



Effect of *Piper longum* Fruit Extract on Mortality of *Gallus gallus* Chick Embryo at Early Stage of Development

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Abstract

Piper longum dried fruits are used in various indigenous medicines that are effective against many diseases and disorders. This study aims at evaluating the effect of *Piper longum* L fruit extracts on mortality of *Gallus gallus* chick embryo at early stage of development with associated deformities if any. Different concentrations of aqueous, alcoholic and acetone extracts of dried fruits of *Piper longum* L were administered by Window method at 48hrs, 72hrs and 96hrs of development and embryos were evaluated for mortality status and associated deformities on 144hrs of development and hatching. Results revealed that aqueous and alcohol extracts at high concentrations were responsible for 80-100% mortality, while acetone extracts with highest dose showed 100% mortality. At low dose influence of administration hours was seen, leading to 20% to 70% mortality. All the mortalities were associated with abnormalities viz. growth retardation, underdeveloped CAM, hemorrhage in various regions and abnormal torsion. But lower doses the animals that survived were normal and their further development was normal.. The results indicated dose dependent toxic effect of *Piper longum* fruits extracts. It causes lethal deformities which might have been associated with bioactive compounds with cytotoxic action.

Keywords

Piper longum L., *Gallus gallus*, Mortality, Window Method, Cytotoxicity



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INTRODUCTION

For any herb or chemical to be implemented clinically against different disorders, claims need to be tested pre-clinically for the toxicity using suitable animal model system. Chick embryo is one of the widely used model for developmental toxicity studies. It provides information of mortality, teratogenicity, growth retardation, metabolism as well as systemic toxicity¹⁻⁶. It is an alternative predictive model in acute toxicological studies of anticancer drugs⁷. In our early laboratory studies, chick embryo at early stage of development was used to assess various plant extract induced toxicity with associated deformities⁸⁻¹⁰; findings of which were used in deciding the possible concentrations doses for induced cardiovascular alterations. Similarly the present study is based on a toxicity testing of *Piper longum* on growth and development of *Gallus gallus* chick embryo through assessment of % mortality and abnormalities during early embryonic stages.

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, pippalayavasavam¹¹. *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 in conditions such as loss of appetite and sleeplessness¹². 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 lipids¹³. Extracts of *Piper longum* have been reported for various biological activities vizantiamoebic, antiangiogenic, immunostimulatory, antiulcer and anti-inflammatory properties¹⁴⁻¹⁷. *Piper longum* is shown to have protective activity against injury, cellular abnormality and cardiotoxicity¹⁸. Piperine being the main constituent shows many properties. It enhances the bioavailability of various drugs and used as bioavailability enhancer¹⁹. It is shown to have anticancer and anti-angiogenic properties in cell culture systems²⁰.

The present study was aimed to investigate mortality and developmental toxicity if any



in *Gallus gallus* chick embryo induced by *Piper longum* fruits extracts using different solvents.

MATERIALS AND METHODS

Plant material and extract preparation:

Properly identified dried fruits of *Piper longum* L. were procured from the local market. The fruits were powdered and strained through muslin cloth. The powder was extracted by routine methods to get aqueous, acetone and alcohol extracts. The yield of aqueous, acetone and alcohol extract was 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 applications.

Experimental Design:

Fertilized eggs of *Gallus gallus* were obtained from Quality Poultry Products, Malgaon (Tal. Miraj, Dist. Sangli, MS, India). The eggs of similar size and weight were selected and checked for damage. The shells were disinfected with 70% alcohol. The eggs were incubated at 37.5°C temperature with relative humidity at 70-75% and maintained until desired stage of development. The treatment doses of varying concentrations of fruit extracts using different solvents were initiated at selected hours (Table 1) and development was continued up to 144 hrs. i.e., on completion of CAM development and capillary networking. Sterile conditions were maintained throughout the experimental period.

Table 1 Exposure schedule of extracts of *Piper longum* at different developmental stages of chick embryos

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

*Dose administration by Window Method*²¹:

After scheduled period of incubations 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. Different concentrations, adjusted in the final volume of 0.5 ml, were spread on the embryonic plate uniformly in different embryos in segregates (Table 1). All the

