



Evaluation of Teratogenicity-Indian Medicine Formulations *Nilavembu Kudineer* and *Mathulai Manapagu* using Zebrafish Model

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ABSTRACT A teratogen is a drug or other substance capable of damaging the DNA and interfering with the development of an embryo and it may lead to birth defects or developmental malformations. The present study is carried out to evaluate the Indian Medicines *Nilavembu Kudineer* and *Madulai Manapagu*. In Siddha system, *Nilavembu Kudineer* is given to treat Dengue fever whereas *Madulai Manapagu* is to improve the hemoglobin content and the management of hormonal imbalance in women. The results exemplified the overall percentage mortality, hatchability, and deformities were observed as low in *Madulai Manapagu* than *Nilavembu Kudineer*. The teratogenic index of *Nilavembu Kudineer* is higher than 1 (1.33), whereas LC_{50} EC_{50} values were observed as 80 µg/ml and 60 µg/ml respectively. Hence, *Nilavembu Kudineer* is considered as teratogenic when compared to *Madulai Manapagu*.

INTRODUCTION

The usage of herbal medicines had increased for the management of various ailments. Most developing countries uses Traditional Medicine as it is an accessible and affordable treatment (World Health Organization 1999a, 1999b; 2002), while Complementary Alternative Medicine is popular in many developed countries (World Health Organization 1998; United Nations Conference on Trade and Development 2000). The World Health Organization (WHO) estimates that 80 percent of the world's population relies on traditional medicinal system to meet the needs of primary health care (Farnsworth et al. 1985). In spite of such widespread and ancient use of traditional medicine, only since 1978 has the WHO recognized the use of herbal medicines for prophylactic, curative, palliative or diagnostic purposes (Ministério 2001). This problem arises since they are often used recklessly due to their belief that they are natural and they are free of adverse reactions and toxic effects (Gallo and Koren 2001). However it has been found that some constituents of the plants have been shown

to be potentially toxic, carcinogenic and teratogenic (Gadano et al. 2002; Gadano et al. 2000; Gadano et al. 2006; Effraim et al. 2001). The embryonic toxicity and teratogenicity focuses on chemical substances that damage embryos and fetuses and lead to death, growth retardation, and/or malformation of offspring. The considerable information has been documented whereas very ample information was known about teratogenicity of herbal products (Seo and Thomas 2017).

Siddha system of medicine is one of the oldest traditional medical system of India along with Ayurveda, Unani, Homeopathy and naturopathy system of medicine. It is originated from the southern peninsular of India particularly in Tamilnadu. The Phytochemical Evaluation, Embryotoxicity, and teratogenic effects of *Curcuma longa* Extract on Zebrafish (*Danio rerio*) was studied by Akinola et al. (2019). The present was carried out to evaluate the teratogenicity of *Nilavembu Kudineer* (NK) and *Madhulai Manapagu* (MM). *Nilavembu Kudineer* (NK) is a classical siddha formulation commonly used in the prophylactic/ treatment of Dengue and chickengunya fever. It is a polyherbal Siddha formulations consisting of equal portions of whole plant of *Andrographis paniculata* (Nilavembu), rhizomes of *Zingiber officinale* (Chukku), roots of

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Vetiveria zizanioides (Vetiver), root of *Plectranthus vettiveroides* (Vilamichuvar), fruits of *Piper nigrum* (Milagu), tubers of *Cyper rotundus* (Korai), heartwood of *Santalum album* (Chandanam), whole plant of *Trichosanthes cucurbitina* and whole plant of *Hedyotis corymbosa*. Traditionally, all these plants are used in the treatment of fever, inflammation, arthritis, gastric ulcer, jaundice, arthralgia and general debility conditions (Varier 1994; Varier 1995; Varier 1996). *Madhulai Manapagu* is an herbal formulation that is used for treating different types of anemia. It has a natural iron and calcium tonic especially for pregnant women. The ingredients of *Madhulai Manapagu* (MM) includes Pomegranate juice (*Punica granatum*), Honey (*Apis mellifera*) and Sugarcane (*Saccharum officinarum*). Though there is enormous therapeutic values of NK and MM demonstrated clinically there was no study on teratogenic effect of these two siddha medicines. Since Zebra fish model is inexpensive, embryonic development is similar to that mammals and many molecular pathways are evolutionary conserved between zebrafish and human, this model is considered as a suitable model for teratogenic screening.

