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EFFECTS OF *PIPER LONGUM* FRUIT EXTRACTS ON CHICK CAM VASCULOGENESIS

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ABSTRACT: Three extracts of *Piper longum* fruits viz, aqueous, alcohol and acetone are evaluated using CAM assay, for embryo growth and CAM vasculogenesis. Each of the extracts was administered in HBSS at 48, 72 and 96 hrs of incubation and studying results at 144hrs. The growth of embryo and CAM was determined using their weights while vasculogenesis was evaluated by measuring the number of secondary vitelline vessels (SVV). Results indicate that all the extracts affected the growth of CAM and embryo and showed significant anti-vasculogenic properties by inhibiting the development of secondary vitelline vessels, aqueous extract being the most potent one. Results are discussed to predict the mode of action of the extracts.

KEYWORDS: *Piper longum*, Chorioallantoic membrane (CAM) assay, secondary vitelline vessels (SVV), vasculogenesis.

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1.INTRODUCTION

Chick embryo development is helpful to use them as models to study vasculogenesis and angiogenesis. They provide set of developmental patterns that can also be used to test drugs of any origin or form so that information of mortality, teratogenicity, growth retardation, different metabolisms as well as systemic toxicity [1-6] can be obtained. It is an alternative, predictive model in acute toxicological studies of anticancer drugs [7] in addition to other testing systems. Our earlier

work includes [8-10] testing of drugs for their influence during cardiovascular development and more precisely of chorioallantoic membrane vasculogenesis and angiogenesis. Such studies may be predominantly helpful to analyze the pro and anti vasculogenic or angiogenic effects of drugs, herbal formulations or others. A study of toxicity testing of *Piper longum* on growth and development of chick embryo through assessment of percent mortality and abnormalities during early embryonic stages has been carried out [11]. The present study assesses the effect of *Piper longum* extracts on vascular development of CAM. *Piper longum* L. belongs to Piperaceae family, commonly known as Indian long pepper and is widely used in traditional system of medicines like Ayurveda and folk medicine. The plant parts most used in Ayurvedic preparations are dried fruits and roots. To list a few the medicinal preparations using *Piper longum* are trikatu, Abhayaristam, pippalayasavam [12]. *Piper longum* is used for the treatment of respiratory tract diseases like cough, bronchitis, asthma, cold, as counter irritant and analgesic. It is used as carminative, usually to treat loss of appetite and sleeplessness [13]. As crude powder, it is applied locally in the form of paste for muscular pain and inflammation. It has been used for chronic and malarial fever, arthritis, gout, allergic conditions of skin, muscular atrophy, goiter, urinary disorders and excess lipids [14]. Extracts of *Piper longum* have also been assessed for various biological activities viz antiamoebic, antigiardial, immunostimulatory, antiulcer and anti-inflammatory properties [15-18]. It is shown to have protective activity against injury, cellular abnormality and cardiotoxicity [19]. Piperine, the main constituent od fruits, shows many properties. It enhances the bioavailability of various drugs and used as a bioavailability enhancer [20]. It is shown to have anticancer and anti-angiogenic properties in cell culture systems [21].

2. MATERIALS AND METHODS

Plant material and extract preparation:

Properly identified dried fruits of *Piper longum* L. were collected from the local market. The powdered fruits are strained through muslin cloth. Routine methods were used to get aqueous, acetone and alcohol extracts of the powder. The yield of aqueous, acetone and alcohol extract were 10.5%, 8.9 % and 7.9 % respectively. The dried extracts were dissolved in Hanks Balanced Salt Solution (HBSS-HIMEDIA, India) to prepare the stock solutions so that suitable concentrations were used for the treatments.

Dose selection:

Based on the evaluation of the mortality of eggs and abnormality studies with different concentrations of the extracts as discussed earlier [11], 0.1mg/egg of aqueous extract, 0.025mg/egg of alcohol extract and 1mg/egg of acetone extract were selected for their vasculogenic and angiogenic response. Minimum effective doses with full survival and without any abnormalities or deformities were selected for the present study.

Extract administration hours:

At 48 hrs, begins the CAM vasculature formation, at 72 hrs vitelline vein have been well established and at 96 hrs, it is the pre-terminal phase of vasculature development of CAM. Therefore, these three are major intervals in CAM development and vascularization takes place until 144 hrs when CAM is fully developed and hence selected for the present study.