**Table 2** Average mortality for different extracts of dried fruits of *Piper logum*

Sr. no	Extract treatment	Experimental conditions of treatment and percent mortality at 144 hrs						Abnormalities at 144hrs
		Normal	Sham	HBSS	48 hrs	72 hrs	96 hrs	
Aqueous extract								
1	0.1 mg	10% at all	6-9% at all	5-6% at all	20	42	20	Normal
2	0.3 mg	hrs of	hrs of	hrs of	40	40	30	Normal
3	0.5 mg	treatment	treatment	treatment	60	80	60	Growth retard
4	1.0 mg	(Normal embryos)	(Normal embryos)	(Normal embryos)	80	75	65	Brain development hampered, abnormal torsion
5	1.5 mg				100	100	80	Abnormal torsion
Acetone extract								
1	0.3 mg	10% at all	6-9% at all	5-6% at all	75	42	50	Growth retard
2	0.5 mg	hrs of	hrs of	hrs of	100	70	0	Growth retard
3	1.0 mg	treatment	treatment	treatment	50	17	50	Normal
4	2.0 mg	(Normal embryos)	(Normal embryos)	(Normal embryos)	75	70	70	Growth retard, underdeveloped CAM, hemorrhage, abnormal torsion
5	3.0 mg				100	100	100	
Alcohol extract								
1	0.025 mg	10% at all	6-9% at all	5-6% at all	50	0	30	Normal
2	0.05 mg	hrs of	hrs of	hrs of	70	0	35	Normal
3	0.1 mg	treatment	treatment	treatment	70	25	50	Normal
4	0.5 mg	(Normal embryos)	(Normal embryos)	(Normal embryos)	100	80	80	Growth retard, Brain development hampered, abnormal torsion

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. The HBSS and all the treatment doses were brought to 37°C before administration. The window made for administration was sealed with sterilized adhesive tape and the embryos were immediately transferred to incubator adjusting the experimental time slot until completion of 144 hrs.

After 144 hrs of incubation, the shells were removed and all the embryos were examined for survival and any external deformities. Similarly, abnormalities were also observed at hatching as well.

RESULTS AND DISCUSSION

Mortality study is the ultimate biomarker of toxic effect of any drug or biomaterials, as it indicates lethal action of active bio-compounds present in it, while abnormalities among embryos indicate the teratogenic potency of compounds²²⁻²⁴. In the present investigation, single dose treatment of



various concentrations of *P. longum* fruits using different solvents such as aqueous, alcohol and acetone extracts were initiated at 48, 72 and 96 hrs of development (Table 1) to determine the toxicity leading to mortality and morphological abnormalities if any. Total weights and survival on hatchability and abnormalities were noted. Results are presented in Table 2.

Results revealed 8-10 % mortality with 92-90 % survival in untreated embryos with no morphological abnormalities at 144 hrs of development. While Sham operated control group showed 10.3-10.7% mortality with 89.7-89.3% survivals. All the embryos of operative control groups were normal. HBSS control embryos showed 4.5-5% mortality at all developmental hours studied. These results indicated that % mortality was decreased with HBSS treatment by 3.5-5% as compared to the normal and 5.8-5.7 % as compared with Sham/operative control at 144 hrs of development with no indication of any morphological abnormalities/malformations.

The administration of aqueous extract exhibited linear dose dependent mortality pattern at 48, 72 and 96 hours of treatment. The abnormality/deformity analysis of dead embryos showed that the embryos with

lower doses (0.1 mg, 0.3mg) were normal and without any morphological deformity. The embryos treated with higher doses (0.5 mg and 1.0 mg) showed only retardation in growth. They appeared normal in overall development but smaller in size; whereas in embryos treated with higher doses of *Piper longum* fruits extracts in every solvent (1.5 mg and 2.0 mg), exhibited increased mortality with gross morphological damage and deformities such as abnormal torsion and twisting, hampered brain development and vascular damage. All these deformities are associated with hemorrhage in embryo and on CAM. Few embryos showed reduced allantois.

The administration of acetone extracts of *Piper longum* fruits was more lethal at lower concentration (0.3 mg and 0.5 mg) and also at very high concentration (2.0 mg and 3.0 mg). All the doses showed more mortalities at early hours of development than at later hours of development. When the dead embryos were observed for lethal abnormalities, it was observed that the embryos at lower doses were normal but with earlier developmental stages (HH stage 26-28, HH stage at 144 hrs should be 28-29). The weights of these embryos were found to be less than normal (unpublished



data). Embryos with higher doses treatments significantly showed retarded growth, abnormal torsion and twisting, hampered brain development, underdeveloped CAM with hemorrhage.

Alcohol extract was most potent among the tested extracts. Concentration of 0.5 mg itself was 100% lethal and no embryo survived when treatment was initiated at lower hours of development. At lower concentration (0.025 mg and 0.05 mg) the cause of death was total growth retard. Treatments of higher concentrations (0.1 mg and 0.5 mg) also showed higher number of mortalities with lethal deformities such as abnormal torsion and twisting, vascular damage, hampered brain development and hemorrhage in embryo as well as on CAM.

The death causing abnormalities in all the three extracts viz. aqueous, acetone and alcohol were of similar kind (Figure 1). It is a well known fact that any change, sufficiently abrupt to interfere with the circulation of blood and hence oxygen and nutrients would be a disaster for the embryo²⁵. The lethal deformities observed in dead embryos upon administration of extract can be correlated with the interference of a/or mixture of active principles with the circulatory system through cell signaling