Objectives

In the present study, the researchers use these two formulation that is, *Nilavembu Kudineer* and *Madhulai Manapagu* to investigate the teratogenic potential using Zebrafish embryo as the animal model. The teratogenic index of both NK and MM were calculated to determine the teratogenicity.

METHODOLOGY

The method was adapted from Brand et al. (2002) and Dahm (2002).

Zebrafish Husbandry

Adult, wild type Zebrafish, *Danio rerio*, were obtained from a commercial supplier (Chromepet, Chennai, Tamilnadu) and housed separately by gender at least 3 week prior to the first intended spawning. The adult fish were maintained in large 70-80 L aquaria with a constant light-dark (14-10h) cycle. The water temperature

in the tank was maintained at 28 ± 1 and pH 7. Dry food flakes were fed twice daily and a continuous aeration was supplied to the aquaria.

Collection of Eggs

The day before egg collection, males and females were placed in breeding tank with 1:2 female: male ratio and they were left undisturbed overnight. Next morning, the eggs could be collected 1 hour after the light had been turned on. The eggs were washed two times with tap water and transferred in a petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl_2 , 0.33 mM MgSO_4 , 0.00001% Methylene Blue).

Test Drugs

E3 medium was used as the medium for all solutions during the experiment. The pH for all solutions was checked and adjusted to 7-8 when necessary by adding Sodium Hydroxide or Hydrogen chloride solution.

Eight concentrations (10, 50, 100, 200, 400, 600, 800 and 1000 $\mu\text{g/ml}$) of the formulations, *Nilavembu Kudineer* and *Madhulai Manapagu* were prepared. Stock solutions were prepared by diluting to the desired concentrations in E3 medium.

Stock Preparation for *Nilavembu Kudineer*

10 grams of the sample was weighed in a 250 ml beaker and to it 150 ml of sterile distilled water was added. Then it was boiled till the decoction is concentrated to 50 ml. the liquid was then drained into falcon tubes and centrifuged at 2000 rpm for 2 minutes. The supernatant was then used for the preparation of working concentrations.

Stock Preparation for *Madhulai Manapagu*

100 mg of the sample was weighed in an eppendorf tube and then it was made up to 1 ml by sterile distilled water. Centrifuged 2000 rpm for 2 minutes.

Embryo Exposure

The zebrafish embryo were exposed in 24-well plates with 2 ml medium per well as it pro-

vides more oxygen supply to the developing embryos and in each well 20 embryos were used (Buschmann 2013). The embryos were exposed to test sample from the initiation of gastrula, that is, 5 hours post fertilization (hpf) ²⁰ till 120 hpf. The temperature is maintained at 26p C with 12:12 hour's light/dark cycle. There are two controls were maintained in this experiment, one is E3 medium (Negative Control) and 1% and 2% ethanol (Positive Control).

Evaluation of Teratogenic Effects

At four selected time points, namely 24, 48, 72 and 120 hpf, the teratogenic effects on the embryos were evaluated using an inverted microscope. The embryos were examined and evaluated for presence of morphological and teratogenic end-points, that is, Mortality, Hatchability and PE-Pericardial Edema; YSE-yolk sac edema; TM-Tail Malformation; YED-yolk extension detached,, MH-malformation of head and GR-growth retardation. All the embryos were examined at all time points and the observations were recorded in Microsoft excel.

Mortality, Hatchability and Deformity Evaluation

Hatchability, malformation and mortality rates were recorded at 24, 48, 72 and 120 hpf, and death was defined as coagulated embryos and no visual heartbeat.

Teratogenicity Index (TI)

For the calculation of LC and EC values, Origin 8 software was used. In order to determine the teratogenic potential of the drug, Teratogenic Index (TI), which is defined as the quotient of LC_{50} and EC_{50} , was calculated. If TI of a substance is greater than 1, then the substance is considered to be teratogenic and if TI is below than, the substance can be considered as embryo lethal.

