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IN-VIVO ASSESSMENT OF EMBRYO TOXICITY POTENTIAL OF IMMUNE BOOSTING SIDDHA FORMULATION NELLIKAI LEGIUM DURING GESTATION PERIOD

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ABSTRACT

Zebrafish model provides valuable insight into teratogenic risk assessment of drugs, chemicals, traditional medicines and other pharmaceuticals. This model has also been recommended as an alternative to fish acute toxicity. Embryo toxicity assay is most reliable model for predicting the teratogenic potential of the formulation and other new drug entities. Zebrafish (*Danio rerio*) attains greater importance in the field reproductive biology due to it's close genetical resemblance with respect to that of the humans. The main aim of the present investigation is to evaluate the embryo toxicity profiling of the siddha formulation Nellikai Legium using Zebrafish embryos. The concentration of the test drug ranging from 10µg to 320 µg/ml upon 24 to 96 hours post fertilization (HPF) exposure at 30 embryos per concentration. The results obtained from the present investigation has clearly shown that there is no significant difference in movement analysis of the drugs treated groups (7.1 ± 1.155 to 7.733 ± 0.90 per min) with that of the control embryos with movement of 7.667 ± 1.184 per min. The data obtained from the present investigation reveals that there is no significant difference in heart rate of the drugs treated groups (124.1 ± 2.255 to 126.5 ± 2.286 beats/min) with that of the control embryos with heart rate of 125.1 ± 2.27 beats/ min. Further there was no abnormalities were detected in observed parameter's such hatching rate, blood circulation,

pigmentation, swimming pattern, balancing and morphogenesis. It was concluded from the results of the present investigation that siddha formulation Nellikai Legium was safe and didn't alter any of the physiology of the developing fetus upon 24 to 96 HPF exposure.

KEYWORDS: Siddha formulation, Nellikai Legium, Zebrafish embryos, Toxicity profiling, Hours post fertilization.

1. INTRODUCTION

Siddha system of medicine holds holistic place in the series of Indian traditional medicines as its pioneering the therapy of treating both communicable and non-communicable diseases. Validation of several siddha formulation is still not attempted as there is a global need of exploration of siddha preparation there is a dire need of safety assessment in more reliable manner. One of the most promising organisms for diagnostic in vivo testing of sediment extracts is zebrafish (*Danio rerio*) embryos, which are a versatile model suitable for high-throughput analysis while keeping several advantages of in vitro approaches (i.e., low-cost, sensitivity, short duration of the test).

The fish embryo toxicity test (FET) with Danio rerio has been considered as a good surrogate for the acute toxicity fish test[1] and was successfully used in several studies for the detection of toxicity and neurotoxicity in sediments samples.[2-4] One of the major advantages of the FET with Danio rerio is the possibility to monitor several toxic endpoints including the modification of molecular processes and malformations which can be related to the exposure to specific pollutants.[5-7] Further, the FET with Danio rerio offers the possibility to monitor changes in behavioral patterns (i.e., swimming activity, early spontaneous movements), which may be relevant also for the population level.[8,9]

The ability to observe effects of toxins in vivo allows for direct assessment of toxicity, as well as measurements of absorption, distribution, metabolism, and elimination. This can be extended for use in screening for treatments that can mitigate toxic effects in live animals as well. Zebrafish express a full range of *cytochrome P450* (*cyp*) genes required for xenobiotic metabolism and biotransformation.[10] In the zebrafish genome assembly (GRCz10), a total of 86 *cyp* genes were identified[11] with many of the metabolic characteristics of the related human enzymes, demonstrating a strong evolutionary relationship with those found in humans.

Siddha formulation Nellikai Legium widely reported for several indication as per standard literatures despite the widely reported safe and therapeutically applications of this novel formulation, there is no research finding reporting the embryotoxic and teratogenic effects of this drugs. Hence the main aim of the present study is to evaluate the teratogenic potential of the formulation using fish embryo toxicity assay as per OECD guideline.

2. MATERIALS AND METHODS

2.1. Selection of Species

Healthy male and female zebrafish continuously monitored for two successive generations were been used for breeding to collect the embryos for the present investigation. Fishes are free from malformation, sings of infections and other illness.