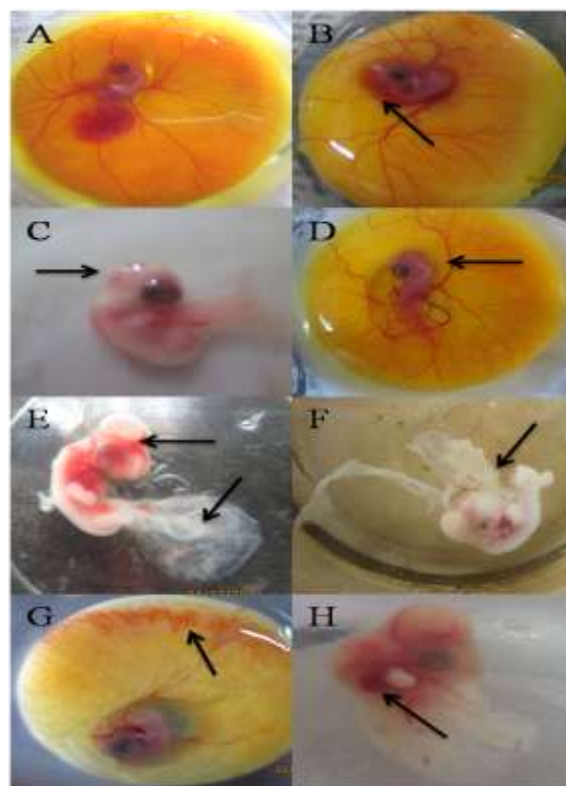


Figure 1: Images showing the comparison of normal embryo and abnormalities observed during early development of chick embryo due to toxicity of various extracts of dried fruits of *Piper longum* L (A; Normal embryo, B-H, Abnormalities associated with embryos survived and/or found dead at various doses) B: hemorrhage inside the embryo sac and growth retards (P.I. aqueous 0.5mg at 96 hrs); C: retarded development of brain (P.I. aqueous 0.5mg at 96 hrs, dead embryo); D: abnormal torsion and twisting of the embryo (P.I. acetone 2mg at 48 hrs treatment); E: huge internal hemorrhage in the brain and the body, no blood vessel to the allantoise, (P.I. aqueous 0.5mg at 96 hrs, embryo dead) F: retarded growth, no blood vessels either to CAM or the embryo (P.I. acetone 0.5 mg at 72 hrs, embryo dead), G: Hemorrhage on CAM surface with deformed vasculature (P.I. alcohol 0.1mg at 72 hrs, embryo dead), H: retarded growth, internal hemorrhage, no differentiation at the lower body of the embryo giving a tumor like appearance, (P.I. aqueous 1.5 mg at 48 hrs, embryo dead), arrow



marks in each image point towards the reported abnormality

pathways. Vascular damage and hemorrhage observed in embryo as well as on CAM strongly support such correlation. Any alterations in vitelline hemodynamics lead to implications in cardiovascular development²⁶. The above stated dose dependent toxic effects of *Piper longum* fruits extracts could be due to its biologically active compounds more possibly by the phytochemicals having cytotoxic action. A cell cycle inhibitory activity with cytotoxic action of *Piper longum* has been reported as well²⁷. A major active component of *piper longum* fruits is piperine. Piperine is shown to have cytotoxicity on inflamed macrophages²⁸, against HeLa cells²⁹. It induces neurotoxicity with possible involvement of lipid peroxidation³⁰. Piperine is shown to promote DNA damage and cytotoxicity induced by benzo[*a*]pyrene (B[*a*]P) in cultured V-79 lung fibroblast cells³¹. Piperine and piperlongumine, both, show toxic effects in brine shrimp and affects development of sea urchin eggs³². Piperine is shown to inhibit proliferation of human mononuclear blood cells and hence cytokine production³³. Another component

piperlongumine is also shown to have cytotoxicity as it selectively kills the transformed cells and not the normal cells³⁴. Piperine, the main constituent of fruit used as bioavailability enhancer³⁵. Growth factors, hormones and other proteins present in amniotic fluid are essential for embryo development^{36,37}. These factors may have been altered by the active compounds leading to reported spectrum of abnormalities. However, embryo groups treated with 0.1 mg and 0.3 mg of aqueous extracts, 0.025 mg, 0.05 mg and 0.1 mg of alcohol extract and 1.0 mg of acetone extract were free of any abnormalities. No abnormality and minimum mortality observed in aqueous and alcoholic extract doses (at lower concentrations) may be related to the counter acting anti-damage principle/s responding to differentiating status of embryo.

These findings suggested that *Piper longum* fruit extracts with different solvents have dose dependent teratogenic effects causing lethal deformities in early stage of embryonic development indicating sensitivity of early developing embryos toward extracts. It might be because of cytotoxic action of bioactive compounds present in fruits. These bioactive compounds



can be analyzed further for their specific action. Thus, mortality and toxicity data is helpful to decide influences of components of extracts and possibility of drug development using further isolation of effective components. Besides, the observed, associated and possible metabolic and teratogenic effects being already known, further drug development process can be cautioned. High concentration doses also indicate specific extract sensitive organs/metabolisms/developmental status which is also important in further steps of drug development. This data that gives safe doses also forms basis for further analysis of components on various metabolisms and developments. Present data forms the basis of further studies of the influence of integrated components of extracts on anti/pro angiogenic effects as well cardiac development.

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