RESULTS

In this experiment the control, that is, E3 medium, fulfilled the acceptance criteria, specifi-

cally ≥ 90 percent fertilization rate and ≤ 10 percent teratogenic effect.

Teratogenic Effect of Zebra Fish Treated with *Nilavembu Kudineer* (NK) and *Madhulai Manapagu* (MM)

The photos of drug treated and control embryos were taken at an interval of 24 hours (Fig. 1 and Fig. 2). The photos were processed using image J software (<http://imagej.nih.gov/ij/>). The dose dependent teratogenic effect was observed in *Nilavembu Kudineer* whereas there was very less or negligible effect of *Madhulai Manapagu* at very higher doses (1000 $\mu\text{g/ml}$). The diverse malformations were observed in *Nilavembu Kudineer*. The percentage of mortality, hatchability and deformity of *Nilavembu Kudineer* and *Madhulai Manapagu* were calculated. From the calculated data the researchers found that *Nilavembu Kudineer* has the higher potential of causing malformation to the Zebrafish embryo, whereas *Madhulai Manapagu* is safe and does not cause any harmful effects to the embryo compared to *Nilavembu Kudineer* and Ethanol (Positive control).

Teratogenic Effects of *Nilavembu Kudineer* (NK) and *Madhulai Manapagu* (MM)

The teratogenic endpoints (Malformation of head, tail, yolk and growth retardation) were evaluated under a microscope (Magnus Inverted Microscope) at 72 hpf. The data were shown in Table 1 and Table 2.

Teratogenic Index

From the above data it was observed that LC_{50} of *Nilavembu Kudineer* is 80 $\mu\text{g/ml}$ and EC_{50} of *Nilavembu Kudineer* is about 60 $\mu\text{g/ml}$. Hence, Teratogenic Index ($TI = LC_{50}/EC_{50}$) of *Nilavembu Kudineer* is 1.33. In case of *Madhulai Manapagu* the mortality percentage is 22 percent at 1000 $\mu\text{g/ml}$ for 72 hpf and the deformity percentage is 44 percent at 1000 $\mu\text{g/ml}$ at 72 hpf. The teratogenic index was less than 1 which is not teratogenic.

DISCUSSION

For gaining global popularity, it is very essential to demonstrate the teratogenicity of tra-