Experimental Design:

Fertilized eggs of *Gallus gallus* were purchased from Quality Poultry Products, Malgaon (Tal. Miraj, Dist. Sangli, 416416, MS, India). It was ensured that the eggs were of similar size and weight and were checked for damage if any. The shells were disinfected by standard procedure (using 70% alcohol). The incubation temperature for embryos was 37.5ºC and relative humidity was 70-75%. This was maintained until desired stage of development. The selected doses of different extracts were initiated at selected hours (Table 1) and development was continued up to 144 hrs. i.e., till the completion of CAM development and capillary networking. Sterile conditions were maintained throughout the experimental period.

Dose administration by Window Method [22]:

On scheduled period of incubations for the desired treatments, the windows were prepared in embryos under aseptic conditions and extracts of *Piper longum* L. were spread on the embryonic plates in the final volume of 0.5 ml HBSS. The concentrations were adjusted in the final volume of 0.5 ml HBSS and were spread on the embryonic plate uniformly in different embryo segregates (Table 1). In each segregate, six eggs were included. All the treatments were given in final volume of 0.5ml of HBSS with appropriate pH and composition. Normal group of embryos were maintained as normal group. Embryos of operative control were sham operated for window preparation and embryos of HBSS control received 0.5ml of HBSS each. The HBSS used and all the treatment doses were brought to 37ºC before administration. Each of the windows made for administration were sealed with sterilized adhesive tape and the embryos were immediately transferred to incubator adjusting the experimental time slot until completion of 144 hrs. All the sterility precautions were followed and maintained during the experimental work.

Table 1: Exposure schedule of extracts of *Piper longum* at different developmental stages of chick embryo.

Group (each containing 6 eggs)	Developmental stage in hrs	Corresponding HH stage	Time of exposure to treatment of doses in			Final observation at hrs
			48	72	96	
I	48	12	√	---	---	144
II	72	20	---	√	--	144
III	96	24	---	---	√	144

Quantification of growth of embryo

Growth of embryos and development of CAM were assessed by taking embryo weight and CAM weight. A fine balance (Shimadzu BL-220H) was used for the weight measurements. Weights of embryos and CAM after different treatments are given in Table 2

Quantification of vasculogenesis

Vasculogenesis was quantified by using morphometry and microscopic observations. For CAM analysis, computerized image analysis system was used. Number of primary vitelline vessels (largest) were measured. Total number of secondary vitelline vessels (branches of primary vitelline vessels) were measured (initiation of branching point to capillary network branching). The observed alterations are presented in Table 2, and Figs 2 (a& b).

Statistical analysis

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

3. RESULTS 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 weights of embryos in Normal, Sham operated and HBSS treated groups were very marginal and statistically not significant. Weight of embryo on treating with HBSS at 48 hrs was reduced by 9.9%, at 72 hrs was reduced by 8.8 % and at 96 hrs was reduced by 10.0% ($p>0.05$ at each level). Embryo weight was increased by 1.02-folds at 48 hrs on administration of aqueous extract. The weight was reduced by 16.9% ($p<0.001$) and 11.2 % ($p<0.01$) at 72 and 96 hrs respectively. Reduction in the weight of embryo on administration of alcohol extract was 9.8%, 13.3% and 12.4% respectively at 48, 72 and 96 hrs of dose administration. On administration of acetone extract, the weight of embryo was reduced by 20.8% ($p<0.001$), 24.4% ($p<0.001$) and 7.4% ($p>0.05$) at 48, 72 and 96 hrs of dose administration respectively. The alterations in the weights of embryo and CAM at different hours of dose administration are presented in Figure 2 The differences in the weight of CAM in normal and sham operated eggs were statistically insignificant. On administration of HBSS alone, weight of CAM was increased by 1.11-folds at 48 hrs, 1.07-folds at 72 hrs and 1.08-folds at 96hrs respectively. These changes were statistically not significant. On administration of aqueous extract at 48, 72 and 96 hrs, the weight of CAM was decreased by 8.9%, 9.2% and 5.3%. The alcohol extract administration showed decrease in the CAM weight by 7.7%, 5.2% and 6.5% at 48, 72 and 96 hrs respectively, when compared with HBSS controls. These changes were not significant. Administration of acetone extract initiated at 48 hrs showed 2.5% decrease, at 72hrs and 96 hrs the reduction was 23.7% each ($p<0.001$).

