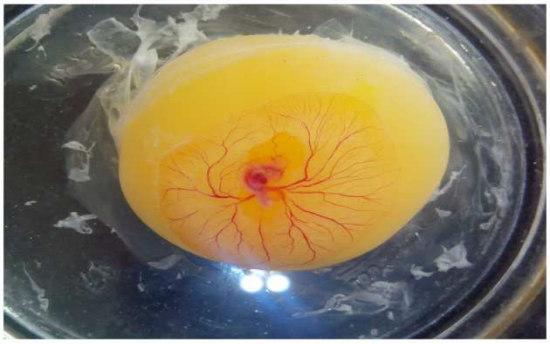


Fig. I Normal

Fig. II Control (HBSS)

Fig. III Treated at 48Hrs

Fig. IV Treated at 48 Hrs

Fig. V Treated at 72 Hrs

Fig. VI Treated at 72 Hrs

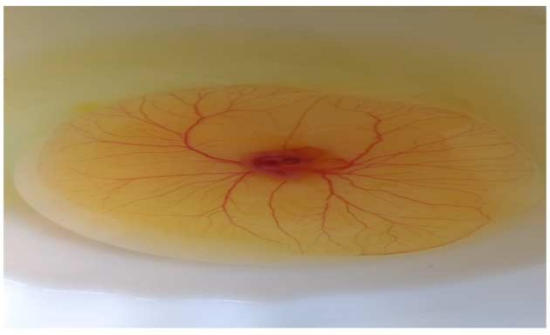


Fig. VII Treated at 96 Hrs

Fig. VIII Treated at 96 Hrs

Fig. I- Normal – Normal Angiogenesis in CAM of chick embryo after 144 hrs of development.

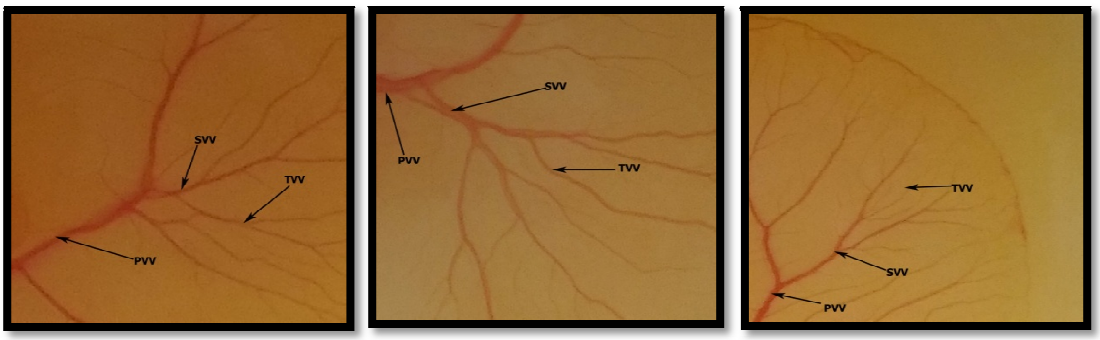
Fig. II- Control (HBSS) - Figure shows increase in the angiogenesis than the normal and increasein the area covered by the primary, secondary and tertiary vitelline veins than normal.

Fig. III & IV –70 µg / ml alcohol extract of M.alba– Figureshows 48 hrs development slight increase in the angiogenesis of CAM than normal and also increase in number and covered by the vitelline veins.

Fig. V & VI –70 µg / ml alcohol extract of M.alba– Figureshows 72 hrs reductions in the normal angiogenesis of CAM than normal and also reduction in number and area covered by the vitelline veins.

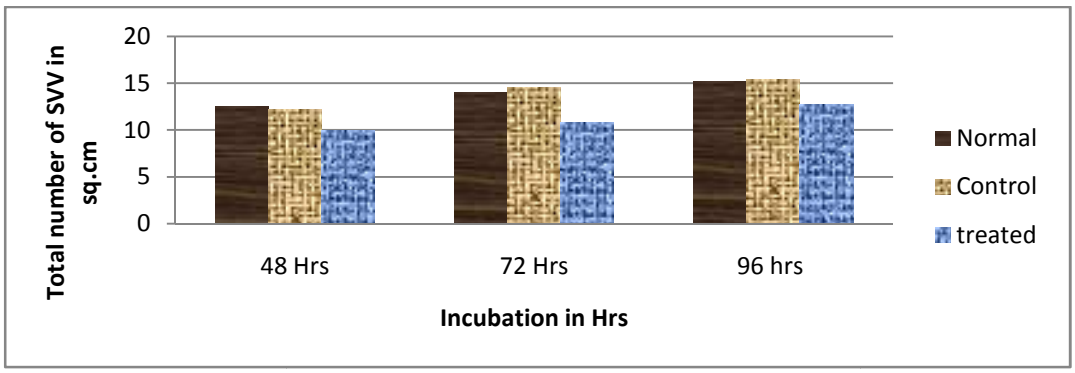
Fig. VII & VIII –70 µg / ml alcohol extract of M.alba— Figureshows 96 hrs reduction in the normal angiogenesis of CAM than normal and also reduction in number and area covered by the vitelline veins.