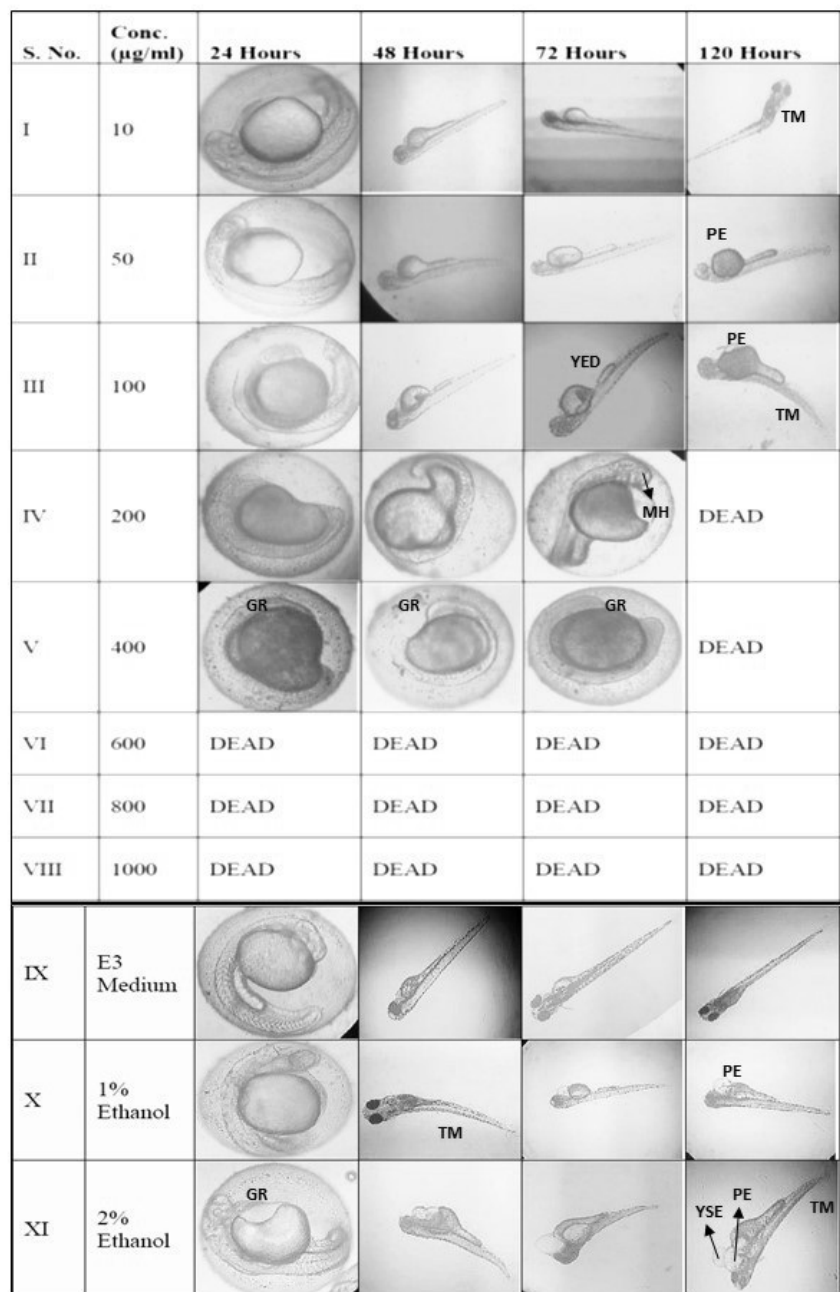


Fig. 1. Teratogenic effect of *Nilavembu Kudineer* on Zebrafish embryos at 24, 48, 72 and 120 hours of post fertilization (hpf)

PE-Pericardial Edema YSE-Yolk Sac Edema; TM-Tail Malformation; YED-Yolk Extension Detached MH-Malformation of Head; GR-Growth Retardation