2.2. Guideline[12]

Toxicity tests was referred to the Organization of Economic Cooperation and Development (OECD) guideline; No. 236: Fish Embryo Acute Toxicity (FET) Test.

2.3. Embryo Stock

Sterilized E3 Medium was utilized for maintenance of liver embryos. 8 ml of the sterilized medium dissolve to one liter to get 1X stock with inclusion of 100 µL of 1% methylene blue used as fungicide.

2.4. Test Condition

Semi-static renewal test is a test with regular renewal of the test solutions after defined periods (every 24 hours).

2.5. Dosing of Test Drug[13]

Every day fresh medium was being added to the plates containing embryo. Desired concentration of the test drug was added to the respective plates classified according to the strength.

2.6. Embryo Collection and Incubation

Fertilized embryos are subjected to the surface sanitation and were transferred into culture plates (30 embryos/plate/ Dose concentration) containing test drug ranging from 10µg to 320 µg/ml. Medium without drug served as control. Exposure involved semi-static renewal condition, 25°C ± 1°C and 14 light: 10 dark cycle period. Plates were sealed to minimize evaporation. Embryo development was monitored 24 h interval of 96 h. 29 Parameters as

listed in Table 1,2 and 3 were evaluated. Inverted Microscope equipped with phase contrast function, camera and software for image optimization. Mortalities if any are recorded at 24, 48, 72 and 96 hours.

2.7. Statistical Method

The statistical analysis was carried by one-way analysis of variance ANOVA (GRAPH PAD PRISM 5 computer program). Results are expressed as ± SD.

3. RESULTS

3.1. Effect of Nellikai Legium on Toxicity Profiling in Zebrafish Embryo with the exposure of 24 – 96 HPF

In the present investigation, zebrafish embryos were used to analyze the possible toxicity including movement behavior at 24, 48, 72 and 96 h post fertilization (HPF), after treatment with Nellikai Legium at the concentration ranges from 10µg to 320µg/ml. The observation of the study reveals no abnormality of the test drug with respect to hatching rate, pigmentation development, hatching rate, posture analysis, organogenesis etc. As shown in Table 1 and Figure 1.

Table 1: Effect of Nellikai Legium on Toxicity Profiling in Zebrafish Embryo with the exposure of 24 – 96 HPF.

		Concentration						
S.No	**Parameters**	**Control**	**10 µg**	**20 µg**	**40 µg**	**80 µg**	**160 µg**	**320 µg**
1.	Developmental Abnormality	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
2.	Loss of equilibrium	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
3.	Pigmentation	**N**	**N**	**N**	**N**	**N**	**N**	**N**
4.	Abdominal distension	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
5.	Migration of melanophores from neural crest	**AN**	**AN**	**AN**	**AN**	**AN**	**AN**	**AN**
6.	Puffy	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**
7.	Bent tails/body axes	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
8.	Pericardial edema	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**
9.	Peritoneal edema	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**
10.	Percentage survival	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
11.	Hatching rate	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
12.	Heart beat	**N**	**N**	**N**	**N**	**N**	**N**	**N**
13.	Yolk sac edema	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**	**NSA**
14.	Hyper pigmentation	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
15.	Pectoral fin malformation	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**

3.2. Effect of Nellikai Legium on Developmental abnormality analysis on Zebrafish Embryo with the exposure of 24 – 96 HPF

Result analysis of the present study clearly reflects that there is no abnormality detected with respect to the following parameters such as Trunk axis, Spontaneous Movement, Heart formation, Curved or Bent axis, Eye malformation, Blood circulation, Mortality, Arrested growth, Craniofacial malformations, Peripheral ischemia and disruption of erythropoiesis, Accumulation of fluid around heart, Response to stimuli, Coagulation of eggs, Tail detachment etc. As shown in Table 2 and Figure 1.

Table 2: Effect of Nellikai Legium on Developmental abnormality analysis on Zebrafish Embryo with the exposure of 24 – 96 HPF.