Plate No. 2: Morphometric evaluation of chick CAM (10X)

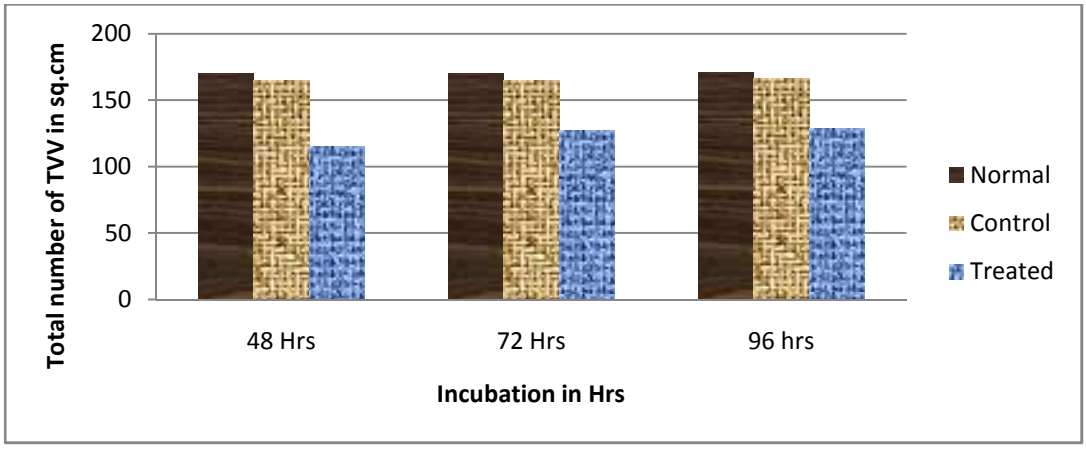


a)Normal b)Control c)M.alba treated

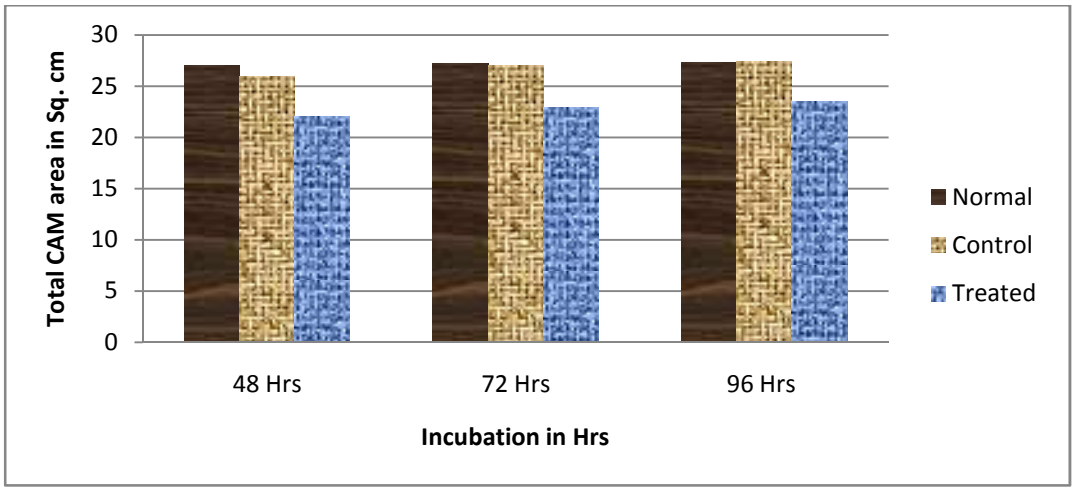
PVV- Primary Vitelline Veins**SVV**- Secondary Vitelline Veins**TVV**-Tertiary Vitelline Veins (zoom 5x 10x)



Graph 1: M. alba leaf extract influenced alterations in number of secondary blood vessels (on 144 Hrs of development)



Graph 2 : M. alba leaf extract influenced alterations in number of tertiary blood vessels (on 144 Hrs of development)



Graph 3: M. alba leaf extract influenced alterations in total area of CAM (On 144 hrs of development)

DISCUSSION:-

Chick embryo study reflects valuable information in further development; it is used as valuable tool to study angiogenesis. Antiangiogenic therapies are being employed to fight cancer and malignancies (e). In present study M.alba plant extract had significant anti angiogenic activity which reduces neovascularization of CAM. The extract of M.alba inhibit normal sprouting of blood vessels in CAM in all treated hrs, but it showed more significant effect in early hours as compared to late of development of CAM. This effect may be due to alkaloids, flavonoids, tannins, cardiac glycosides, carbohydrates, proteins and steroids in the M.alba extract.

A similar study were observed in aqueous root extract of Pulsatilla koreana on hepatocellular carcinoma cells of mouse reported by Sang-Won Hong et. al; 2012. Various plant extract showed anti-angiogenic property reported by N. Boghani and M. Pithawala in 2013. It has also been reported that; acetonic, alcoholic and benzene extract of whole plant of Boerrhavia diffusa on chick chorioallontoic Membrane showed antiangiogenic effect (g).

Aqueous leaf extract of Caesalpenia bonducella showed anti-angiogenic effect on mammary carcinoma cells of mouse and chorioallontoic membrane (CAM) assay of chick by Shankar Jayarama et. al; in 2013.Aqueous leaf extract of Tridax procumbens indicates antiangiogenic effect on CAM assay reported by Mhaske and Gonjari in 2016. Javad Baharara et. al; in 2015 also reported antiangiogenic activity of methanolic extract of Brittle star on CAM in chick.

From the above study it has been noticed that further necessary investigation of phytochemical constituents of extract of M.alba which will inhibit development of secondary and tertiary vitelline veins of CAM. The work in this line is in progress in our laboratory.

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