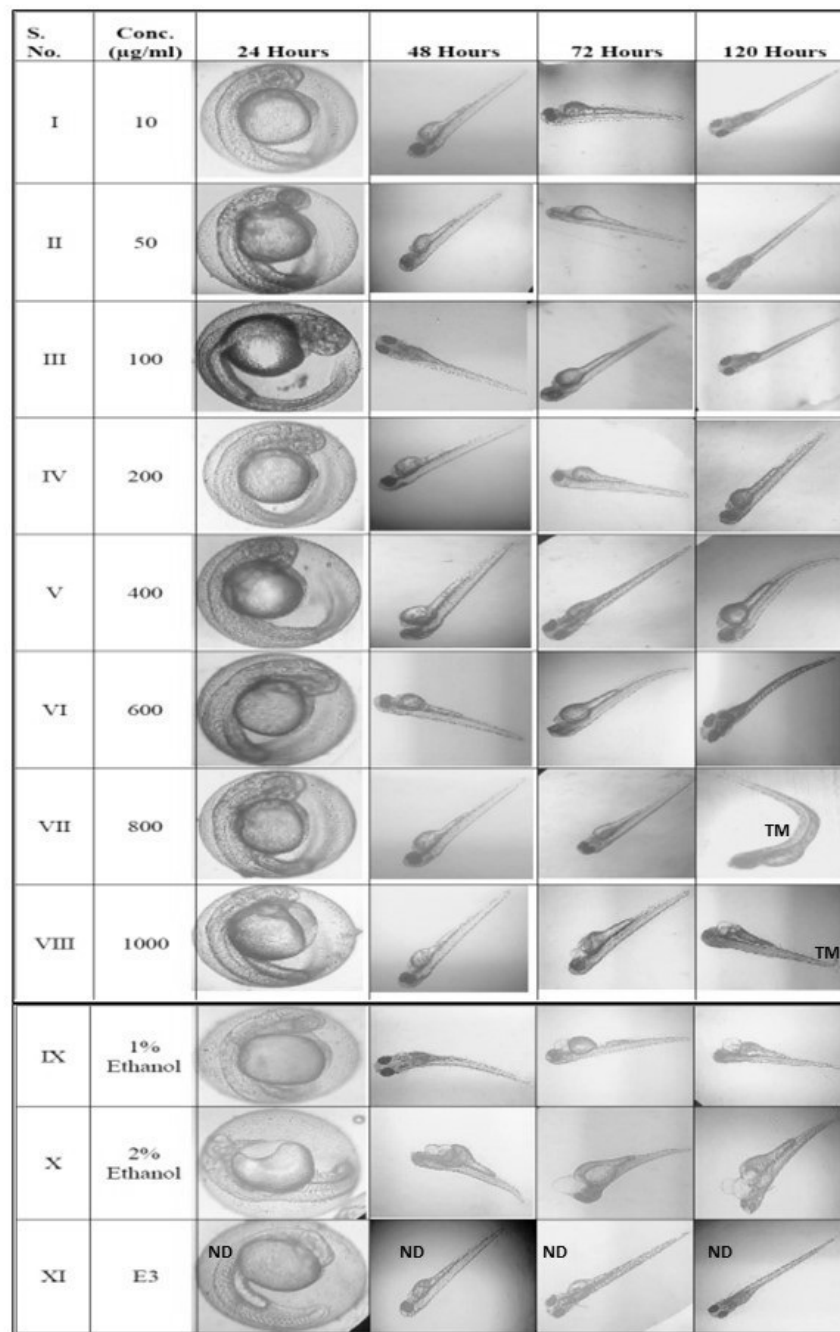


Fig. 2. Teratogenic effect of *Madhulai manapaggu* on Zebrafish embryos at 24, 48, 72 and 120 hours of post fertilization (hpf)

TM-Tail Malformation; ND-Normal Development

Table 1: Effect of Nilavembu Kudineer on zebrafish embryo

Concentration (µg/ml)	Total No. treated	Lethal	Deformed	Normal	Head	Tail	Yolk	Growth retardation
10	20	6 (30%)	2 (14%)	12 (86%)	1 (50%)	-	1 (50%)	1 (50%)
50	20	9 (43%)	5 (45%)	6 (54%)	2 (40%)	1 (20%)	2 (40%)	3 (60%)
100	20	11 (56%)	7 (78%)	2 (22%)	2 (28%)	1 (14%)	3 (43%)	4 (57%)
200	20	16 (80%)	4 (100%)	0%	2 (50%)	1 (25%)	3 (75%)	4 (100%)
400	20	18 (90%)	2 (100%)	0%	2 (100%)	2 (100%)	2 (100%)	2 (100%)
600	20	100%	0%	0%	-	-	-	-
800	20	100%	0%	0%	-	-	-	-
1000	20	100%	0%	0%	-	-	-	-
E3	20	1 (7%)	0%	19 (93%)	-	-	-	-
1% Ethanol	20	3 (13%)	9 (53%)	8 (47%)	3 (33%)	2 (22%)	6 (67%)	5 (55%)
2% Ethanol	20	7 (33%)	13 (100%)	0%	5 (38%)	4 (31%)	9 (69%)	11 (85%)

Table 2: Effect of Madhulai Manapagu on zebrafish embryo

Concentration (µg/ml)	Total No. treated	Lethal	Deformed	Normal	Head	Tail	Yolk	Growth retardation
10	20	1 (3%)	0 (0%)	19 (100%)	-	-	-	-
50	20	1 (3%)	2 (10%)	17 (89%)	1 (50%)	-	-	1 (50%)
100	20	1 (7%)	2 (10%)	17 (90%)	1 (50%)	-	-	1 (50%)
200	20	2 (10%)	3 (17%)	15 (83%)	1 (33%)	-	1 (33%)	2 (67%)
400	20	2 (10%)	3 (17%)	15 (83%)	1 (33%)	-	1 (33%)	3 (100%)
600	20	2 (10%)	4 (22%)	14 (78%)	2 (50%)	1 (25%)	2 (50%)	2 (50%)
800	20	3 (13%)	5 (29%)	12 (71%)	2 (40%)	2 (40%)	1 (20%)	3 (60%)
1000	20	4 (22%)	7 (44%)	9 (56%)	2 (29%)	2 (29%)	3 (43%)	4 (57%)
E3	20	1 (7%)	0 (0%)	20 (100%)	0%	0%	0%	0%
1% Ethanol	20	3 (13%)	9 (53%)	8 (47%)	3 (33%)	2 (22%)	6 (67%)	5 (55%)
2% Ethanol	20	7 (33%)	13 (100%)	0 (0%)	5 (38%)	4 (31%)	9 (69%)	11 (85%)