S.No		Concentration						
	Parameters	**Control**	**10 µg**	**20 µg**	**40 µg**	**80 µg**	**160 µg**	**320 µg**
1.	Trunk axis	**AN**	**AN**	**AN**	**AN**	**AN**	**AN**	**AN**
2.	Spontaneous Movement	**N**	**N**	**N**	**N**	**N**	**N**	**N**
3.	Heart formation	**N**	**N**	**N**	**N**	**N**	**N**	**N**
4.	Curved or Bent axis	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
5.	Eye malformation	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
6.	Blood circulation	**N**	**N**	**N**	**N**	**N**	**N**	**N**
7.	Mortality	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**
8.	Arrested growth	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
9.	Craniofacial malformations	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
10.	Peripheral ischemia and disruption of erythropoiesis	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
11.	Accumulation of fluid around heart	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
12.	Response to stimuli	**N**	**N**	**N**	**N**	**N**	**N**	**N**
13.	Coagulation of eggs	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**
14.	Tail detachment	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**	**NO**

NO-None Observed, N-Normal, AN-Appears Normal, NSA-No such Abnormality were observed.

3.2. Effect of Nellikai Legium on Developmental abnormality analysis on Zebrafish Embryo with the exposure of 24 – 96 HPF

Zebrafish embryos have very stringent locomotive behavior which typically resembles with that of the movement behavior in rodents. After coming out of the chorionic sac at 48 HPF zebrafish exerts a dynamic change in their motility pattern. The results obtained from the present investigation has clearly shown that there is no significant difference in movement analysis of the drugs treated groups (7.1 ± 1.155 to 7.733 ± 0.90 per min) with that of the control embryos with movement of 7.667 ± 1.184 per min. The data obtained from the

present investigation reveals that there is no significant difference in heart rate of the drugs treated groups (124.1 ± 2.255 to 126.5 ± 2.286 beats/min) with that of the control embryos with heart rate of 125.1 ± 2.27 beats/ min. As shown in Table 3.

Table 3: Effect of Nellikai Legium on Embryo Movement and Heart Beats at 24 and 48 HPF.

Parameters	Concentration						
	Control	10 µg	20 µg	40 µg	80 µg	160 µg	320 µg
Embryo Movement at 24 HPF	7.667 ± 1.184	7.233 ± 0.8976	7.567 ± 0.9714	7.5 ± 1.167	7.733 ± 0.9072	7.1 ± 1.155	7.033 ± 1.066
Hear Beats/ Min at 48 HPF	125.1 ± 2.27	124.1 ± 2.255	125.1 ± 2.113	124.5 ± 2.36	125 ± 2.671	124.5 ± 2.751	126.5 ± 2.286