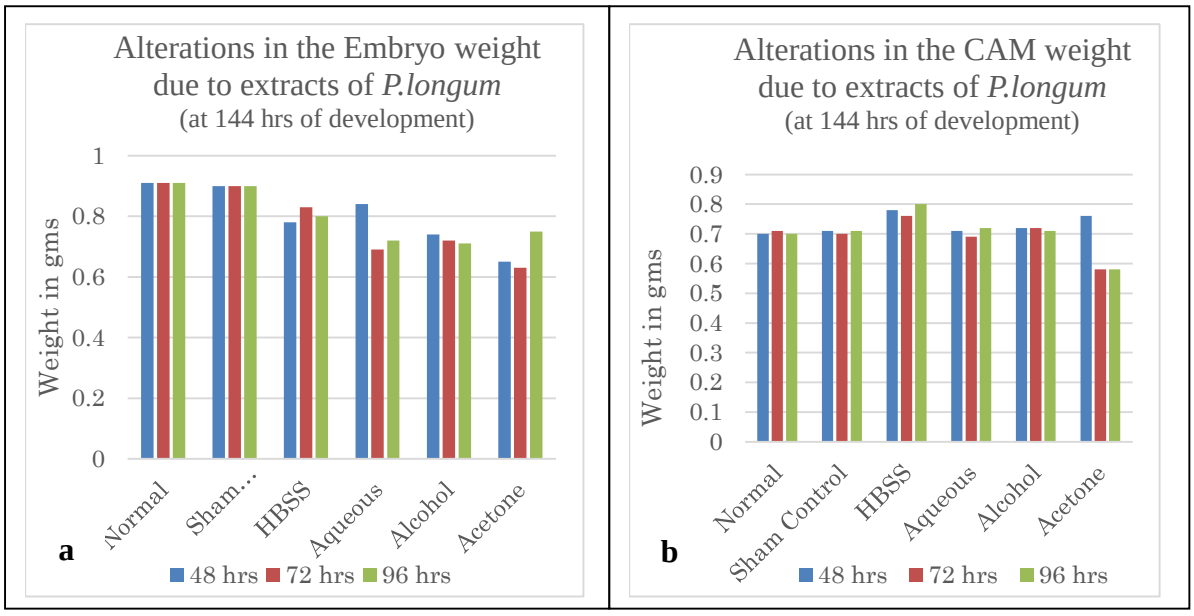

Figure 2 a, b: Alterations in the weights of embryos and CAM at 144 hrs due to administration of Piper longum extracts (aqueous, alcohol and acetone) at 48, 72 and 96 hrs of incubation

Analysis of CAM for blood vessels:

The analysis of whole mounts of embryos was done using visual images of the CAM development as shown in Figure 1. The observation of normal and sham control CAM at 144 hrs of development showed normal vasculogenesis and angiogenesis with dendritic branching pattern of blood vessel formation. Vasculogenesis was quantified by numerical analysis of total CAM blood vessels. Primary vitelline veins and secondary vitelline veins were studied for antivasculogenic response. The primary vitelline veins in all these experimental groups, that included normal and sham operated controls remained unchanged (hence data not represented here). The dendritic pattern of blood vessel formation was undisturbed in all these experimental groups and the number of secondary vessels were studied. The number of secondary vitelline veins were changed insignificantly in the corresponding embryos of normal, sham operated and HBSS controls at all hours of treatment initiation. When aqueous extract was administered, the secondary vitelline veins were decreased by 37% ($p<0.001$) at 48 hrs, by 43% ($p<0.001$) at 72 hrs of incubation while at 96 hrs of incubation the number was decreased by 52% ($p<0.001$) as compared to number of veins in HBSS treated embryos. Alcohol extract administration at 48 hrs, 72 hrs and 96 hrs of incubation affected the number of secondary vitelline vessel by 26%($p<0.001$) 33% ($p<0.001$) and 28.5 % ($p<0.001$) respectively. The acetone extract administration at 48 hrs reduced the number of secondary vessels by 26% ($p<0.001$). This reduction was 19% ($p<0.01$) at 72hrs and 14% at 96hrs. Quantitative data is represented in Table 2 and Figure 3.