ditional medicines which are claiming therapeutic importance. The present research revealed that teratogenic effect of Indian medicines, *Nilavembu Kudineer* and *Madhulai Manapagu*. The teratogenic potential of the siddha drugs can be predicted quantitatively by ranking zebrafish embryos based on scoring system for phenotypic changes. In this experiment, *Nilavembu Kudineer* was observed as potential teratogenic drug than *Madhulai Manapagu*. But this is lower teratogenic effect when compared to antiepileptic drugs studied by Lee et al. (2013). In case of *Nilavembu Kudineer*, the malformed embryos can be seen clearly starting from 48 hpf at 200 µg/ml, whereas, in *Madhulai Manapagu*, the number of malformed embryos can be clearly seen in 72 hpf at concentration 600 µg/ml. The former was found to exhibit no teratogenic effect on the embryos, whereas, the latter shows a high teratogenic effect (1% shows teratogenicity from 48 hpf with and 2% shows at 24 hpf).

The teratogenic potential of the drugs can be described based on the scoring system for phenotypic changes that is similar to the morphological assessments conducted using in vivo embryo-lethal development of mammals (Chapin et al. 2008). The phenotypic changes observed in embryos treated with *Nilavembu Kudineer* are head deformities, tail malformation, yolk sac edema and growth retardation (Table 1). At 10 µg/ml majority of the changes observed is growth retardation (50%) and head deformation (50%), in 50 µg/ml, growth retardation (60%) can be seen in majority of the embryos, in 100 µg/ml yolk edema (43%) and growth retardation (57%) were observed, at 200 µg/ml head deformation (50%), yolk edema (75%) and growth retardation (100%) were observed in majority of the fishes and in 400 µg/ml, head deformation (100%), tail malformation (100%), yolk edema (100%) and growth retardation (100%). In *Madhulai Manapagu*, phenotypic changes can be observed from 50

µg/ml to 1000 µg/ml. However, since the morphological effect induced by the drug is very low compared to *Nilavembu Kudineer* (Table 2). This dose dependent malformation of both drugs were corroborated with the study on teratogenic effects of *curcuma longa* on zebra fish (Akinloa et al. 2019). The results also suggested that the prolonged exposure of NK and MM extracts leading to phenotypic malformations and finally lethality.

Based on EC₅₀ and LC₅₀ a teratogenic index was calculated. The *Madhulai Manapagu* showed a low range of LC₅₀ and EC₅₀ whereas in *Nilavembu Kudineer* showed higher range as LC₅₀ 80 µg/ml and EC₅₀ is 60 µg/ml. The teratogenic index (TI) was calculated, as 1.33. Since the TI value is greater than 1, *Nilavembu Kudineer* can be considered as teratogen. The observation was made that the teratogenic drug that produces wide separation between lethality and malformations. It showed that the possibility of toxic drug that causes severe malformations not mortality conversely a potential embryonic toxic drug can be lethal that malformations are not observed (Reimers 2004). The TI values of Siddha drugs used here for ranking the teratogenic effects. Hence the higher TI of NK would display the greater teratogenic effect and irreversible.

CONCLUSION

The present study has shown that the traditional medicines with therapeutic effect could still possess embryonic toxicity at higher doses. In this experiment, *Madhulai Manapagu* is safe even at higher doses whereas *Nilavembu Kudineer* could be lead to malformation of the fetus.

RECOMMENDATIONS

Madulai Manapagu could be taken during pregnancy for anemia whereas *Nilavembu Kudineer* should not be consumed.

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