Values represents mean ± standard deviation for n=30

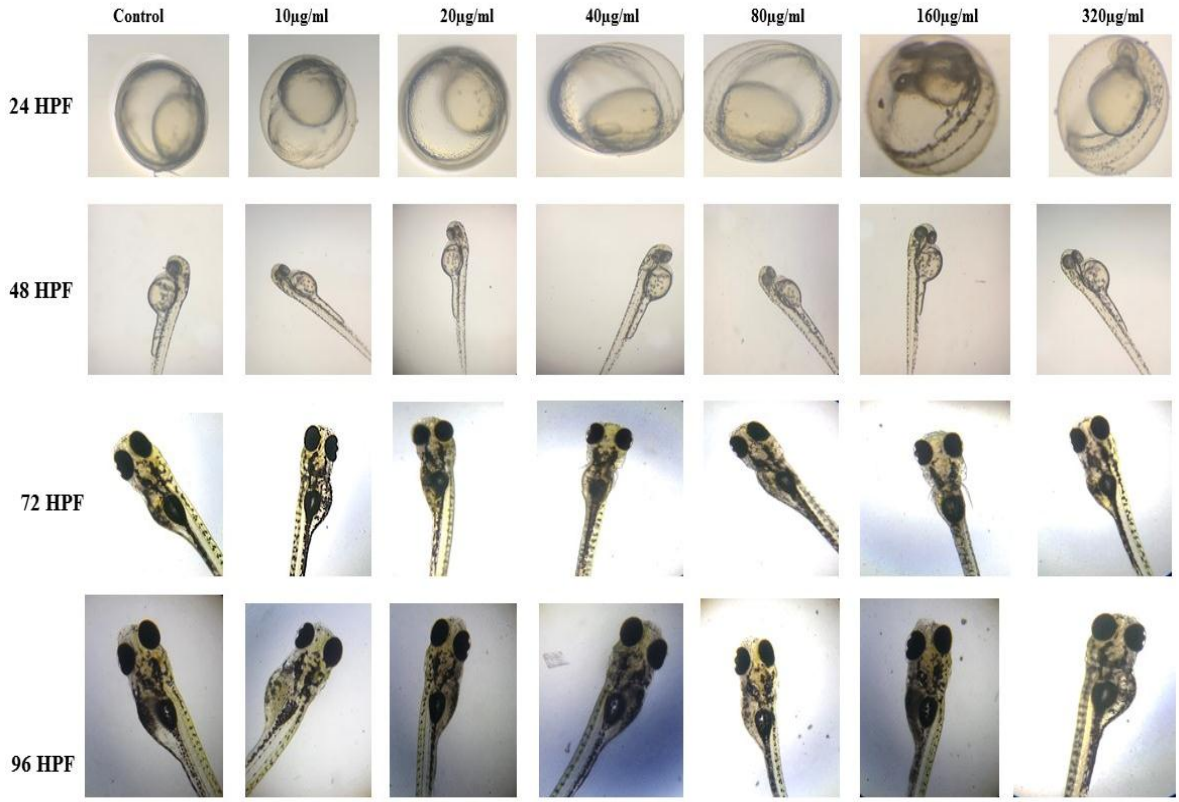

Fig. 1: Effect of Nellikai Legium on Different stages of Developmental Embryo from 24 to 48 HPF.

4. DISCUSSION

The zebrafish, also known as *Danio rerio*, is a model in drug screening assays that are also promising for studying human diseases.[14] An increasing number of academic institutions and pharmaceutical companies have recognized the use of monitoring zebrafish embryo

development, over the past decade, as a powerful tool in drug discovery, which has potential to speed up screening processes of new therapeutic drugs for humans.[15] As a research subject, its application possesses many critical advantages over other traditional vertebrate models.[16,17]

In the present investigation, zebrafish embryos were used to analyze the possible toxicity including movement behavior at 24, 48, 72 and 96 h post fertilization (HPF), after treatment with Nellikai Legium at the concentration ranges from 10µg to 320µg/ml. The observation of the study reveals no abnormality of the test drug with respect to hatching rate, pigmentation development, hatching rate, posture analysis, organogenesis etc.

There are several inherent advantages of utilizing zebrafish for drug screening [reviewed in.[18] They are easily bred in large numbers year-round, with a single spawning producing ~200 eggs. Additionally, embryos can live up to a few days after fertilization without external feeding. Drugs can diffuse through the skin and gills of embryos/larvae, and enter the body orally when zebrafish begin to swallow at around 72 h post-fertilization (hpf). In addition, the transparency of zebrafish for several days' post-fertilization (dpf) enables the *in vivo* observation of internal organ development.

In the present study there is no abnormality detected with respect to the following parameters such as Trunk axis, Spontaneous Movement, Heart formation, Curved or Bent axis, Eye malformation, Blood circulation, Mortality, Arrested growth, Craniofacial malformations, Peripheral ischemia and disruption of erythropoiesis, Accumulation of fluid around heart, Response to stimuli, Coagulation of eggs, Tail detachment etc.

The embryos develop rapidly *ex utero*, with most organs becoming fully functional at 2–5 dpf.[19] Furthermore, the organization of the genome and the genetic pathways controlling signal transduction and development are highly conserved between zebrafish and humans.[20] The results obtained from the present investigation has clearly shown that there is no significant difference in movement analysis of the drugs treated groups (7.1 ± 1.155 to 7.733 ± 0.90 per min) with that of the control embryos with movement of 7.667 ± 1.184 per min. The data obtained from the present investigation reveals that there is no significant difference in heart rate of the drugs treated groups (124.1 ± 2.255 to 126.5 ± 2.286 beats/min) with that of the control embryos with heart rate of 125.1 ± 2.27 beats/ min.

5. CONCLUSION

Zebra fish embryo model offers numerous advantage as its physiology simulates more of human with respect to it genetically and enzymatic compatility. Screening model like embryo toxicity studies provides more reliable data's with in short span of time. It was observed from the present study that treatment with Nellikai Legium does not induce any toxicity in the tested embryos and hence this research outcome reveals the safety of the formulation in the developing embryos.

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