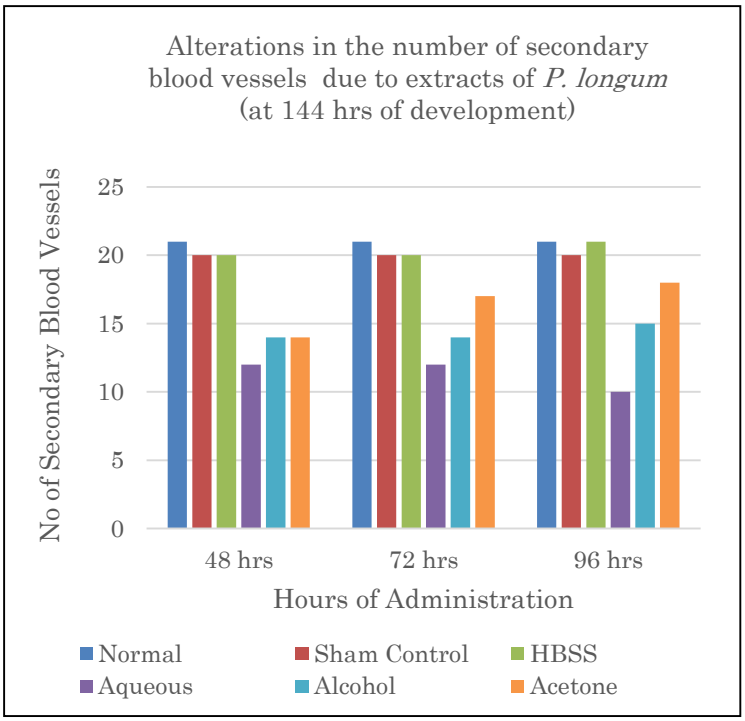

Figure 3: The alterations in the secondary blood vessels at 144hrs of development after 48, 72 and 96 hrs of treatment initiation of *P. longum* extracts (aqueous, alcohol and acetone)

DISCUSSION:

The weight of embryos and CAM if compared in experimental animals shared interesting pattern. At 48hrs, the alterations in embryo and CAM weights share inverse relationship while at 72 hrs, they share very close parallel relationship and at 96 hrs same pattern is followed by aqueous and alcohol extract mediated changes. With the acetone extract they shared an inverse relationship between CAM weight and embryo weight. Secondary vitelline vessels (SVV) at 48 hrs dropped in number with all extracts and was parallel with the trend of CAM weight alterations. At 72 hrs, the number of SVV dropped significantly with aqueous and alcohol extracts but with acetone extract there was no change. With the initiation of treatments at 96hrs, the trend was similar with the 72hrs initiation observations of number of SVV. 30% loss in the number of SVV was common for alcohol extract at all hour of dose initiation. Secondary vessel inhibition was highest with aqueous extract whereas acetone extract did not show any change. All the three extracts show a similar pattern of inhibition of SVV at 48, 72 and 96 hrs of incubation. The same level of inhibition in the SVV due to alcohol extract at 48, 72 and 96 hrs of treatment initiations could be due to piperine which is more soluble in alcohol. Its effect seems to be independent of treatment initiation. Maximum inhibition due to aqueous extract indicates its increased potency and could be exercised by other extract components. Acetone extract seem to contain variable components that differ in their action at different hours of treatment initiation. Risau summarized the process of vasculogenesis and angiogenesis. Fibroblast growth factor family is crucial in inducing paraxial and lateral plate

mesoderm to form angioblasts and hematopoietic stem cells [23]. Angioblast cells form the primitive blood vessels at or near the site of origin. The differentiation of angioblast cells from mesoderm and the formation of primitive blood vessels are the two distinct steps of vasculogenesis. Vascular endothelial growth factor receptor-2 (VEGFR-2, also known as flk-1 in mouse and KDR in humans) acts as an important regulator of embryonic and adult blood vessel development [24, 25]. VEGF receptors and sufficient levels of their ligands are necessary for vasculogenesis. Induction of VEGFR-2 may initiate angioblast differentiation but quantity and activity of VEGF ligands is responsible for angioblast survival [23]. Regulatory mechanisms of VEGF-receptor and VEGF induction and commitment of cells remain to be revealed. Once the primary plexus formation is established further vasculature is developed by sprouting and intussusceptive mechanisms of angiogenesis. Pruning and remodeling takes place rapidly to resemble a mature system with larger and smaller vessels. Thus, inhibition of secondary blood vessels only, by extracts of *Piper longum*, may be due to component/s of extracts in independent or synergistic action. They possibly interfere the pruning and remodeling of the structure at the development of secondary blood vessels.

Table 2: Effect of extracts of *Piper longum* at various hours of incubation on growth parameters of chick embryo

Initiation Hours	Groups	No of secondary blood vessels	Weight of embryo	Weight of CAM
48	**Normal**	21±3	0.91±0.08	0.70±0.081
	Sham Control	20±2	0.90±0.09	0.71±0.083
	HBSS	19±3	0.82±0.08	0.79±0.040
	Aqueous extract	12±1.2	0.84±0.09	0.70±0.81
	Alcohol Extract	14±1.3	0.74±0.06	0.82±0.10
	Acetone extract	14±1.1	0.64±0.06	0.84±0.09
72	**Normal**	21±2	0.91±0.08	0.70±0.12
	Sham Control	20±1.5	0.90±0.09	0.71±0.11
	HBSS	21±1.5	0.83±0.03	0.75±0.02
	Aqueous extract	12±1.0	0.69±0.08	0.59±0.08
	Alcohol Extract	14±1.1	0.72±0.029	0.60±0.008
	Acetone extract	17±1.6	0.59±0.14	0.57±0.08
96	**Normal**	21±1.9	0.91±0.08	0.70±0.12
	Sham Control	20±2.1	0.90±0.09	0.71±0.11
	HBSS	21±1.6	0.81±0.08	0.81±0.06
	Aqueous extract	10±1.1	0.72±0.19	0.74±0.11
	Alcohol Extract	15±1.2	0.71±0.11	0.58±0.04
	Acetone extract	18±1.91	0.75±0.09	0.58±0.04

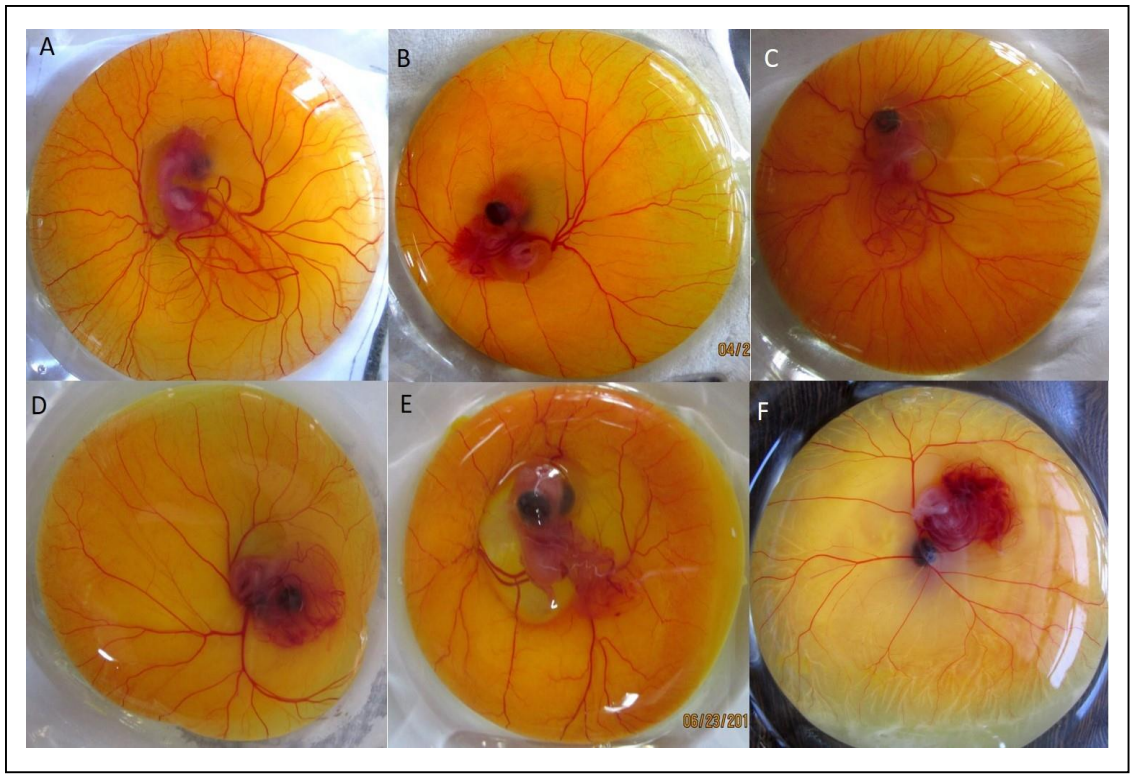

Figure 1: Images showing comparison of vascular development of different treatment groups of eggs. The groups are normal (A), Sham operated for window (B) and HBSS treated (C). The images (D-F) show CAM development on treatment with aqueous extract at 48 hrs, 72 hrs and 96 hrs respectively.

4. CONCLUSION

Aqueous, acetone and alcohol extracts do not affect the growth of embryo at any hours of dose administration which indicates that these extracts do not disturb the general growth of the embryo. Similarly, CAM growth is also not affected by these extracts at all hours of development except at 96 hrs where only acetone extract has caused significant decrease in CAM weight. The effect of aqueous, acetone and alcohol extracts at corresponding hours of dose administration on vasculogenesis is significant and the loss of number of secondary vitelline vessels due to aqueous extract is comparatively more. Thus, the aqueous extract of *Piper longum* is more potent anti-vasculogenic agent and the effect could be due to water soluble components present in the fruits.

6. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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